SOY ISOFLAVONES MAY REVERSE BONE LOSS IN AN OVARIECTOMIZED RAT MODEL OF POSTMENOPAUSAL OSTEOPOROSIS

By

LATHA DEVAREDDY

Bachelor of Science
Madras University
Chennai, India
1996

Master of Science
Oklahoma State University
Stillwater, OK
2002

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 2005

SOY ISOFLAVONES MAY REVERSE BONE LOSS

IN AN OVARIECTOMIZED RAT MODEL OF

POSTMENOPAUSAL OSTEOPOROSIS

Dissertation Approved:

Dr. Bahram H.Arjmandi

Dissertation Adviser

Dr. Brenda J. Smith

Dr. Edralin A. Lucas

Dr. Carey N. Pope

Dr. A. Gordon Emslie

Dean of the Graduate College

DEDICATION

To my dearest Naina,
who would have been
very proud of this
accomplishment

ACKNOWLEDGEMENTS

Pursuing graduate education has been a very rewarding experience. A number of special people have supported me in achieving my aspirations. First of all, I would like to thank Dr. Bahram H. Arjmandi for his guidance, encouragement and making me believe in what I am doing. My sincere thanks to my committee members, Dr. Edralin A. Lucas, Dr. Carey N. Pope and Dr. Brenda J. Smith, for their input towards this dissertation and my education.

I would like to thank my father, Dr. D.B. Reddy for making me realize the importance of education and supporting me, and my mother, Prabha for her love and encouragement. I am grateful to my husband, Prakash for his continued love, motivation, and patience through my graduate education. I also would like to acknowledge my sister, Sudha, and brother-in-law, Vijay, for their kind support and guidance. I thank God for blessing me with an exceptionally supportive and lovingfamily.

Special thanks to Dr. Dania A. Khalil, Lisa Hammond, Dr. Shanil Juma, Dr. Do Y. Soung, Kiranmayi Korlagunta, Shirin Hooshmand and the staff of the Nutritional Sciences Department for their assistance in completion of this research work.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

CHAPTER III

CHAPTER IV

LIST OF FIGURES

Figure Page

CHAPTER III

CHAPTER IV

LIST OF ABBREVIATIONS

ALP	Alkaline Phosphatase
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMP	Bone Morphogenetic Protein
COL	Collagen Type I
Dpd	Deoxypyridinoline
DXA	Dual Energy X-Ray Absorptiometry
FDA	Food and Drug Administration
FOS	Fructooligosaccharides
HRT	Hormone Replacement Therapy
IGF-I	Insulin Like Growth Factor-I
IL-1	Interleukin-1
IL-6	Interleukin-6
MAPK	Mitogen-Activated Protein Kinase
MCSF	Macrophage Colony Stimulating Factor
NF-κB	Nuclear Factor-kappaB
NOF	National Osteoporosis Foundation
OC	Osteocalcin
OPG	Osteoprotegerin

OVX	Ovariectomized
PGE_2	Prostaglandin E_2
PTH	Parathyroid Hormone
RANK	Receptor Activator of Nuclear Factor-kappaB
RANKL	Receptor Activator of Nuclear Factor-kappaB Ligand
Runx2	Runt-Related Gene 2
Tb.Th	Trabecular Thickness
Tb.N	Trabecular Number
Tb.Sp	Trabecular Separation
TGF-β	Transforming Growth Factor- β
TNF-α	Tumor Necrosis Factor-α
TRAP	Tartrate Resistant Acid Phosphatase
WHO	World Health Organization
μCT	Micro-Computerized Tomography

CHAPTER I

INTRODUCTION

Osteoporosis is a debilitating disease characterized by decrease in bone mass and deterioration of bone microarchitecture which results in increased fragility and susceptibility to fracture (Center & Eisman 1997). It afflicts about 10 million people in the US, 80% of whom are women with an additional 44 million people being at the risk of developing this disease. Postmenopausal osteoporosis is the most prevalent type of osteoporosis and it is predicted that one out of every two American women will have an osteoporosis related fracture in her lifetime (NOF, 2002). For many years, hormone replacement therapy (HRT) has been used to prevent osteoporosis as it has been shown to improve bone mineral density and lower the risk of fractures in postmenopausal women (Komulainen *et al.* 1998; Hart *et al.* 1998). Nonetheless, the results of the Women's Health Initiative Trial (Rossouw *et al.* 2002) and the Heart and Estrogen/Progestin Replacement Study (HERS) (Grady *et al.* 2002) have clearly indicated that the risks associated with HRT outweigh its benefits making it clinically an unacceptable option for preventing or treating osteoporosis. Though, several treatment options have been approved by the Food and Drug Administration (FDA), the incidence of the disease has not declined (Biskobing *et al.* 2002). Several studies (McCombs *et al.* 2004; Kotzan *et al.* 1999) have reported that this may be due to the lack of long-term adherence to treatment.

This raises the need for therapies that are feasible, inexpensive, with minimal or no side effects.

Postmenopausal women are more attracted towards natural alternative therapies as they are perceived to have fewer side effects (Heaney 2000). In terms of dietary supplements, soy isoflavones have received considerable attention in the prevention and treatment of osteoporosis due to their structural similarity to estrogen and their ability in binding estrogen receptors. However, the effects of soy protein and its isoflavones on bone in both women (Potter *et al.* 1998; Dalais *et al.* 1998; Ho *et al.* 2001; Gallagher *et al.* 2000) and ovarian hormone deficient animal models of osteoporosis (Arjmandi *et al.* 1998; Arjmandi *et al.* 1996; Anderson *et al.* 1998; Picherit *et al.* 2001) are uncertain. These differences may be due variations in bioavailability of isoflavones among individuals and presence or absence of favorable intestinal microflora (Xu *et al.* 1995). The gut microflora is influenced by compounds known as prebiotics in the diet and play an important role in colon cancer prevention, reducing blood glucose and cholesterol, and boost the immune system of the host (Nettleton *et al.* 2005). For instance, fructooligosaccharide (FOS) is classified as a prebiotic and is a non-digestible oligosaccharide (Burns & Rowland 2004). The bioavailability and the absorption of soy isoflavones e.g., genistin and daidzin, have been reported to increase in the presence of FOS in the diet (Tokunaga 2004). Absorption of calcium and magnesium is also enhanced by FOS, thereby having the potential to improving skeletal health. In support of this notion, Mathey et al., (Mathey *et al.* 2004) indicated that soy isoflavones in combination with FOS prevented bone loss. Therefore, it is reasonable to postulate that the combination of soy isoflavones and FOS would be able to reverse bone loss.

Nonetheless, there are no reports on the extent to which the combination of soy and FOS reverses bone loss and, therefore it merits investigation. Therefore in the present research, in addition to investigation the dose dependent of soy isoflavones in the reversal of bone loss, the efficacy of combining soy with FOS on rebuilding bone in Ovx rats has been evaluated

Research Objectives

The principal objective of this study was to examine the role of soy with varying levels of isoflavones in the reversal of bone loss in an ovariectomized rat model of postmenopausal osteoporosis. Another objective of this study was to examine the synergy between soy protein with normal levels of isoflavones and FOS on reversal of bone loss.

The ***specific aims*** of experiment I were as follows:

1. To examine the dose-dependent effects of soy isoflavones in ***reversing*** bone loss by assessing bone mineral density (BMD) and bone biomechanical properties by utilizing an ovariectomized rat model of postmenopausal osteoporosis.

2. To determine the dose-dependent role of soy isoflavones in ***restoring*** bone structure byassessing trabecular microstructural properties of the proximal tibia and the fourth lumbar vertebra.

The ***specific aims*** of experiment II were as follows:

1. To examine the efficacy of Soy, FOS and their combination in ***reversing*** bone loss by assessing bone mineral density (BMD) and bone biomechanical properties by utilizing an ovariectomized rat model of postmenopausal osteoporosis.

2. To determine the role of Soy, FOS and their combination in ***restoring*** bone structure byassessing trabecular microstructural properties of the proximal tibia and the fourth lumbar vertebra.

Hypothesis

The *central hypothesis* of this study was that soy protein with its isoflavones reverses bone loss in an ovariectomized (Ovx) rat model of postmenopausal osteoporosis. The *ancillary hypothesis* of this study was that soy isoflavones and FOS exert synergistic effects in the reversal of bone loss in osteopenic ovx rats.

Format of Dissertation

The experiments are organized in twoindividual manuscripts for publication. Chapters III and IV are written in journal article format using the journal guidelines for Bone, and Menopause journals. The other chapters follow the Oklahoma State University format.

CHAPTER II

REVIEW OF LITERATURE

Osteoporosis is a major health concern as it increases the risk of hip and other fragility fractures. It is characterized by decrease in bone mass and deterioration of microarchitecture of bone. The World Health Organization defines osteoporosis as condition where the BMD is $\geq$ 2.5 Standard Deviations below the young adult reference mean (WHO, 1994). According to National Institutes of Health, osteoporosis is a condition of compromised bone strength that leads to increased fracture risk (NIH, 2000). The National Osteoporosis Foundation estimates that 10 million Americans are already afflicted with osteoporosis and another 34 million individuals have low bone mass, putting them at risk of osteoporosis-related fractures. The annual expenditure for treating osteoporosis related fractures was $17.5 billion in 2002 (Melton, III 2003) and is projected to exceed $60 billion by the year 2020 (Tucci 1998).

The skeleton is a highly specialized and dynamic organ that undergoes continuous remodeling (Manolagas 2000). Three main functions of the skeleton include 1) mechanical support and site of muscle attachment for movement, 2) protection for vital organs and bone marrow, and 3) reserve of ions like calcium and phosphorus (Baron 1993). Anatomically, bone is divided into two distinct types namely trabecular bone and compact bone (Martin *et al.* 1998). Compact bone (cortical bone) is dense bone found in

the shafts of long bones as well as forming a cortex or shell around vertebral bodies and other spongy bones. The cortical bone primarily provides the mechanical and protective function. Spongy trabecular or cancellous bone is porous bone found in the cuboidal bone (e.g., vertebrae), the flat bones, and the ends of the long bones. The trabecular bone primarily provides the metabolic function (Baron 2003).

Mature bone has an outer shell of compact bone known as the cortex, which encloses a meshwork of trabecular bone, with interconnecting spaces containing myeloid or fatty marrow (Marks & Odgren 2002). The cortical bone is covered by a periosteal membrane, which contains arterioles and capillaries that pierce the cortex, entering the medullary canal. These vessels along with larger structures enter one or more nutrient canals, which provide the blood supply to bone (Resnick *et al.*1995) .

Constituents of bone include collagen, hydroxyapatite, proteoglycans, and noncollagenous proteins, bone marrow and water (Baron 2003). Collagen is a structural protein and the predominant collagen in bone is type I, which is a rigid, rod-like, molecule composed of two alpha chains consisting of repeating amino acids with glycine in every third position and a high content of proline and lysine (Martin *et al.* 1998). These chains form a triple helix that is stabilized by the hydroxylation of proline and lysine residues by ascorbic acid (Raisz *et al.* 1998). The inorganic mineral of bone consists of hydroxyapatite crystals that contain carbonate, citrate, sodium, and magnesium (Jee W.S.S. 1983). Proteoglycans' specific role in bone is not clear; however, they may play an important role through their calcium-binding properties (Martin *et al.* 1998). The most abundant noncollagenous protein is osteocalcin, which is secreted by the osteoblasts and may play a role in bone mineralization (Martin *et al.*

1998). Bone marrow lies between the spaces in the trabeculae of all bones (Resnick *et al.* 1995). It provides a continuous supply of red blood cells, white blood cells, and platelets to meet the tissue's demands for oxygenation, immunity, and coagulation (Resnick *et al.* 1995; Martin *et al.* 1998).

The calcified bone matrix is not metabolically inert and there are cellular components that are very important for the formation and maintenance of bone (Raisz *et al.* 1998). The major cellular components include osteoclasts, osteoblasts, and osteocytes (Raisz *et al.* 1998). The osteoclasts are the "resorbers" that are closely related to the macrophage cells that remove debris or pathologic material throughout the body. Osteoblasts or the "formers" are closely related to the fibroblasts, which are cells that produce structural molecules in other tissues (Martin *et al.* 1998). The osteoclasts are responsible for bone resorption and the osteoblasts are responsible for bone formation (Raisz 2004). Another cell type in bone is known as the osteocyte, which is a former osteoblast that has become buried in bone and sits in the cavities called lacunae. These cells communicate among themselves and osteoblasts through tunnels called canaliculi ("canals") (Martin *et al.* 1998). The cellular activity is primarily devoted to an orderly sequence of bone resorption and formation (Raisz 2004).

The cellular components develop and differentiate through the control provided by growth factors, cytokines, and systemic hormones (Manolagas 2000). The exact details of this operation are not clear; however, a few mechanisms have been proposed (Manolagas & Jilka 1995). These include, 1) growth hormone and cytokines form positive and negative feedback loops; 2), some of the same factors influence both osteoclasts and osteoblasts; and 3) systemic hormones influence the formation of

osteoclasts and osteoblasts through their ability to control the production and/or action of local mediators (Manolagas 2000).

Precursors of osteoclasts are hematopoietic cells of the monocyte/macrophage lineage. A large group of cytokines and colony-stimulating factors are involved in hematopoiesis and also affect osteoclast development (Schinke T & Karsenty G 2002; Manolagas 2000). These cytokines include interleukins (IL) IL-1, IL-3, IL-6, IL-11, tumor necrosis factor (TNF)-α, and granulocyte macrophage-colony stimulating factor (M-CSF). The cytokines that inhibit osteoclast development are IL-4, IL-10, IL-18 and interferon-γ (Raisz 2004).

The precursors for osteoblasts are mesenchymal stem cells (Raisz *et al.* 1998). The formation of osteoblasts, osteoblastogenesis, is initiated by bone morphogenetic proteins (BMPs) from uncommitted progenitors (Rosen *et al.* 1996). Other factors such as transforming growth factor β (TGFβ), platelet-derived growth-factor (PDGF), insulin-like growth factors (IGFs), and members of the fibroblast growth factor (FGF) family can all stimulate osteoblast differentiation (Yanovski *et al.* 2000; Raisz 2004).

Bone Modeling and Remodeling

During development and growth, the skeletal size and shape is obtained by the removal of old bone and deposition of new bone, a process called modeling (Raisz 2004). As the skeletal grows, during childhood and adolescence, bone formation dominates. Once the skeleton has reached maturity, regeneration continues via a process known as remodeling (Marks & Odgren 2002). Remodeling is a life long process; however, the rate of activity varies depending on the age. Remodeling results in complete regeneration

of bone every 10 years (Manolagas 2000). The purpose of remodeling is thought to repair fatigue damage and maintain calcium homeostasis (Martin *et al.* 1998). At the beginning of the third decade of life, there is a steady decrease in bone mass due to the higher rate of resorption (Raisz 2004). Bone remodeling becomes uncoupled and the osteoclast activity becomes greater than the osteoblast activity resulting in bone loss. This phenomenon was described by Albright et al., as early as 1941 (Albright *et al.* 1941).

Humoral Regulation of Bone Metabolism

Remodeling can be activated by both systemic and local factors. One of the main systemic factors is the parathyroid hormone (PTH), which is secreted by the parathyroid gland. Parathyroid hormone has a direct effect on bone to regulate bone remodeling and enhance the mobilization of calcium from the skeleton (Resnick *et al.* 1995). The final product of vitamin D, $1,25(OH)_2$ vitaminD_3, is another humoral factor, which regulates intestinal mineral absorption and maintains skeletal growth and development (DeLuca & Cantorna 2001). However, the exact role it plays in remodeling is unknown. Calcitonin appears to play a small role in regulating bone turnover even though it inhibits bone resorption by acting directly on the osteoclasts (Raisz *et al.* 1998). Another systemic hormone is growth hormone, which increases both circulating and local levels of insulin – like growth factor–I (IGF-I). Growth hormone (GH) directly stimulates cartilage cell proliferation and both hormones increase bone remodeling (Raisz *et al.* 1998). Bone cells contain both estrogen and androgen hormone receptors (Raisz *et al.* 1998). Estrogens and androgens are critical for skeletal development and maintenance. Studying

the physiology of bone and the mechanisms through which bone remodeling occurs can help us better understand the etiology of postmenopausal osteoporosis.

Postmenopausal Osteoporosis

Menopause is defined as the period after 12 months of amenorrhea with no pathological reason (NAMS). The postmenopausal period typically occupies one-third of a woman's lifespan (Barrett-Connor, 1993), and currently there are more than 46 million postmenopausal women in the U.S. This number is predicted to increase to more than 50 million by the year 2020 (NAMS, 2000). Postmenopausal osteoporosiswas first defined by Albright (Albright & Reifenstein 1948) and later expanded by Riggs et al., (Riggs *et al.* 1982) as bone loss caused by the decreased levels of endogenous estrogen. Postmenopausal osteoporosis is the most common form of osteoporosis and is also known as primary osteoporosis. According to the National Osteoporosis Foundation, one half of the women will suffer from an osteoporotic fracture in their lifetime (NOF, 2002). The annual cost for treating osteoporosis-related fractures in the U.S. is currently estimated at $17 billion (Melton, III *et al.* 2003) and is projected to exceed $60 billion by the year 2020 (Tucci 1998). Therefore, osteoporosis-related fractures are enormous public health problems with immense socioeconomic implications.

Estrogen deficiency causes an increase in bone turnover with rates of resorption exceeding bone formation. The rate of bone loss is about 5% for cancellous bone and 1 to 3% for cortical bone per year in early postmenopausal women. It is estimated that postmenopausal women lose up to 50% of trabecular bone and 30% of cortical bone after 20 years of menopause. Estrogen protects against bone loss mainly by blocking bone

resorption, (Manolagas & Jilka 1995), but some studies have also shown that it may also play a role in bone formation (Chow *et al.*1992; Bain *et al.*1993) . The antiresorptive role of estrogen is mainly through decreasing osteoclastogenesis, which is the differentiation of bone resorbing cells, and the activity of mature osteoclasts. Estrogen also down-regulates the synthesis of numerous factors that enhance osteoclastogenesis, e.g., IL-1, IL-6, M-CSF, TNF-α, and prostaglandin (PG) E_2 (Schinke T & Karsenty G 2002). These effects will be discussed in the section on estrogen and bone.

In addition to preventing bone resorption, estrogen plays an important role in calcium absorption in the gut (Gennari *et al.* 1990) and its reabsorption in the kidney (McKane *et al.* 1995). The presence of estrogen receptors in the intestine has been reported and estrogen has been shown to increase intestinal calcium absorption both in rats (Arjmandi *et al.*1993; Arjmandi *et al.*1994) and humans (Gennari *et al.* 1990).

In summary, postmenopausal bone loss occurs by at least two mechanisms, the first mechanism is by enhanced osteoclastogenesis which leads to increased bone resorption, resulting in an early rapid phase of bone loss (Riggs *et al.* 1998). This causes a rapid influx of calcium from bone into circulation resulting in suppressed parathyroid hormone secretion and vitamin D production. During the late phase of menopause, bone loss occurs predominantly by a second mechanism, which is due to decreased intestinal calcium absorption. This decrease in calcium absorption causes secondary hyperparathyroidism and increased bone resorption (Riggs *et al.* 1998). Although postmenopausal osteoporosis is a widely researched area, the incidence of the fracture has not been reduced considerably and therefore there are still needs for understanding its etiology and developing efficacious therapies for postmenopausal osteoporosis.

The Ovariectomized Rat Model of Postmenopausal Osteoporosis

The FDA guidelines state that in addition to testing for toxicity, therapies used for the prevention and treatment of osteoporosis should be tested in preclinical models before their use in humans (Thompson *et al.* 1995). According to those guidelines, osteoporosis studies should be conducted using an Ovx rat model to examine whether a test agent is effective in preventing or treating osteoporosis. In prevention studies using an Ovx animal model, treatment should be initiated immediately after ovariectomy. In a bone loss reversal study, significant bone loss after ovariectomy should be demonstrated prior to initiation of treatment (Thompson *et al.* 1995).

Ovariectomized rat model is the most commonly used animal model of postmenopausal bone loss and has been reported to be an appropriate model to study cancellous bone changes in humans (Jee & Yao 2001). Additionally, rats are relatively inexpensive, easy to handle, changes in bone can be seen in shorter time frame and variability in studies can be minimized as genetically specific strains are available (Turner 2001). As rats do not experience natural menopause, ovariectomy has been used as a method to induce estrogen deficiency in young but skeletally mature rats (Kalu 1991; Wronski *et al.* 1985). Rapid loss of trabecular bone mass and strength occurs shortly after ovariectomy and these changes are similar to those that occur in postmenopausal women (Westerlind *et al.* 1997). The rapid bone loss following ovariectomy is caused by increased rate of bone turnover, with higher rate of resorption than formation. Other similarities between postmenopausal bone loss and ovariectomy-induced osteopenia in rats include; 1) higher loss of trabecular than cortical bone; 2) impaired intestinal absorption of calcium; and 3) similar effects of treatments such as estrogen, tamoxifen,

bisphosphonates, parathyroid hormone, calcitonin and exercise on bone (Kalu 1997). Laib et al. (2001) reported that cancellous bone volume decreased rapidly by about 40%, following ovariectomy and the rate of decrease slowed considerably around 60 days post-surgery. Similar observations were made by Wronski et al., (1985) who reported that a two fold decrease in tibial cancellous bone volume occurred following ovariectomy.

The use of rat as model of postmenopausal osteoporosis does have some limitations. These include; 1) longitudinal bone growth in long bones occurs transiently after ovariectomy in rats, however this limitation can be minimized by using 9 to 12 month-old rats (Wronski & Yen 1991); 2) rats lack or have poorly developed Haversian systems (Wronski & Yen 1991); and 3) rats do not experience fragility fractures. However, these can be assessed by biomechanical testing of the lumbar vertebra and the long bones (Kimmel 2002). In spite of these shortcomings, Ovx osteopenic rats are considered the most appropriate small animal model to study the efficacy of a treatment for postmenopausal osteoporosis. Therefore, in the present study, 9-month old female Sprague-Dawley rats were used to study the extent to which dietary treatments reverse bone loss.

Estrogen and Bone

The fundamental effects of estrogen on the skeleton include; 1) inhibition of bone remodeling by halting the activation of new bone remodeling units); 2) suppression of bone resorption; and 3) a possible stimulatory effect on bone formation.

As discussed earlier, bone loss occurs in estrogen deficiency mainly due to an increase in bone remodeling brought about by basic multicellular units (BMUs). BMUs are temporary anatomic structures comprising of osteoclasts and osteoblasts (Parfitt 1994). Loss of estrogen causes a marked increase in osteoclastic precursor cells from homatopoietic cells known as colony forming units-granulocytes/macrophages (CFU-GMs) in the marrow (Jilka *et al.* 1998). Estrogen also suppresses both osteoclast and osteoblast precursors. Since both osteoblasts and the stromal/osteoblastic cells play an important role in osteoclast development, the inhibition of these cells may be a key mechanism by which estrogen suppresses bone remodeling (Di Gregorio *et al.* 2001).

In addition to its action in reducing osteoblast and osteoclast precursors, estrogen plays an important role in osteoclast development, activity, and apoptosis. Receptor activator of nuclear factor-kappaB ligand (RANKL) is a nuclear factor that is expressed on the surface of bone marrow stromal/osteoblast precursor cells, T-cells, as well as B-cells (Eghbali-Fatourechi *et al.* 2003). RANKL is an essential molecule in the development of osteoclasts (Lacey *et al.* 1998). Receptor activator of nuclear factor-kappa B (RANK) is its cognate receptor that is seen on osteoclast lineage cells (Hsu *et al.* 1999). Osteoprotegerin (OPG) is the decoy receptor that binds to RANKL and is produced by osteoblastic lineage cells (Simonet *et al.* 1997). Estrogen suppresses RANKL production by osteoblastic, T- and B-cells (Eghbali-Fatourechi *et al.* 2003) and simultaneously increases OPG production (Hofbauer *et al.* 1999). This results in inhibition of bone resorption. The other antiresorptive effects of estrogen are modulated through its effects on cytokines that stimulate bone-resorption, such as IL-1, IL-6, TNF-α, M-CSF, and PGE_2. Although several studies have demonstrated the role of these

cytokines in resorption of bone, it is seen that multiple cytokines act together in inducing bone resorption in the absence of estrogen and seem to have synergistic effects on bone resorption. Estrogen also causes an increase in the production of TGF-β (Oursler *et al.* 1991) which has been shown to induce apoptosis of osteoclasts (Hughes *et al.* 1996). Estrogen influences all the aspects of osteoclast development, activity, and lifespan, and these facts explain why estrogen deficiency results in a marked increase in bone resorption.

From the bone formation point of view, estrogen is found to prolong the lifespan of the osteoblast by inhibiting osteoblast apoptosis at the cellular level, thereby increasing the function of osteoblast (Kousteni *et al.* 2001). Dang et al., (2002) reported that estrogen stimulated the differentiation of progenitor cells through the osteoblast lineage and not adipocyte lineage, as seen by elevated alkaline phosphatase activity and increased nodule formation.

More recently, it has been reported that estrogen may also act directly on osteocytes. Estrogen deficiency has been shown to induce apoptosis of osteocytes (Tomkinson *et al.* 1997) in iliac bone. The apotosis of osteocytes has been shown to be inhibited by estrogen in ovariectomized mice (Kousteni *et al.* 2001). As osteocytes may play a role in sensing mechanical loading, the loss of these cells can exacerbate bone loss similar that that seen in weightless conditions (Pitsillides *et al.* 1995). In summary, these are some proposed mechanisms by which estrogen influences bone remodeling and plays a role in maintaining bone mass.

Treatment Options for Postmenopausal Osteoporosis

In general, treatments for postmenopausal osteoporosis are aimed at either decreasing bone resorption or increasing bone formation. There have been several treatment options that have been approved in the recent years by the Food and Drug Administration (FDA), however, their effects on reduction of incidence of fractures remains to be seen as the time between starting treatment and assessing its effects on bone mass and fracture is several years. Some of the drugs approved by the FDA are discussed below.

Bisphosphonates: Bisphosphonates are class of compounds that are derivatives of pyrophosphate. They act by inhibiting hydroxyapatite formation, and thereby decreasing bone resorption (Akesson 2003). There are several different bisphosphonates that are available, however, the major drugs for osteoporosis are alendronate, etidronate, and risedronate. Bisphosphonates interfere with numerous actions of osteoclasts (Russell & Rogers 1999) including the disruption of the formation of cytoskeletal actin ring in polarized resorbing osteoclasts (Murakami *et al.* 1995); inhibition of protein tyrosine phosphatases (Schmidt *et al.* 1996); and induction of osteoclast apoptosis (Hughes *et al.* 1995).

Etidronate was the first bisphosphonate to be tested for osteoporosis (Storm *et al.* 1990; Watts *et al.* 1990). Later a large randomized controlled trial was published with the bisphosphonate, alendronate (Liberman *et al.* 1995). Alendronate was approved by the FDA in 1996 and is marketed by Merck Phamaceuticals with the trade name Fosamax, for the prevention and treatment of osteoporosis. In a recently published, large five-year long clinical trial involving more than 3000 women, alendronate (10 mg/day)

for 5 years of treatment significantly reduced fracture risk in women with low bone mass (Quandt *et al.* 2005). However, alendronate is associated with numerous side effects which include nausea, constipation, diarrhea, and abdominal pain (Doggrell 2004). The third bisphosphonate used for the treatment and prevention of osteoporosis was risedronate (actonel™) was approved by the FDA in April 2000. Risedronate has been shown to have fewer side effects than alendronate and has also been demonstrated to reduce the risk of non-vertebral fracture in women with severe osteoporosis (Adachi *et al.* 2001). However, currently the data are insufficient to compare fracture rates with alendronate and risedronate (The Medical Letter, 2005).

Calcitonin: Calcitonin is a polypeptide hormone made in the C cells in the thyroid (Bennet et al., 1984; Raisz et al., 1998). This hormone acts on the osteoclast by inhibiting the proliferation of progenitors as well as the differentiation of committed precursors (Price et al., 1980). It is available with the trade name of Miacalcin that is usually administered as a nasal spray. Miacalcin (Kaskani *et al.* 2005) has been shown to increase BMD in postmenopausal women, however its effects on fracture risk reduction are yet to demonstrated?. The possible side-effects include runny nose, nose bleeds and nose pain and are considered to be mild (Thamsborg *et al.* 1991; Lyritis *et al.* 1995).

Raloxifene: Raloxifene is a benzothiophene derivative that was approved by the FDA in December 1997 for the treatment of osteoporosis. Raloxifene is a new generation SERM that has demonstrated estrogen-like effects on the skeleton and cardiovascular system, but anti-estrogen effects on the breast and endometrium (Licata et al., 2000, Jordan 2001; Setchell 2001). In the MORE (Multiple Outcomes of Raloxifene Evaluation) study

(Cummings SR, et al., 1998), 7705 postmenopausal women aged 31–80 years with osteoporosis, raloxifene increased lumbar spine and femoral neck BMD by 2%–3%, reduced the risk of vertebral fractures by 30%–50%, and decreased the incidence of breast cancer. However, raloxifene may have potential adverse effects, such as an increase in hot flashes, an increase in risk for blood clots in the leg veins and/or the lungs (similar to estrogen), leg cramps and fluid retention (Deitcher & Gomes 2004; Vogelvang *et al.* 2004).

Parathyroid Hormone: Parathyroid hormone is an analog of human PTH approved for the treatment of osteoporosis in postmenopausal women and men who are at high risk for fracture. Depending on the duration of dosing and the mode of administration, PTH can either increase bone formation or resorption. Continuous administration leading to persistent higher levels of the hormone result in increase bone resorption leading to bone loss, whereas, intermittent PTH injections cause a transient peaks in serum hormone levels, leading to increased bone formation and BMD. Therefore, intermittent PTH acts as an anabolic agent. The studies investigating the role of PTH as a treatment option were started as early as 1970s by Reeve et al., (1976) and showed that PTH increased bone density in postmenopausal women. The findings of a large two-year, clinical trial involving 1637 postmenopausal osteoporotic women who were receiving PTH indicated that PTH increased lumbar spine BMD by 9%–13% and there was 65% reduction in vertebral fracture risk (Neer *et al.* 2001). The most common side effects are dizziness and leg cramps. Elevations in blood calcium and urine calcium can also occur. The cost of administering teriparatide, recombinant human parathyroid hormone (1-34), available by the trade name FORTEO®, is $600 per month and the duration of treatment is between

18-24 months (Eriksen & Robins 2004). Side effects include marrow fibrosis, tunneling resorption, nausea, and headache (Jiang *et al.* 2003). One of the concerns of PTH use is that its safety has not been evaluated beyond two years.

There are other numerous other agents that are not yet approved by the FDA but have undergone or currently undergoing clinical trials for the treatment of osteoporosis. These potential treatment options include tibolone , strontium ranelate, OPG, cathepsin K inhibitors, etc (Akesson 2003).

In summary, though there are numerous options for the prevention and treatment of osteoporosis, they are associated with certain risks. Therefore, there is a need for continuous search for an alternative/adjunctive therapy which can reduce the incidence of fracture without the side-effects. Among natural compounds, phytoestrogens have shown immense promise in the prevention and treatment of osteoporosis.

Phytoestrogens

As the name suggests phytoestrogens are plant estrogen-like compounds that may have beneficial effects on the cardiovascular system and may help in alleviating some of the symptoms that are attributed to menopause such as osteoporosis and breast cancer (Wroblewski & Cooke 2000). Isoflavones, lignans and coumestans are the major types of phytoestrogens (Brezinski & Debi 1999; Knight & Eden 1996). Common and significant edible source of isoflavones are soybeans and to a lesser extent other legumes such as chick peas and black-eyed peas. The richest source of lignans, are, oilseed and cereals.

Broccoli and alfalfa sprouts are rich in dietary coumestans. Coumestans are more potent in their estrogenic activity than isoflavones (NAMS, 2000). Phytoestrogens are heterocyclic compounds that have structural similarities to estrogenic steroids (Murkies *et al.* 1998).

Plant isoflavones and lignans are converted to heterocyclic phenols in the gut and these compounds are similar in structure to estrogen and they exhibit weak estrogenic properties (Murkies *et al.* 1998). Phytoestrogens have varying effects on different tissues and various types of phytoestrogens have varying affinities to estrogen receptors (Anderson *et al.* 1995).

Phytoestrogens have shown both estrogenic and anti-estrogenic properties. They are estrogenic because they have a tendency to bind to estrogen receptors (Miksicek 1993). They are anti-estrogenic because, unlike estrogen, they inhibit the activity of aromatase and the proliferation of the breast cells (Morito *et al.* 2001). In addition to acting by binding to estrogen receptors, phytoestrogens may impart additional health benefits by acting as anti-oxidants. This conclusion is based on the oxidative resistance of the LDL-cholesterol obtained from participants consuming high levels of phytoestrogens (Tikkanen *et al.*1998) .

Scientists in various countries are beginning to explore the health benefits of numerous phytoestrogens in chronic diseases including cancer, heart disease and osteoporosis. The incidence of chronic diseases, e.g. cardiovascular disease, cancer, osteoporosisand stroke is much less in countries consuming high amounts of soy when compared with countries that do not traditionally consume soy (Clarkson 2000). Japanese

women are found to have significantly lower rates of deaths related to cardiovascular disease, osteoporosisand cancers (Boring *et al.*1994) .

Soy Isoflavones

The three major isoflavones that are present in soy are genistein, daidzein, and glycetein (*see figure 1 for structures*). One gram of soy contains about 1 to 3 mg of diadzin, genistin, glycetin and their corresponding glucosides (Barnes & Messina, 1991). Genistein and diadzein comprise the major portion of isoflavones in soy and have been shown to bind to estrogen receptors, a property explained by their structural similarity with estrogens (Knight & Eden 1996b). The phenolic ring is the essential structural element that enables these compounds to bind to estrogen receptors (Setchell 1998). The findings by Kuiper et al. (Kuiper *et al.* 1998) showed that the binding affinity of genistein to ER-β was about 20 times greater than binding ER-α. ER-β is expressed more in non-reproductive tissue, such as bone and the vascular system. This may explain some of the bone-protective effects of soy isoflavones without the side-effects on uterine tissue.

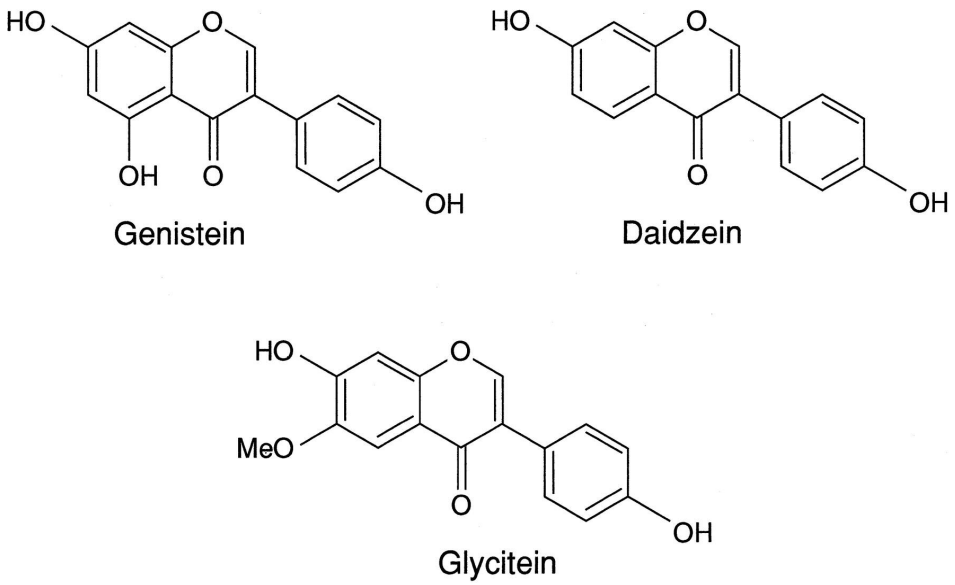


Figure 1: Major soy isoflavones, genistein, diadzein and glycetein.

Isoflavones are conjugated substances, when hydrolysed by β-glucosidases in the jejunum, release bioactive aglycones, daidzein and genistein. These aglycones have been shown to have affinity for estrogen receptors and have other non-hormonal effects on the cellular mechanisms (Setchell 1998). By looking at the pharmacokinetics of soy isoflavones, it is seen that maintaining high steady state of plasma concentrations can be maintained only by the daily intake of phytoestrogens throughout the day (Setchell 2000). Since soy isoflavones can be easily incorporated into daily diet in various forms, it is relatively easy to maintain the plasma concentrations of isoflavones and their metabolites. Thus, soy isoflavones can be used as a feasible and inexpensive adjunctive/alternative therapy for osteoporosis. The following sections will discuss the efficacy of soy isoflavones in prevention and reversal of bone loss and their mechanisms of action.

Soy and Bone – Animal Studies

The role of soy isoflavones in modulating bone have been examined using various animal models including ovariectomized rats (Arjmandi *et al.* 1996; Arjmandi *et al.* 1998), mice (Fonseca & Ward 2004) and cynomolgus monkeys (Register *et al.* 2003). Arjmandi et al., (Arjmandi *et al.* 1996) were one of the first groups to report that soy protein-based diet was efficacious in preventing bone loss as it attenuated loss of vertebral bone density and positively modulated biomarkers of bone formation and resorption. In a follow up study (Arjmandi *et al.* 1998), the same investigators examined whether soy protein or its isoflavones are responsible for the bone protective effects of soy. Ovx rats were fed soy protein based diet with normal isoflavone or soy protein based-diet deplete of isoflavones. The findings of that study indicated that rats that

received soy with normal isoflavones content had significantly greater femoral bone density than rats that received isoflavone-deplete soy diet. These findings were similar to those of Picherit et al., (Picherit *et al.* 2001) who reported that isoflavones prevent bone loss in Ovx rats by increasing bone formation and reducing bone resorption. Blum et al., (2003) also reported the bone-sparing effects of soy isoflavones indicated by increased endocortical and cancellous bone formation as measured by histomorphometric analyses

Although prevention is better than cure, a large number of postmenopausal women are already suffering from osteoporosis, and it is desirable to have a treatment option that would reverse bone loss with minimum or no side effects. Therefore, the role of soy isoflavones in the reversal of bone loss has also been examined and there have been conflicting results. Isoflavones have shown to have modest effects on the reversal of the ovarian hormone deficiency-induced bone loss (Arjmandi *et al.* 1998). In contrast to this study, Picherit et al., (2001) indicated that although soy isoflavones prevent bone loss, they do not reverse bone loss in Ovx rats. Similar observations were also made in ovariectomized monkeys (Lees & Ginn 1998; Register *et al.* 2003). The differences in these findings may be due to the variations in the age of the animals, duration of the study, and the dose of isoflavones used in these studies. This raises the need for studies that are appropriately powered using relevant animal models with a good experimental design.

Soy and Bone – Human Studies

A few clinical studies (Arjmandi *et al.* 2005; Potter *et al.* 1998; Alekel *et al.* 2000) have examined the effects of soy isoflavones on bone mineral density and the

results of these studies are inconclusive. One of the earlier studies were conducted by Potter et al., (1998) in which 66 postmenopausal women receiving 90 mg of isoflavones with 40 g soy protein had a significant increase in spine, but not hip BMD after six months of treatment. In a similar study by Alekel *et al.* (2000) of perimenopausal women receiving 80 mg isoflavones/day, isoflavones prevented the loss of lumbar spine BMD or BMC, whereas, significant losses in BMD and BMC occurred in the control group. One year supplementation of 80 mg/d isoflavones have been shown to increase trochanter BMC, but not BMD, in postmenopausal Chinese women with low initial bone mass (Chen *et al.* 2003). The findings of a recently published one-year study by Arjmandi et al., (2005) indicated that supplementation of 25 g soy protein with 60 mg isoflavones positively modulated markers of bone formation, but is unable to prevent the loss of lumbar and whole body BMD in postmenopausal women.

The effects of soy supplementation on biomarkers of bone formation and resorption are also contradictory. In a study by Alekel et al. (2000), the positive effects of soy supplementation by changes in serum bone-specific alkaline phosphatase activity, a marker of bone formation, or urinary N-telopeptide (NTX), a marker of bone resorption, as these markers were not affected by treatment. Similar observations were made by Wangen et al (2000), where soy isoflavones had minimal effects on bone biomarkers. In a short term clinical trial by Arjmandi et al. (2001), consumption of 40 g soy protein with 90 mg isoflavones daily significantly reduced urinary Dpd excretion, a specific marker of bone resorption and concurrently increased serum IGF-I concentrations in postmenopausal women. These conflicting reports may be due to the fact that biochemical markers of bone turnover are highly variable and studies assessing these

should have large sample sizes (Weaver & Cheong 2005). Bone is a tissue which is undergoing constant remodeling and to observe treatment effects on bone studies have to be long enough to accommodate several remodeling cycles (Weaver & Cheong 2005). Therefore, there is a need for long-term, large clinical studies to confirm the bone protective effects of soy isoflavones.

Mechanism of Action of Soy Isoflavones on Bone

Several mechanisms have been proposed by which soy isoflavones exert beneficial effects on bone including their structural similarity with estrogen. In the early 1970s, Shutt and Cox (1972) determined that phytoestrogens have a binding affinity to estrogen receptors and more recently Kuiper et al., (1997) demonstrated that genistein has a particular binding affinity for estrogen receptor-β, (Kuiper et al. 1998). Thus, the binding of isoflavones to estrogen receptor-β is postulated as the possible mechanism by which isoflavones modulate bone (Burke *et al.* 2000). The binding affinity of isoflavones to ER-β, but not ER-α (Kuiper *et al.* 1997), causes beneficial effects on bone without the undesirable side-effects on breast and uterine tissue. Estrogen independent effects of isoflavones have also been reported (Akiyama *et al.* 1987; Blair *et al.* 1996). An *in vitro* study by Akiyama et al. (1987), showed that genistein inhibits the tyrosine-specific protein kinase activity of the epidermal growth factor (EGF) receptor and therefore, results in decreased osteoclastic protein synthesis (Blair *et al.* 1996). The effect of soy on calcium absorption, in part, may protect against bone loss as reported by Arjmandi et al. (2002), where similar to estrogen, soy treated Ovx rats experienced enhanced intestinal calcium absorption. Additionally, soy supplementation has been reported to increase the

circulating and mRNA levels of insulin-like growth factor-1 (IGF-1) in postmenopausal women (Arjmandi B.H. *et al.* 2003) and Ovx rats (Arjmandi *et al.* 1998), respectively. Several studies (Langlois *et al.* 1998; Bauer *et al.* 1997) have reported the close correlation between circulating levels of IGF-1 and BMD and isoflavones may positively modulate bone via the IGF-1 dependent pathway. Soy isoflavones have also been shown to exhibit antioxidant properties by inhibiting the production of hydrogen peroxide and activating antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase (Wei *et al.* 1995). Antioxidants prevent oxidative damage and thereby prevent bone resorption and stimulate bone formation (Basu *et al.* 2001; McBride J 1999). Though these are some plausible ways by which soy isoflavones may exhibit bone protective effects, their mechanism of action remains to be clarified.

Fructooligosaccharides

Traditionally, the main functions of colon are described as water and electrolyte absorption, and storage and excretion of waste (Marfarlane & McBain 1999). It also plays an important role in nutrient absorption, which is mainly due to the metabolic activities of intestinal microflora (Berg 1996). *Lactobacillus* and *Bifidobacterium* species are the two important types of bacteria that constitute the intestinal microflora (Reid & Burton 2002). The gastrointestinal tract is a dynamic ecosystem where these microflora ferment the foods and their components that are present in the colon of the host. The composition and activities of these bacteria can be modified by diet to enhance their beneficial effects. These beneficial effects include 1) energy generation by fermenting the carbohydrates and proteins (Cummings & Macfarlane 1991); 2) synthesis of certain

vitamins such as vitamin B and K (Berg 1996); 3) production of short chain fatty acids, that lower the pH of the colon and increase water absorption (Gibson & Roberfroid 1995); 4) synthesize antimicrobial compounds (Yildirim & Johnson 1998); and 5) enhancement of gut barrier function as these microorganisms compete with disease causing pathogens for adhesion receptors in the intestinal mucosa and thereby improve host's immunity (Cebra 1999; Cunningham-Rundles & Lin 1998).

Certain dietary components stimulate the growth of these beneficial bacteria and are defined as prebiotics. These include, FOS and inulin, which are short chain carbohydrates that are resistant to digestion in the upper GI tract by human enzymes (Gibson & Roberfroid 1995). FOS is fermented in the colon by the intestinal microflora to short chain fatty acids (SCFAs), hydrogen, and carbon dioxide (Cummings & Macfarlane 1991). Wiechmann (1996) has reported an increase in a calcium binding receptor, recoverin, during the SCFA-induced differentiation in cells. Additionally, the absorption of calcium and magnesium are shown to increase in rats that are fed FOS (Ohta *et al.*1994) .

In terms of soy isoflavones, as mentioned earlier, a study by Mathey et al., (2004) demonstrated the efficacy of soy isoflavones is enhanced in the presence of FOS in preventing bone loss in Ovx rats. The beneficial effects of this combination may be due to the increased bioavailability of the isoflavones by FOS. The primary isoflavones, genistein and diadzein commonly occur as glyconated forms and the glycosidic bonds have to be hydrolyzed by an enzyme, β-glucosidase before they can become bioactive (Setchell 1998). The activity of β-glucosidase increases with FOS administration and thereby promotes the bioavailability of isoflavones. Additionally, equol, a metabolite of

diadzein is synthesized by intestinal bacteria and FOS increases the growth of these beneficial bacteria (Setchell 1998). In summary, in addition to the benefits of either soy or FOS alone on bone, these may be some of the mechanisms by which FOS in combination with soy exerts beneficial effects in reversal of bone loss. Therefore, the efficacy of this combination in the reversal of bone loss merits investigation.

CHAPTER III

Soy moderately improves bone mass and microstructural properties in an ovariectomized rat model of osteoporosis

Latha Devareddy[a], Brenda J. Smith[a], Dania A. Khalil[a], Edralin A. Lucas[a], Do Y. Soung[a], Denver D. Marlow[b], and Bahram H. Arjmandi[a]

[a]Department of Nutritional Sciences and [b]College of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma 74078-6141 USA

Corresponding author and reprint requests should be directed to: Bahram H. Arjmandi, PhD, RD, Department of Nutritional Sciences, 301 Human Environmental Sciences, Stillwater, OK 74078-6141; (405) 744-4437; FAX (405) 744-1357; E-mail: arjmand@okstate.edu

Keywords: Estrogen; Isoflavones; Microcomputed tomography

Abstract

Soy protein is reported to prevent bone loss in both women and rat models of osteoporosis. However, the role of soy isoflavones on the trabecular microarchitectural properties needs to be explored. In the present study, we examined whether soy protein with graded doses of isoflavones reverses loss of bone mineral density (BMD), bone mineral content (BMC), and trabecular microstructure in an ovariectomized (Ovx) osteopenic rat model. Seventy-eight 9-m old female Sprague-Dawley rats were either sham-operated (Sham; 1 group) or Ovx (5 groups) and fed a semi-purified casein-based diet. After 90 days the occurrence of bone loss was confirmed using dual energy x-ray absorptiometry. Thereafter, rats were assigned to the following treatments: Sham, Ovx (control), Ovx+17β-estradiol (E_2; 10 μg/kg body wt. twice per wk), Ovx + soy protein depleted of isoflavones (Soy-; 0.06 mg isoflavones/g protein), Ovx + soy protein with normal isoflavone content (Soy; 3.55 mg isoflavones/g protein), and Ovx + soy protein enriched isoflavones (Soy+; 7.10 mg isoflavones/g protein). After 125 days of treatment, rats were euthanized and tissues were collected for the assessment of BMD and BMC, and tibial and 4^{th} lumbar trabecular micro-architectural properties via x-ray microcomputed tomography. Soy significantly increased tibial BMC and BMD by 10 % and 4.5% in comparison with Ovx control, whereas the effects of Soy-, Soy+ and E_2 were less pronounced. All of the soy-based diets, irrespective of their isoflavone content, had a modest effect on the lumbar BMD. However, only the Soy+ diet positively affected the tibial architectural properties including trabecular thickness, separation, and number. None of the treatments had any effect on trabecular microarchitectural properties of the fourth lumbar vertebra. In summary, our findings suggest that isoflavones may exert a

biphasic effect on tibial BMD, and higher doses of isoflavones may be required to reverse the loss of tibial microstructural properties.

Introduction

Among the edible plants, soybeans are the richest source of isoflavones which may improve bone health. Lately, certain estrogen-like compounds of plant origin, such as soy isoflavones, have been characterized as naturally occurring selective estrogen receptor modulators (SERMs) with similar beneficial effects to raloxifene on bone [1-3]. Hence, similar to synthetic SERMs, soy isoflavones have been suggested to exert the beneficial effects of estrogen without its side effects [2]. However, the effects of soy protein and its isoflavones on bone in both women [4-7] and ovarian hormone deficient animal models of osteoporosis [8-11] are uncertain.

Decreased bone mass is only one of the many factors jeopardizing bone integrity, resulting in reduced bone strength and increased susceptibility to fractures. Other important factors that influence bone health include architectural arrangement, presence or absence of microfractures, and abnormalities in bone matrix or mineralization [12-14]. In support of this notion, emerging data [15,16] raise the issue of whether treatment-induced changes in BMD are predictive of fracture risk reduction. For instance, a study by Sarkar et al. [15] showed that raloxifene reduced the risk of fracture without a corresponding increase in BMD. Findings from a meta analysis by Cummings et al. [16] also indicated that assessing BMD alone is not adequate and suggested that other parameters, not measured by standard densitometry, are necessary to be evaluated. In terms of bone density, there was significant overlap between the subjects who have experienced osteoporotic-related fractures and those who remained fracture free [17]. These studies [15,16] strongly suggest a need for assessment of other parameters such as bone quality and trabecular microstructural properties to predict future fractures.

Because the trabecular architecture is anisotropic [18], its most accurate evaluation requires 3-dimensional (D) imaging. Although histomorphometry has been in use for a number of years, it provides only limited 2-D information about trabecular structure. Trabecular integrity is compromised as a result of aging and estrogen deficiency [17]. Therefore, the aim of present study was to examine the ability of graded doses of isoflavones in the context of soy protein to improve the 3-D architecture of trabeculae lost due to ovariectomy.

Materials and methods

Animal care, diets and bone density assessment

Seventy-eight 9-month old female Sprague-Dawley rats (Harlan; Indianapolis, IN) were housed in an environmentally controlled laboratory upon arrival and acclimatized for five days. The animals were either ovariectomized (Ovx; 5 groups, N=12-13) or sham-operated (Sham; 1 group N=13) and were fed AIN-93M (Teklad Madison, WI) diet for 3 months. Rats were pair-fed to the average food intake of Sham group and had free access to deionized water. Food intake was recorded every three days and body weights were measured weekly. After bone loss was confirmed using a dual energy x-ray absorptiometry (DXA;QDR-4500A Elite; Hologic, Waltham, MA), rats were assigned to following treatments: Sham, Ovx (control), Ovx + 17β-estradiol (E_2; 10 μg E_2/kg body wt. twice per wk subcutaneously), Ovx + soy protein depleted of isoflavones (Soy-; 0.06 mg isoflavones/g protein), Ovx + soy protein with normal isoflavone content (Soy; 3.55 mg isoflavones/g protein), and Ovx + soy protein enriched isoflavones (Soy+; 7.10 mg isoflavones/g protein). The diets were isonitrogenous and isocaloric experimental powdered diets. Rats in the Sham, Ovx, and E_2 groups were fed

casein-based diet that contained 0.4% calcium, 0.3% phosphorus, and 0.195 nmol/g vitamin D_3 and the rats in the soy groups were fed a similar diet in which casein was replaced with soy protein isolate (22.7 g/100 g diet; The Solae Company, St. Louis, MO). The calcium and phosphorus levels were adjusted to the casein-based diet (Table 1). The calcium and phosphorus contents of proteins were 0.015 and 0.743 g/100 g casein, respectively and 0.342 and 0.826 g/100 g soy protein, respectively. The proximate analyses of the diets confirmed that the diets were similar in macronutrients, calcium and phosphorus contents.

All conditions and handling of animals were approved by the Institutional Animal Care and Use Committee. At the end of a 125-day treatment period, rats were anesthetized with a mixture of ketamine and xylazine (70 mg and 3 mg/kg body weight, respectively) to measure whole body BMD and BMC using DXA and then sacrificed and bone specimens were collected.

The BMD and BMC of the tibiae, and 4th lumbar vertebrae were measured using DXA equipped with appropriate software for bone density assessment in small laboratory animals as reported elsewhere [19].

Microcomputed tomography (μCT) analysis of tibia and 4th lumbar vertebra

The treatment effect on trabecular structure of the right tibial metaphysis and 4th lumbar vertebra were evaluated using μCT 40 scanner (Scanco Medical, Switzerland). All specimens obtained at sacrifice had been frozen at -20°C until the time of scanning. The tibia was scanned from the proximal growth plate in the distal direction (16 μm/slice). This region included 350 images obtained from each tibia using 1024 x 1024

matrix resulting in an isotropic voxel resolution of 22 μm^3 [20]. An integration time of 70 milliseconds per projection was used, with a rotational step of 0.36 degrees resulting in a total acquisition time of 150 minutes/sample. The volume of interest (VOI) was selected as a region twenty five slices away from the growth plate at the proximal end of the tibia to 125 slices. The 3D images were also obtained for visualization and display.

Lumbar vertebra were scanned from the caudal to the dorsal end (530 slices;16μm/slice). This region included 530 images obtained from each vertebra using the same isotropic voxel resolution and integration time as described with the tibia. The VOI selected 25 slices away from the appearance of the growth plate at each end of the vertebral body resulted in approximately 300 slices. Bone morphometric parameters including bone volume over total volume (BV/TV), trabecular number (Tb.N.), separation (Tb.Sp.), thickness (Tb.Th.), connectivity density, and structure model index (SMI) were obtained by analyzing VOI. The operator conducting the scan analysis was blinded to the treatments associated with the specimen.

Statistical analyses

The data analysis involved estimation of means and SEM using the Statistical Analysis System (SAS) version 8.2 (Cary, NC). Analysis of variance (ANOVA) was performed to determine whether there were statistically significant ($P < 0.05$) differences among the groups. When ANOVA indicated any significant differences among the means, Fisher's Least Significant Difference follow-up multiple comparison test was used to determine which means were significantly different ($P < 0.05$).

Results

Body and organ weights

In spite of pair feeding the animals, the final body weights of Ovx controls were significantly higher than the sham animals (Table 2). Estrogen completely prevented the Ovx-induced weight gain as the mean weight of rats in the E_2 group was not different from that of the Sham. While Soy+ had an intermediary effect in preventing the weight gain due to Ovx, Soy and Soy- had no such effect on body weight. As expected, Ovx caused atrophy of uterine tissue, indicating the success of the surgical procedure and administering E_2 significantly increased the uterine weight compared to Ovx controls (Table 2).

Bone mineral content and density

There were no differences in the whole body BMC among any of the treatment groups; however, ovariectomy significantly lowered the whole body BMD (Table 3). Whereas, Soy increased the tibial BMC by 10.3%, bringing it up to the level of sham, Soy-, Soy+ and E_2 had no such an effect. Soy also increased tibial BMD by 4.5%, which was significantly higher than Ovx controls, but was still lower than mean tibial BMD of sham animals. The other treatments were not able to reverse tibial loss as indicated by BMD due to Ovx. Ovariectomized animals also experienced loss of lumbar BMC and BMD. While none of the treatments were able to influence fourth lumbar BMC, E_2 was able to increase the 4^{th} lumbar BMD but not to the level of sham animals. The Soy-, Soy, and Soy+ tended to have a positive effect on 4^{th} lumbar BMD, but the values were not statistically different from Ovx controls.

μCT Analysis

Three-dimensional images of proximal tibia showed differences in trabecular architecture among the various treatment groups as represented in Figs. 1A-1F and Fig. 2. Analysis of data indicated that Ovx decreased proximal tibial and lumbar trabecular BV/TV (Fig. 1A) by 30% when compared to the sham-operated animals. Ovx decreased Tb.N. (Fig. 1B), but increased Tb.Th. (Fig. 1C) and Tb.Sp. (Fig. 1D) in the proximal tibia. In contrast, Tb.Th. in the vertebra was decreased in response to ovariectomy. Tibial and vertebral Tb. N. (1B) were decreased by 25% and 20%, respectively. Tb.Sp. (Fig. 1D) was increased in both bones by 26% compared to the sham animals. Ovx also significantly reduced connectivity density (Fig. 1F) in the tibia and vertebra. Interestingly, SMI, which quantifies the pattern of trabeculae as either more rod- or plate-like was 1.42 in the sham group (Fig. 1E), while in the Ovx animals there was a shift to less favorable rod-like (i.e. SMI=2.4) trabecular morphology. There were no significant differences in the SMI values of the 4th lumbar vertebrae as a result of Ovx.

Neither the soy-based diets nor E_2 were able to restore trabecular bone volume or thickness in these osteopenic rats. E_2 administration reduced the Tb.Th. of the tibia in comparison with all the other groups including sham (Fig. 1C). Soy-, Soy+ and E_2 further reduced Tb.Th. of the vertebra to the levels below the Ovx control animals. Soy+ and E_2 treatments were able to restore Tb.Sp. and Tb.N. to sham levels (Fig. 1 B and D) in the tibia but not in the vertebra. In the lumbar bone, Tb.Sp. was significantly higher in the Soy- and E_2 groups, but not in the Ovx, Soy and Soy+. None of the soy-based diets or E_2 were able to improve the ratio of rods and plates or connectivity density (Fig. 1 E and F) induced by Ovx in both the bones analyzed. The increase in trabecular number and

decrease trabecular seperation indicate some beneficial effects of Soy+ in restoring microarchitectural properties in the tibia. The effects of soy were less pronounced in the lumbar vertebra.

Discussion

In agreement with our previous observations [9,21] the excess body weight gain due to Ovx was completely prevented by E_2 administration as expected [21]. Similar to E_2, Soy+ was able to significantly reduce the Ovx-induced body weight gain by 6%, indicating that isoflavones at certain dosage behave like estrogen at least in terms of influencing body weight. Our earlier findings [8] and those of Blum et al. [22] also support that soy isoflavones are able to prevent excess body weight gain in ovarian hormone deficiency.

Estrogen and isoflavones may be involved directly in energy metabolism by binding to estrogen receptors within the abdominal, subcutaneous, and brown fat pads [23,24]. In humans, it has been shown that soy supplementation results in an increase in hip lean mass indicating a role for soy isoflavones in promoting lean mass and reducing adipose tissue [25]. In the present study, the observed reductions in body weight may be due to changes in energy metabolic pathways as all rats consumed similar amounts of food. Isoflavones may also influence body composition by altering serum levels of leptin, a hormone that regulates energy expenditure as suggested by our earlier animal study [26].

In regards to bone, an earlier study [27] by our laboratory demonstrated that soy protein had a slight reversal effect on femoral bone loss in a young mature (i.e. four month old) osteopenic rat model. The rats in the present study were three times older than that of the

earlier study (twelve- vs. four-month old at the initiation of treatments), hence the response to treatment may differ when compared to younger rats. As Kalu et al. [28] stated, the changes in skeletal characteristics of this age rat (i.e. 12 month-old) when ovariectomized are primarily due to ovarian hormone deficiency, and are uncomplicated by continued rapid bone growth and age-related bone loss due to other factors. Hence, the observations of the present study may be similar to that which occurs in postmenopausal women who have already experienced significant bone loss [29].

In this study, we have determined the effects of soy isoflavones on different bone sites including tibia and lumbar vertebra. Bone loss due to Ovx occurred at all sites with varying degrees. Similar to our earlier observations [27], the findings of this study indicate a modest bone modulating effects of soy and its isoflavones in these older rats. In our earlier observations soy diets were somewhat effective in reversing the femoral but not the fourth lumbar bone-density loss. In that study, we speculated that the bone protective effect of soy was due to its isoflavone content and its ability to increase bone insulin-like growth factor-1 mRNA transcripts which is known to correlate with both bone mineral density and the rate of bone formation [30,31]. In the present study, although none of the treatments were able to reverse whole body BMD, tibial BMD was significantly increased in the Soy group by 4.5% in comparison with Ovx controls. Soy-, Soy+, and E_2 had an intermediary effect, in that tibial BMD was increased, but not to that of the sham level. Therefore, our data suggest that in order to examine the effects of soy or its isoflavones, as therapeutic agents, it may be necessary to assess various bone sites to determine their efficacy in reversing bone loss. However, our findings were in contrast to those of Picherit et al., [32] who reported that isoflavones were not able to reverse bone

loss in osteopenic rats. In that study [32], isoflavones were not fed in the context of soy protein and the treatment period was shorter than the present study (84 vs. 125 days). These differences in the study design and the protein sources may explain the discrepancies in the results. In terms of tibial BMD, our observations coincide with the findings of Anderson et al. [33] who reported that soy isoflavones have a biphasic effect, where too little or too much would not be ideal. In terms of BMC, Soy was able to completely reverse the loss in the tibia. Alekel and colleagues [34] have made similar observations in premenopausal women where soy with normal isoflavone content had a significant effect on percent change in BMC, but not on percent change in BMD. However, in the present study, the effect on 4^{th} lumbar vertebrae was less pronounced. These findings are in contrast to those of human studies [4,34]which have shown soy with isoflavones improves 4^{th} lumbar BMD and BMC more so than at other sites. At this point, we cannot offer a reasonable explanation for our findings in this study.

BMD has been described as only a surrogate measure of bone strength [35] and additional parameters such as trabecular micro-architectural properties are necessary to evaluate the true impact of a treatment on quality of trabecular bone. Although low bone mass is a major risk factor for fracture [36], the preservation of trabecular bone architecture significantly contributes to bone strength and may reduce fracture risk beyond BMD and BMC as demonstrated by a number of studies which have reported close correlations between microstructural properties and biomechanical strength of bone [37-39]. Since trabecular bone is more readily lost due to Ovx in this animal model [40], it is reasonable to assume that the trabeculae would be more responsive to treatment. As shown by other investigators [41,42], μCT evaluation of the trabecular bone in the

metaphyseal region of tibia and the fourth lumbar vertebra indicated that Ovx significantly reduced BV/TV and Tb.N., while increasing Tb.Sp. Restoration of Tb.N. is an important step towards improving bone strength and our results showed that Soy+ and E_2 were able to reverse the detrimental effects of Ovx in the tibia. This increase in Tb.N. may be a reason for seeing a decrease in Tb.Sp. A number of studies [42,43] have reported a decrease in Tb.Th. following Ovx. In contrast to these findings [39,42,43], in the present study Tb.Th. of the tibia was increased in the Ovx animals. We speculate that this discrepancy may be due to the age of animals and the number of days post Ovx. Laib et al. [44] had also shown that Tb.Th. decreased 35 days after Ovx, but as Ovx period continued there was a gradual increase in Tb.Th. Our findings and those of Laib et al. [44] are in agreement with the observations made in osteoporotic women, where the number of trabeculae is reduced while their thickness is increased [45,46]. As it has been suggested [47], this increase in trabecular thickness may be a compensatory mechanism to make up for the lost trabecular connectivity.

SMI of 0 and 3 represent bone that consists purely of plate- or rod-like structures, respectively. Values observed in the sham group represent bone with even distribution of plate like- and rod like- structures [44]. After ovariectomy the trabeculae become more rod-like as demonstrated by the increase in SMI. Although Soy+ in the present study had positive effects on the Tb.N. and Tb.Sp. of tibia, none of the treatments including E_2, were able to restore trabecular bone completely. These findings are in agreement to those of other investigators [44] who were unable to observe restoration of trabecular structure after its deterioration has occurred, emphasizing the need for prevention of trabecular bone loss.

In the present study, we did not observe any beneficial effects of treatments on lumbar microarchitectural properties. Our findings are in agreement with those of Kinney et al., [47] who reported that responses to treatment, e.g. estrogen were weaker in the vertebrae than tibiae. Further studies are needed to evaluate whether higher doses of isoflavones are needed to restore the lumbar microstructural properties.

Based on the results of the present study, soy protein and its isoflavones appear to have a modest beneficial effect in established osteoporosis as evident by improvements in tibial BMC and BMD and certain structural parameters. The bone modulating effects of soy isoflavone may be, in part, due to increased bone formation, decreased bone resorption or both. Several studies suggest [22,48] that soy isoflavones, induce bone formation based on at least three lines of evidence: 1) stimulation of activity, proliferation, and differentiation in cells of osteoblast lineage [49-51]; 2) protection of osteoblasts from apoptosis [49,50]; and 3) enhancement of bone formation rate as assessed by bone histomorphometry [22,48]. Additionally, our recent data clearly indicate that isoflavones in the context of soy protein increases mRNA levels of bone specific alkaline phosphatase, an indicator of osteoblastic activity, and several of bone matrix proteins including type I collagen and osteocalcin in ovx rats [52]. Regarding bone resorption, in vitro studies have also indicated that soy isoflavones suppress osteoclast activity [53-55]. We have also demonstrated the antiresorptive effects of soy supplementation in postmenopausal women not on HRT [56]. However, from the review of existing literature it is too early to state that soy protein or its isoflavones should be supplemented to prevent or reverse bone loss induced by ovarian hormone deficiency.

Acknowledgements. This study was funded by the Oklahoma Center for Advancement of Science and Technology, (grant no HR01070). The soy isoflavones used in this study were generously provided by Archer Daniels Midland Company (Decator, IL) and the soy protein preparations used were generously donated by Protein Technologies International (St. Louis, MO).

Table 1: Diet Composition

Ingredient	Control Diet (g/100g)	Soy Diet (g/100g)
Soy protein (normal isoflavones)[1]		22.70
Casein[2]	22.70	
Corn Starch	20.00	20.00
Sucrose	41.76	41.76
Cellulose[4]	5.60	5.60
Corn Oil	5.70	5.70
Mineral mix, Ca-P deficient (TD 79055)[5]	1.34	1.34
Vitamin mix (TD 40060)[6]	1.0	1.0
Calcium carbonate $CaCO_3$	1.16	1.03
Sodium phosphate monobasic $NaH_2PO_4.H_2O$	0.388	0.388
Potassium Phosphate, monobasic	0.238	0.238
Potassium Citrate, monohydrate	0.090	0.090

[1] Teklad diet #88190 (Harlan Teklad, Madison, WI).

[2] Soy protein isolate obtained from The Solae Company (St. Louis, MO).

[3] Alphacel obtained from ICN Biochemicals (Costa Mesa, CA).

[4] Vitamin mixture (g/kg diet; TD 40060) obtained from Harlan Teklad (Madison, WI): p-aminobenzoic acid, 0.1101; ascorbic acid, 1.0166; biotin, 0.00044; vitamin B-12 (0.1% trituration), 0.0297; calcium pantothenate, 0.0661, choline dihydrogen citrate, 3.4969; folic acid, 0.00198; inositol, 0.1101; menadione, 0.0495; niacin, 0.0991; pyridoxine HCl, 0.0220; riboflavin, 0.0220; thiamin HCl, 0.0220; dry retinyl palmitate, 0.0044; dry d,l-α-tocopheryl acetate, 0.2423; corn starch (diluent), 4.6669.

[5] Mineral mixture (TD 79055) obtained from Harlan Teklad (Madison, WI). This mineral mixture is a modification of AIN76 lacking calcium, phosphorus, and sucrose as diluent).

Table 2

Effects of ovariectomy (Ovx), soy protein devoid of (Soy-), with normal (Soy) and added (Soy+) isoflavones, and 17β-estradiol (E_2) on food consumption, and body and uterus weights

Measure	**Sham**	**Ovx**	**Ovx+Soy-**	**Ovx + Soy**	**Ovx+Soy+**	**Ovx+E_2**	**P-Value**
Food Intake	15.35±0.30	14.99±0.36	15.10±0.38	14.91±0.36	14.98±0.36	14.87±0.36	0.9757
Body Weights (g)							
Initial	312±7	314±8	307±8	312±8	311±8	311±8	0.9920
Final	312±7^{c}	372±8^{a}	359± 8^{ab}	372±7^{a}	350±7^{b}	334±7^{c}	<0.0001
Uterus	0.66±0.03^{a}	0.16±0.03^{c}	0.17±0.03^{c}	0.19±0.03^{c}	0.20±0.03^{c}	0.28±0.03^{b}	<0.0001

Values are means ± SEM, Values that do not share the same superscript letters are significantly (P<0.05) different from each other; n = 12 to 13 rats per group.

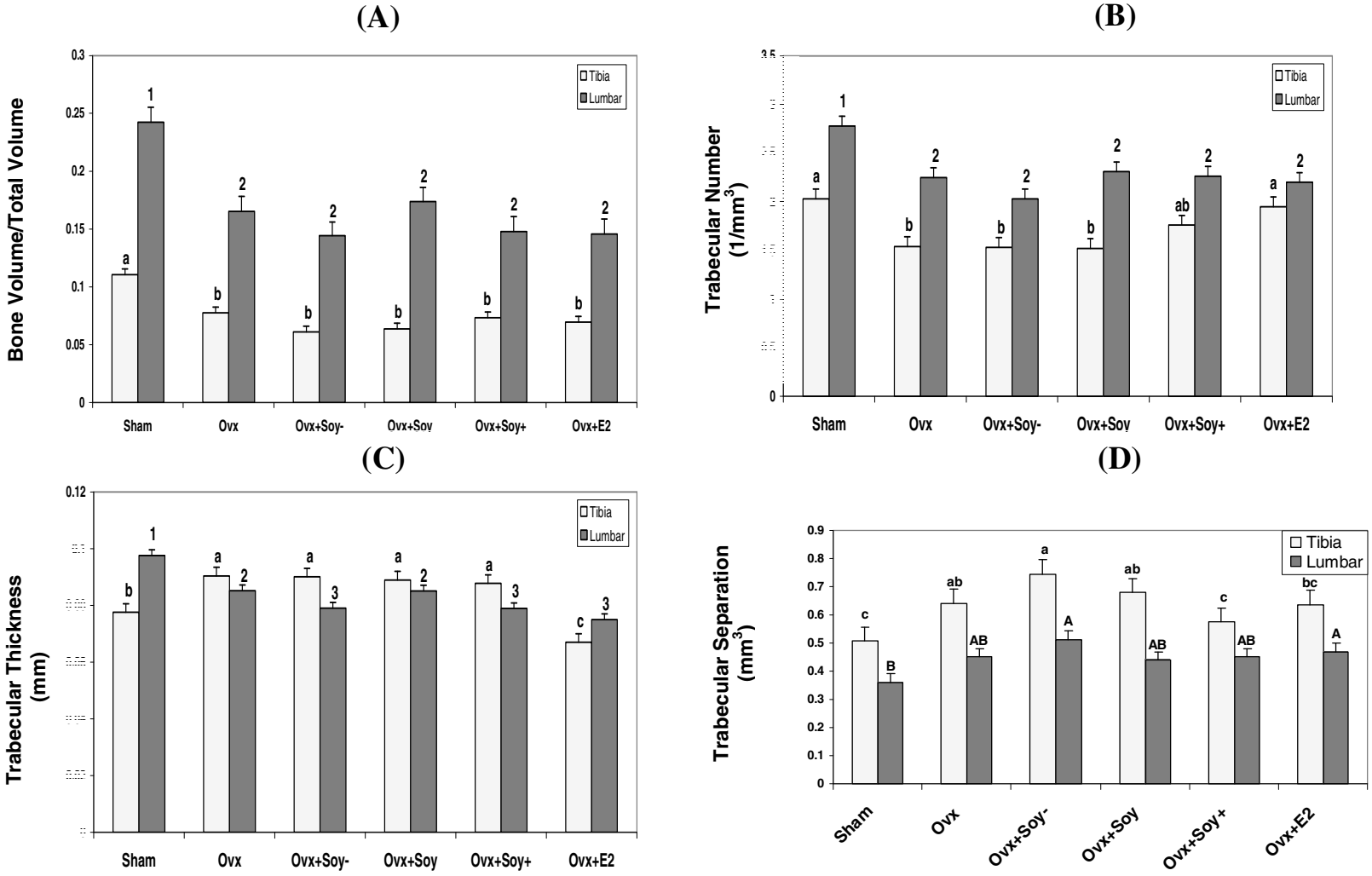
Table 3

Effects of ovariectomy (Ovx), soy protein devoid of (Soy-), with normal (Soy) and added (Soy+) isoflavones, and 17β-estradiol (E_2) on bone mineral content (BMC) and density (BMD)

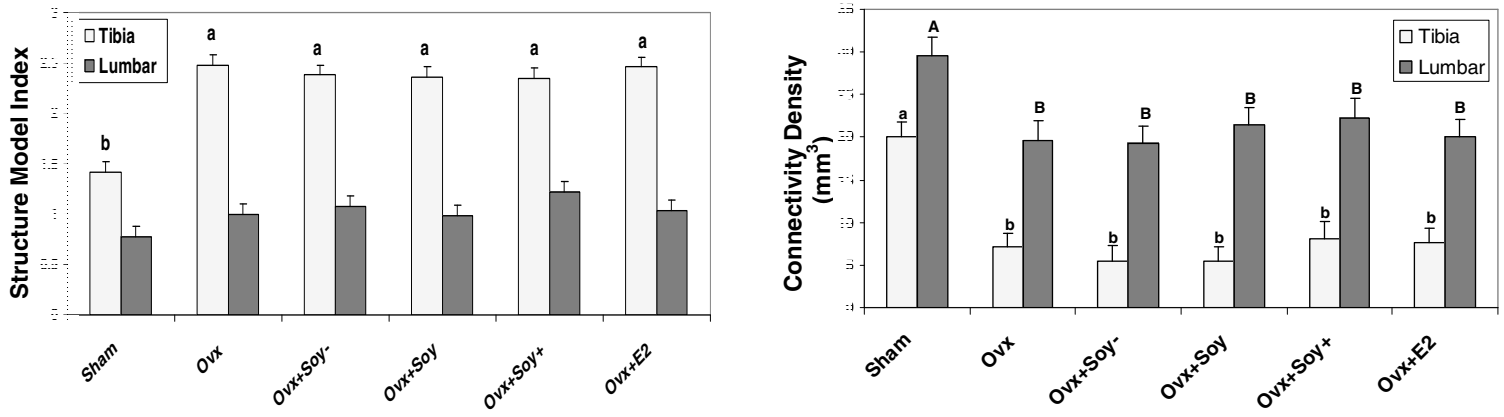
Measure	**Sham**	**Ovx**	**Ovx+Soy-**	**Ovx + Soy**	**Ovx+Soy+**	**Ovx+E_2**	**P-Value**
Whole Body							
Before Treatment							
BMC (g)	10.61±0.21	10.09±0.23	9.99±0.23	10.24±0.23	10.24±0.23	10.16±0.23	0.9612
BMD (g/cm^2)	0.1611±0.002^{a}	0.1569±0.002^{b}	0.1583±0.002^{b}	0.1610±0.002^{b}	0.1592±0.002^{b}	0.1601±0.002^{b}	<0.0001
Final							
BMC (g)	10.90±0.18	10.95±0.19	11.06±0.19	11.25±0.18	10.93±0.18	10.81±0.18	0.6415
BMD (g/cm^2)	0.1662±0.002^{a}	0.1540±0.002^{b}	0.1527±0.002^{b}	0.1568±0.002^{b}	0.1571±0.002^{b}	0.1582±0.002^{b}	0.0002
Tibia							
BMC (g)	0.3583±0.008^{a}	0.3103±0.008^{c}	0.3272±0.008^{bc}	0.3423±0.008^{ab}	0.3296±0.008^{bc}	0.3273±0.008^{bc}	0.0022
BMD (g/cm^2)	0.1993±0.002^{a}	0.1825±0.003^{c}	0.1882±0.003^{bc}	0.1908±0.003^{b}	0.1870±0.003^{bc}	0.1892±0.003^{bc}	0.0006
4th Lumbar Vertebra							
BMC (g)	0.1279±0.003^{a}	0.1071±0.003^{b}	0.1123±0.003^{b}	0.1135±0.003^{b}	0.1116±0.003^{b}	0.1163±0.003^{b}	0.0016
BMD (g/cm^2)	0.2264±0.003^{a}	0.1986±0.003^{c}	0.2040±0.003^{bc}	0.2041±0.003^{bc}	0.2042±0.003^{bc}	0.2091±0.003^{b}	<0.0001

Values are means ± SEM Values that do not share the same superscript letters are significantly (P<0.05) different from each other; n = 12 to 13 rats per group.

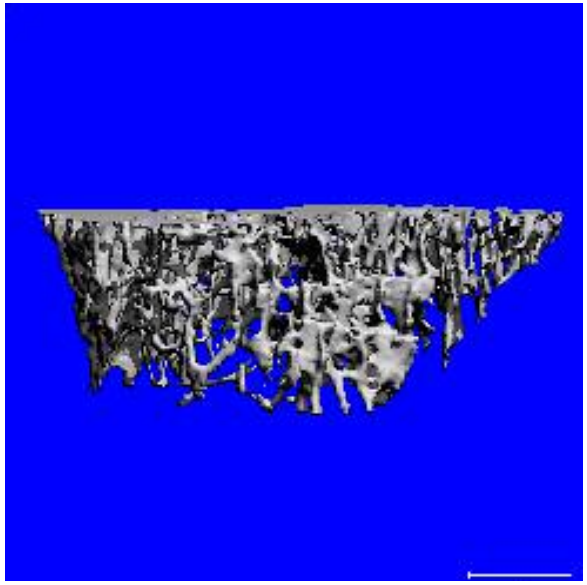
Fig. 1
Effects of ovariectomy (Ovx), soy protein devoid of (Soy-), with normal (Soy) and added (Soy+) isoflavones, and 17β-estradiol (E_2) on tibial μCT parameters



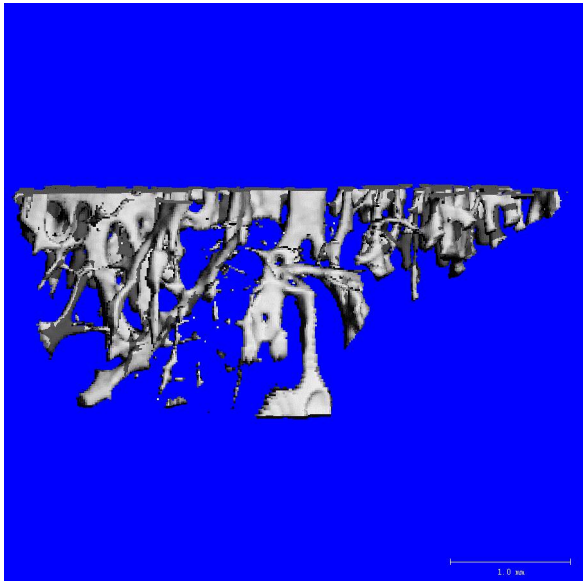
(E) **(F)**



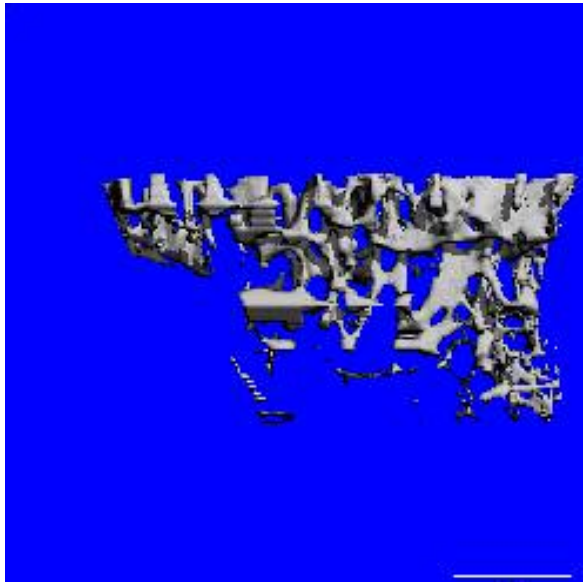
Values are means ± SEM, Bars that do not share the same superscript letters are significantly (P<0.05) different from each other; n = 12 to 13 rats per group



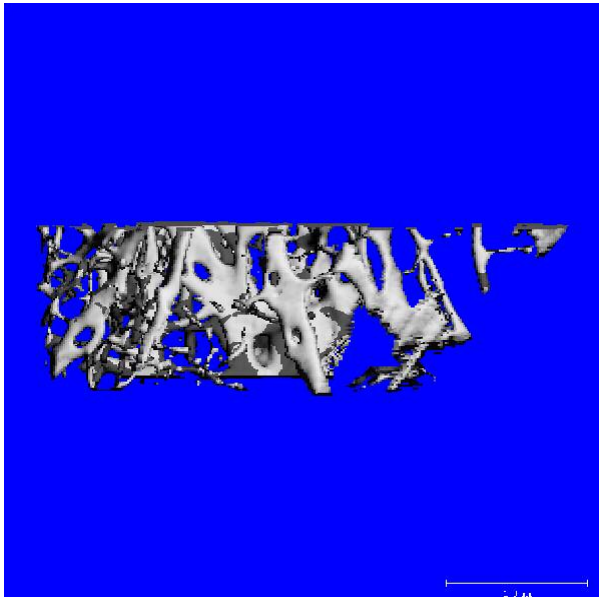
A



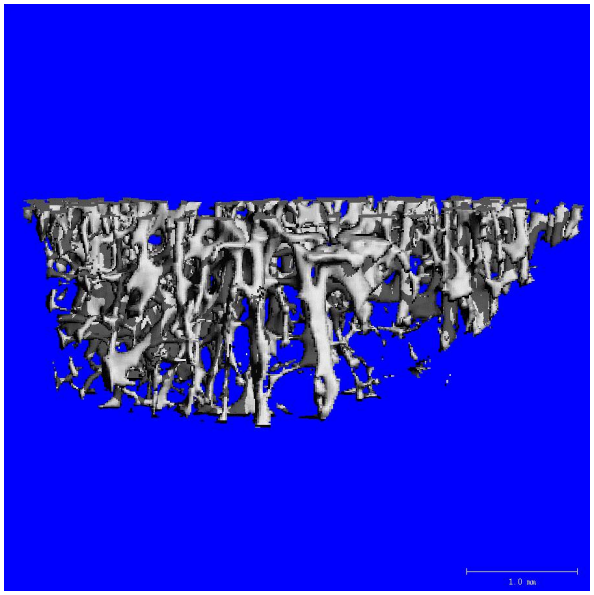
B

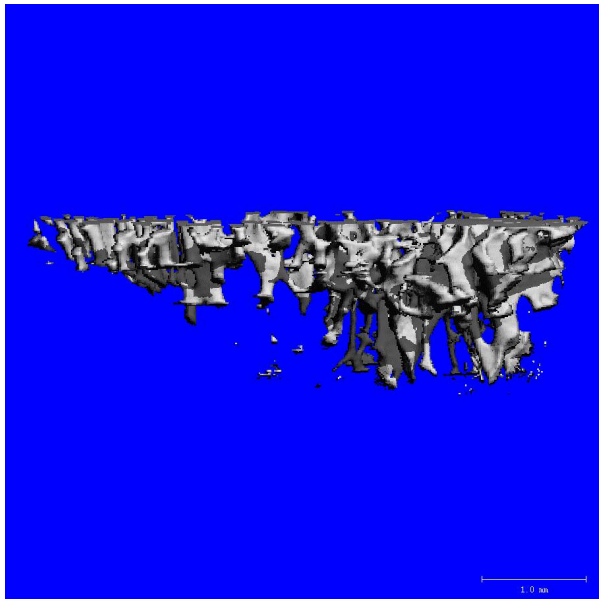


C



D





E **F**

Fig. 2. 3-D trabecular images of proximal tibia using micro-CT. (A) Sham, (B) Ovx control, (C) Soy- , (D) Soy, (E) Soy+, and (F) E_2 (10 μg 17β-estradiol/kg body wt. twice per wk.). Soy-, Soy, and Soy+ contained 0.06, 3.55, and 7.10 mg isoflavones/g protein, respectively. In accord with our data, Soy+ had the greatest effect on reversal of trabecular bone structure.

References

[1] Cosman F and Lindsay R. Selective estrogen receptor modulators: clinical spectrum. End Rev 1999;20:418-434.

[2] Setchell KDR. Soy isoflavones--benefits and risks from nature's selective estrogen receptor modulator (SERMs). J Am Coll Nutr 2001;20:354S-362S.

[3] Brezinski A and Debi A. Phytoestrogens: the "natural" selective estrogen receptor modulators. Eur J Ob & Gyn & Rep Bio 1999;85:47-51.

[4] Potter SM, Baum JA, Teng H, Stillman RJ, Shay NF, Erdman JWJ. Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. Am J Clin Nutr 1998;68 Supp:1375S-1379S.

[5] Dalais FS, Rice GE, Wahlqvist ML, Grehan M, Murkies AL, Medley G, Ayton R, Strauss BJG. Effects of dietary phytoestrogens in postemenopausal women. Climacteric 1998;1:124-129.

[6] Ho SC, Chan SG, Yi Q, Wong E, Leung PC. Soy intake and the maintenance of peak bone mass in Hong Kong Chinese women. J Bone Miner Res 2001;16:1363-1369.

[7] Gallagher JC, Rafferty K, Haynatzka V, Wilson M. Effects of soy protein on bone metabolism. J Nutr 2000;130:667S-

[8] Arjmandi BH, Alekel L, Hollis BW, Amin D, Stacewica-Sapuntzakis M, Gou P. Dietary soy protein prevents bone loss in an ovariectomized rat model of osteoporosis. J Nutr 1996;126:161-167.

[9] Arjmandi BH, Birnbaum R, Goyal NV, Getlinger MJ, Juma S, Alekel L, Hasler CM, Hollis BW, Drum ML, Kukreja SC. Bone-sparing effect of soy protein in ovarian hormone deficient rats is related to its isoflavone content. Am J Clin Nutr 1998;68:1364S-1368S.

[10] Picherit C, Chanteranne B, Bennetau-Pelissero C, Davicco MJ, Lebecque P, Barlet JP, Coxam V. Dose-dependent bone-sparing effects of dietary isoflavones in the ovariectomized rat. Br J Nutr 2001;85:307-316.

[11] Anderson JJ, Ambrose WW, Garner SC. Biphasic effects of genistein on bone tissue in the ovariectomized, lactating rat model. Proc Soc Exp Biol Med 1998;217:345-350.

[12] Kanis JA. Estrogens, the menopause, and osteoporosis. Bone 1996;19:185S-190S.

[13] Judd HL, Meldrum DR, Deftos LJ, Henderson BE. Estrogen replacement therapy: indications and complications. Ann Int Med 1983;98:195-205.

[14] Pattijn V, Van Cleynenbreugel T, Vander SJ, Van Audekercke R, Van der PG, Wevers M. Structural and radiological parameters for the nondestructive characterization of trabecular bone. Ann Biomed Eng 2001;29:1064-1073.

[15] Sarkar S, Reginster JY, Crans GG, Diez-Perez A, Pinette KV, Delmas PD. Relationship between changes in biochemical markers of bone turnover and BMD to predict vertebral fracture risk. J Bone Miner Res 2004;19:394-401.

[16] Cummings SR, Bates D, Black D. Clinical use of bone densitometry: scientific review. JAMA 2002;288:1889-1897.

[17] Kinney JH, Lane NE, Haupt DL. In vivo, three-dimensional microscopy of trabecular bone. J Bone Miner Res 1995;10:264-270.

[18] Mawatari T, Miura H, Higaki H, Kurata K, Moro-oka T, Murakami T, Iwamoto Y. Quantitative analysis of three-dimensional complexity and connectivity changes in trabecular microarchitecture in relation to aging, menopause, and inflammation. J Orthop Sci 1999;4:431-438.

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

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

[21] Arjmandi BH, Khan DA, Juma SS, Svanborg AS. The ovarian hormone deficiency-induced hypercholesterolemia is reversed by soy protein and the synthetic isoflavone, ipriflavone. Nutr Res 1997;17:885-894.

[22] Blum SC, Heaton SN, Bowman bM, Hegsted M, Miller SC. Dietary soy protein maintains some indices of bone mineral density and bone formation in aged ovariectomized rats. J Nutr 2003;133:1244-1249.

[23] Tchernof A and Poehlman ET. Effects of the menopause transition on body fatness and body fat distribution. Obes Res 1998;6:246-254.

[24] Joyner JM, Hutley LJ, Cameron DP. Estrogen receptors in human preadipocytes. Endocrine 2001;15:225-230.

[25] de la Brousse FC, Shan B, Chen JL. Identification of the promoter of the mouse obese gene. Proc Natl Acad Sci USA 1996;93:4096-4101.

[26] Sohn E, Deyhim F, Devareddy L, Arjmandi BH. Soy isoflavones attenuate estrogen deficiency-induced increases in abdominal fat in the hamster. Nutr Res 2004;24:1023-1029.

[27] Arjmandi BH, Getlinger MJ, Goyal NV, Alekel L, Hasler CM, Juma S, Drum ML, Kukreja S. The role of soy protein with normal or reduced isoflavone content in reversing bone loss induced by ovarian hormone deficiency in rats. Am J Clin Nutr 1998;68:1358S-1363S.

[28] Kalu DN, Liu CC, Hardin RR, Hollis BW. The aged rat model of ovarian hormone deficiency bone loss. Endocrinology 1989;126:7-16.

[29] Kalu DN. The ovariectomized rat model of postmenopausal bone loss. Bone and Min 1991;15:175-192.

[30] Barengolts EI, Kouznetsova T, Segalene A, Lathon P, Odvina C, Kukreja SC, Unterman TG. Effects of progesterone on serum levels of IGF-1 and on femur IGF-1 mRNA in ovariectomized rats. J Bone Miner Res 1996;11:1406-1412.

[31] Kalu DN, Arjmandi BH, Liu C, Salih MA, Birnbaum RS. Effects of ovariectomy and estrogen on the serum levels of insulin-like growth factor-I and insulin-like growth factor binding protein-3. Bone Miner 1994;25:135-148.

[32] Picherit C, Bennetau-Pelissero C, Chanteranne B, Labecque P, Davicco MJ, Barlet JP, Coxam V. Soybean isoflavones dose-dependently reduce bone turnover but do not reverse established osteopenia in adult ovariectomized rats. J Nutr 2001;131:723-728.

[33] Anderson JJB and Garner SC. The effects of phytoestrogens on bone. Nutr Res 1997;17:1617-1632.

[34] Alekel DL, St Germain A, Peterson CT, Hanson KB, Stewart JW, Toda T. Isoflavone-rich soy protein isolate attenuates bone loss in the lumbar spine of perimenopausal women. Am J Clin Nutr 2000;72:679-680.

[35] Bouxsein ML. Mechanisms of osteoporosis therapy: a bone strength perspective. Clin Cornerstone 2003;Suppl 2:S13-S21.

[36] Borah B, Dufresne TE, Chmielewski PA, Gross GJ, Prenger MC, Phipps RJ. Risedronate preserves trabecular architecture and increases bone strength in vertebra of ovariectomized minipigs as measured by three-dimensional microcomputed tomography. J Bone Miner Res 2002;17:1139-1147.

[37] Ulrich D, van Rietbergen B, Laib A, Ruegsegger P. The ability of three-dimensional structural indices to reflect mechanical aspects of trabecular bone. Bone 1999;25:55-60.

[38] Muller R, Hannan M, Smith SY, Bauss F. Intermittent ibandronate preserves bone quality and bone strength in the lumbar spine after 16 months of treatment in the ovariectomized cynomolgus monkey. J Bone Miner Res 2004;19:1787-1796.

[39] Lane NE, Kumer JL, Majumdar S, Khan M, Lotz J, Stevens RE, Klein R, Phelps KV. The effects of synthetic conjugated estrogens, a (cenestin) on trabecular bone structure and strength in the ovariectomized rat model. Osteoporos Int 2002;13:816-823.

[40] Wronski TJ, Dann LM, Scott KS, Cintrón M. Long-term effects of ovariectomy and aging on the rat skeleton. Calcif Tissue Int 1989;45:360-366.

[41] Alexander JM, Bab I, Fish S, Muller R, Uchiyama T, Gronowica G, Nahounou M, Zhao Q, white DW, Chorev M, Gazit D, Rosenblatt M. Human arathyroid hormone 1-34 reverses bone loss in ovariectomized mice. J Bone Miner Res 2001;16:1665-1673.

[42] Lark MW, Stroup GB, James IE, Dodds RA, Hwang SM, Blake SM, Lechowska BA, Hoffman SJ, Smith BR, Kapadia R, Liang X, Erhard K, Ru Y, Dong X, Marquis RW, Veber D, Gowen M. A potent small molecule, nonpeptide inhibitor of Cathepsin K (SB 331750) prevents bone matrix resorption in the ovariectomized rat. Bone 2002;30:746-753.

[43] Lark MW, Stroup GB, Dodds RA, Kapadia R, Hoffman SJ, Hwang SM, James IE, Lechowska B, Liang X, Rieman DJ, Salyers KL, Ward K, Smith BR, Miller WH, Huffman WF, Gowen M. Antagonism of the osteoclast vitronectin receptor with an orally active nonpeptide inhibitor prevents cancellous bone loss in the ovariectomized rat. J Bone Miner Res 2001;16:319-327.

[44] Laib A, Kumer JL, Majumdar S, Lane NE. The temporal changes of trabecular architecture in ovariectomized rats assessed by MicroCT. Osteoporos Int 2001;12:936-941.

[45] Seeman E. Reduced bone formation and increased bone resorption: rational targets for the treatment of osteoporosis. Osteoporos Int 2003;14 Suppl 3:S2-S8.

[46] Barger-Lux MJ and Recker RR. Bone microstructure in osteoporosis: transilial biopsy and histomorphometry. Top Magn Reson Imaging 2002;13:297-305.

[47] Kinney JH, Haupt DL, Balooch M, Ladd AJC, Ryaby JT, Lane NE. Three-dimensional morphomoetry of the L6 vertebra in the ovariectomized rat model of osteoporosis: biomechanical implications. J Bone Miner Res 2000;15:1981-1991.

[48] Fanti O, Monier-Faugere MC, Geng Z, Schmidt J, Morris PE, Cohen D, Malluche HH. The phytoestrogen genistein reduces bone loss in short-term overiectomized rats. Osteoporos Int 1998;8:274-281.

[49] Choi EM, Suh KS, Kim YS, Choue RW, Koo SJ. Soybean ethanol extract increases the function of osteoblastic MC3T3-E1 cells. Phytochemistry 2001;56:733-739.

[50] Lee YS, Chen X, Anderson JJB. Physiological concentrations of genistein stimulate the proliferation and protect against free radical-induced oxidative damage of MC3T3-E1 osteoblast-like cells. Nutr Res 2001;21:1287-1298.

[51] Sugimoto T, Nishiyama K, Kuribayashi F, Chihara K. Serum levels of insulin-like growth factor (IGF) 1, IGF-binding protein (IGFBP) -2, and IGFBP-3 in osteoporotic patients with and without spinal fractures. J Bone Miner Res 1997;12:1272-1279.

[52] Devareddy L, Soung D, Khalil DA, Lucas EA, Smith BJ, Arquitt AB, Arjmandi B.H. Soy isoflavones may increase bone formation as seen by enhanced osteoblast specific gene products in ovariectomized rat model of postmenopausal osteoporosis. J Bone Min Res 2004;S218:

[53] Gao YH and Yamaguchi M. Suppressive effect of genistein on rat bone osteoclasts: involvement of protein kinase inhibition and protein tyrosine phosphatase activation. Int J Mol Med 2000;5:261-267.

[54] Gao YH and Yamaguchi M. Suppressive effect of genistein on rat bone osteoclasts: apoptosis is induced through Ca2+ signaling. Biol Pharm Bull 1999;22:805-809.

[55] Amano H, Yamada S, Felix R. Colony-stimulating factor-1 stimulates the fusion process in osteoclasts. J Bone Miner Res 1998;13:846-853.

[56] Arjmandi B.H., Khalil DA, Smith BJ, Lucas EA, Juma S, Payton ME, Wild RA. Soy protein has a greater effect on bone in postmenopausal women not on hormone replacement therapy, as evidenced by reducing bone resorption and urinary calcium excretion. J Clin Endocrinol Metab 2003;88: 1048-1054.

CHAPTER IV

Fructooligosaccharides enhance the efficacy of soy protein to rebuild bone in ovariectomized rats

Latha Devareddy et al.

[a]Department of Nutritional Sciences and [b]Food & Agricultural Products Research & Technology Center, Oklahoma State University, Stillwater, OK 74078

Corresponding author and reprint requests should be directed to: Bahram H. Arjmandi, PhD, RD, Department of Nutritional Sciences, 301 Human Environmental Sciences, Stillwater, OK 74078-6141; (405) 744-4437; FAX (405) 744-1357; E-mail: arjmand@okstate.edu

Keywords: Biomechanical properties; Microcomputed tomography; Isoflavones; Ovariectomy

Abstract

Objective: The intestinal microflora is important in rendering soy isoflavones bioavailable by facilitating their conversion to equol. Hence, substances that can modulate the intestinal microflora could affect the bioavailability of isoflavones. In this study, we examined the effects of fructooligosaccharides (FOS), a prebiotic, on enhancing the effects of soy isoflavones on bone in ovariectomized osteopenic female rats.

Design: Sixty three 9-month old female Sprague-Dawley rats were either sham-operated (Sham; 1 group) or ovariectomized (Ovx; 4 groups) and were fed control diet for three months to induce bone loss. After bone loss was confirmed via dual energy x-ray absorptiometry, rats were placed on dietary treatment for 125 days. The Sham and one Ovx group received a control diet while the remaining Ovx groups received either a soy protein-based diet (Soy), a FOS supplemented diet (FOS), or a soy protein-based and FOS supplemented diet (Soy+FOS). Prior to the termination of the study, whole body bone mineral density (BMD) and bone mineral content (BMC) were assessed under anesthesia. Immediately after euthanasia, bone specimens were collected for the assessments of BMD, BMC, biomechanical and microarchitectural properties.

Results: Whole body BMD values were significantly higher in FOS and Soy+FOS groups in comparison with Ovx controls. The tibial BMC increased by 10, 6, and 4% in Soy, FOS and Soy+FOS groups, respectively when compared to Ovx control group. FOS and FOS+Soy treatments had the most pronounced effects in enhancing lumbar BMC and BMD. The BMC and BMD were increased in the animals fed FOS diet by 12 and 5.7%, respectively when compared to Ovx control groups. The combination of FOS and Soy

effectively improved tibial microarchitectural properties by increasing trabecular number and decreasing trabecular separation compared to control diet. The effects of dietary treatments on lumbar microarchitectural properties were minimal and biomechanical properties of the femur were not affected by any of the dietary treatments.

Conclusion: Overall, our findings suggest that the positive bone modulating effects of soy can be enhanced in the presence of FOS as seen from the improved BMD and microarchitectural properties. The microarchitectural properties of the tibia were improved by Soy+FOS treatment as the trabecular number and separation were bought back to the level of Sham group.

Introduction

Osteoporosis is a debilitating disease that affects millions of Americans. It is estimated that about one in every two women over the age of 50 years will have an osteoporotic fracture in her lifetime.[1] Although the US Food and Drug Administration (FDA) has approved a number of therapies for the prevention of osteoporosis, there has not been a substantial reduction in the incidence of fractures.[2] This may be attributed to many factors including low compliance, undesirable side effects or high cost of the treatment options.[2] Recently, there has been an increased interest in the use of alternative and complementary medicine for the prevention or treatment of chronic diseases. Therefore, an effective alternative/complementary option that can reverse postmenopausal bone loss with no or minimal side effects is desirable.

Numerous studies have investigated the osteoprotective role of soy and/or its isoflavones in both animals[3-5] and humans.[6-8] Previously, we have showed that soy protein prevents loss of bone due to ovariectomy[4] and modestly reverses bone loss in osteopenic female rats.[9] In contrast to this study[9], Picherit et al., [10] indicated that although soy isoflavones prevent bone loss, they do not reverse bone loss in Ovx rats. Therefore, the findings related to the efficacy of soy and its isoflavones are inconclusive. This may be, in part, attributed to the activity of gastrointestinal microorganisms as they influence the bioavailability of soy isoflavones.[11] An "optimal" gut microflora produces a range of metabolites that enhance the bioavilability and absorption of certain nutrients such as isoflavones, and other phytochemicals. Although the role of different species of bacteria that are responsible for these beneficial activities is not fully understood, bifidobacteria and lactobacilli are commonly thought to be the beneficial gut microflora which influence

bioavailabilty of nutrients and phytochemicals.[12] In this regard, there are a number of dietary supplements that can potentially enhance gut microflora including prebiotic substances such as fructooligosaccharides (FOS). FOS is a class of indigestible and soluble fiber that positively modulates intestinal microflora. For instance, Ohta et al.,[13] reported that FOS increases calcium and magnesium absorption in the colon of FOS fed rats. FOS has also been shown to augment the effects of soy isoflavones in preventing bone loss in ovariectomized rats[14] and mice.[15] However, to our knowledge the efficacy of combining soy and FOS in the reversal of bone loss has not been investigated. The objective of the present study was to determine the extent to which soy, FOS and their combination reverses ovarian hormone deficiency-induced bone loss. Additionally, the effects of these dietary treatments in enhancing biomechanical and microarchitectural properties of bone were assessed using three point bending test and microcomputed tomography, respectively.

Materials and methods

Animal care, diets and bone density assessment

Sixty five 9-month old female Sprague-Dawley rats (Harlan; Indianapolis, IN) were housed in an environmentally controlled laboratory upon arrival and acclimatized for five days. The animals were either ovariectomized (Ovx; 4 groups, N=13) or sham-operated (Sham; 1 group N=13) and fed AIN-93M (Harlan Teklad; Madison, WI) diet for 3 months. Rats were pair-fed to the average food intake of Sham group and had free access to deionized water. Food intake was recorded every three days and body weights

were measured weekly. The composition of the different dietary treatments is presented in **Table 1**.

Three months from the time of surgery, bone loss was confirmed via dual energy x-ray absorptiometry (DXA; QDR-4500A Elite; Hologic, Waltham, MA) and rats in the Sham and one Ovx group continued to receive control diet. The remaining Ovx groups were assigned to the following treatments: 1) soy protein-based diet (Soy), 2) casein-based diet plus 5% FOS (FOS) and 3) soy protein-based diet plus 5% w/w FOS (Soy+FOS). The proximate analyses of the diets confirmed that they were similar in macronutrients, calcium and phosphorus contents.

All conditions and handling of animals were approved by the Institutional Animal Care and Use Committee. At the end of a 125-day treatment period, rats were anesthetized with a mixture of ketamine and xylazine (70 mg and 3 mg/kg body weight, respectively) to measure whole body BMD and BMC using DXA. Immediately after euthanasia, bone specimens were collected. The BMD and BMC of the tibiae, and 4th lumbar vertebrae were measured using DXA equipped with appropriate software for bone density assessment in small laboratory animals as reported elsewhere.[16]

Microcomputed tomography (μCT) analysis of tibia and 4th lumbar vertebra

The treatment effect on trabecular structure of the right tibial metaphysis and 4th lumbar vertebra were evaluated using μCT 40 scanner (Scanco Medical, Switzerland). All specimens obtained at sacrifice had been frozen at -20°C until the time of scanning. The tibia was scanned from the proximal growth plate in the distal direction (16 μm/slice). This region included 350 images obtained from each tibia using 1024 x 1024

matrix resulting in an isotropic voxel resolution of 22 μm^3.[17] An integration time of 70 milliseconds per projection was used, with a rotational step of 0.36 degrees resulting in a total acquisition time of 150 minutes/sample. The volume of interest (VOI) was selected as a region twenty five slices away from the growth plate at the proximal end of the tibia to 125 slices. The 3D images were also obtained for visualization and display.

Lumbar vertebra were scanned from the caudal to the dorsal end (530 slices;16μm/slice). This region included 530 images obtained from each vertebra using the same isotropic voxel resolution and integration time as described with the tibia. The VOI selected 25 slices away from the appearance of the growth plate at each end of the vertebral body resulted in approximately 300 slices. Bone morphometric parameters including bone volume over total volume (BV/TV), trabecular number (Tb.N.), separation (Tb.Sp.), thickness (Tb.Th.), connectivity density, and structure model index (SMI) were obtained by analyzing VOI. The operator conducting the scan analysis was blinded to the treatments associated with the specimen.

Biomechanical testing of femur

The biomechanical properties of femoral bones were assessed using a three-point bending test[18] utilizing an Instron Universal Testing Machine (Model 5543; Instron Corp; Canton, MA). Each femur was tested in a three-point bending apparatus (TA.XT2i, Stable Microsystems, Inc.). The femur was placed in a three-point bending fixture such that the posterior surface rests on the lower supports and the upper support touches the anterior surface and the test was performed at a displacement rate of 3 mm/minute.

Throughout the test, the anterior surface was under compression and the posterior surface is under tension. The load-displacement curve was recorded simultaneously during the test and the ultimate load, yield load and stiffness of the specimen were determined. After the mechanical test, the cross-sectional surface at the fracture site was prepared flat for tracing and subsequent second moment of area and area calculations. The ultimate, yield stresses, and modulus of elasticity of the specimen were calculated using beam bending theory. [19;20]

Statistical analyses

The data analysis involved estimation of means and SEM using the Statistical Analysis System (SAS) version 8.2 (Cary, NC). Analysis of variance (ANOVA) was performed to determine whether there were statistically significant ($P < 0.05$) differences among the groups. When ANOVA indicated any significant differences among the means, Fisher's Least Significant Difference follow-up multiple comparison test was used to determine which means were significantly different ($P < 0.05$).

Results

Body and organ weights

Although the food intake was similar among all treatment groups and all of the treatment groups started with similar mean body weights (**Table 1**), at the end of the study, the final body weights of rats in the Ovx groups were significantly higher than those in the Sham group (**Table 2**). However, the mean final body weights of animals in FOS and Soy+FOS groups were lower than the Ovx controls by 2.7% and 7%,

respectively, though difference between Ovx and FOS groups did not reach statistical significance. These lower body weight gains in these two treatment groups, in part, can be explained by reduced body fat (**Table 2**). As expected, the mean uterine weight of each Ovx group was significantly lower than that of the Sham group (Table 1), indicating the success of ovariectomy with neither Soy nor FOS diets imparting estrogen-like effects on the uterus.

Bone mineral content and density

As expected, Ovx control rats had lower whole body BMD than intact rats at the end of the three-month treatment period (**Table 3**).While mean whole body BMD of Soy group was somewhat higher than that of Ovx control group, FOS and Soy+FOS treated animals had significantly higher whole body BMD values. No effects on whole body BMC were observed with any of the treatments.

Tibial BMC was increased in rats in the Soy group by 10.3% to levels similar to those of Sham animals. FOS and Soy+FOS were not able to reverse the loss of tibial BMC. Soy, FOS and Soy+FOS increased the tibial BMD by 4.5, 3.7 and 3.4%, respectively in comparison with Ovx controls, though the values were lower than those of rats in the Sham group. Although, all the dietary treatments were able to improve tibial BMD, soy treatment had the most pronounced effect on this parameter.

Ovariectomy resulted in significant losses of lumbar BMC and BMD and FOS alone was able to ameliorate these losses. FOS increased lumbar BMC and BMD by 12 and 5.7%, respectively in comparison with Ovx controls, however, only lumbar BMC was brought back to Sham level. In contrast to tibial BMC, Soy and Soy+FOS had lesser

effects on lumbar BMC. While soy alone had a minimal effect on lumbar BMD, FOS and Soy+FOS were able to significantly increase lumbar BMD (Table 3).

Biomechanical properties of the femur

Data on biomechanical properties of femur are presented in **Table 4**. Ovariectomy did not alter ultimate and yield load, yield stress, or modulus of elasticity. The mean femoral stiffness was reduced by 12.6% in untreated Ovx rats when compared to sham group. Soy, FOS, and Soy+FOS treatments were all able to increase the stiffness by varying degrees with soy treatment reaching a statistical significance. Ultimate stress was increased as a result of Ovx , and all of the dietary interventions (FOS, Soy, and Soy+FOS) brought this parameter to the Sham level (Table 4).

μCT Analysis

Figures 1A through 1F show the effects of ovariectomy and dietary treatments on microstructural properties of the proximal tibia and 4^{th} lumbar vertebra. As expected, ovariectomy decreased BV/TV, connectivity density, and Tb.N and increased SMI and Tb.Sp in both tibia and the vertebra. Interestingly, trabecular thickness was increased in tibia but not in the vertebra due to ovariectomy. Although none of the treatments could reverse the alterations in BV/TV (Fig. 1A) and connectivity density (Fig. 1B), the mean vertebral SMI value in Soy+FOS group was not different from either Sham or Ovx control group (Fig. 1C). This indicates that Soy+FOS favorably changed trabecular morphology from rod- to plate-like structure in the vertebra, however, this change was not observed in the tibia. Trabecular number (Fig. 1D) was higher in the Soy+FOS group by 17.5 and 10.4% in the tibia and vertebra, respectively in comparison to Ovx controls.

FOS also caused an increase in Tb.N., but only lumbar Tb.N. was up to the level of Sham. Tibial Tb.Th (Fig. 1E) was increased in the Ovx animals, perhaps to compensate for the deterioration of the trabecular microarchitecture, as observed earlier.[21] The average Tb.Th in Soy+FOS was neither different from that of Sham nor from Ovx control group. Dietary treatment had no effect on lumbar Tb.Th (Fig. 1E). Tibial Tb.Sp. (Fig. 1 F) was reduced by FOS and Soy+FOS treatments with Soy+FOS having a more pronounced effect than FOS alone, as the mean value for Soy+FOS group did not differ from Sham. There were no differences in Tb.Sp of the vertebra among the groups. Considering all the μCT parameters, the overall results suggest that soy protein in combination with FOS may be more efficacious in reversing the loss of trabeculae in ovarian hormone deficiency.

Discussion

The higher final mean body weights of the Ovx animals, except for the Soy+FOS group, were significantly greater than those of Sham animals. This increase in body weight is due, in part, to higher body fat. Indeed, the average total body fat of animals in Ovx control group was 85% higher than those of Sham animals as determined by DXA. In the present study, although Soy or FOS treatment alone were unable to reduce the Ovx-induced gains in body weight and fat mass, their combination effectively prevented excess body weight and fat mass gains. Our observations are in accord with those of Mathey and colleagues[14] who reported that FOS enhanced the effects of soy isoflavones in preventing body weight gain due to Ovx. Additionally, we postulate that the observed effects of FOS on body weight are similar to those observed with other dietary fibers

(soluble and insoluble). For instance, a population study by Lissner et al.,[22] reported that fiber intake was inversely related to obesity rate. Dietary fiber also plays a role in weight regulation, by decreasing the rates of gastric emptying and fat absorption.[23] In addition to the effects of fiber, the intake of soy isoflavones has been shown to attenuate abdominal fat gain in Ovx rats[24] and postmenopausal women.[25] Therefore, it is conceivable that the combination of Soy and FOS will have synergistic effects in lowering body weight and fat gain as is observed in this study. Accordingly, in the present study Soy plus 5% FOS in the diet reversed the Ovx-induced gain in both body weight and adipose tissue. Future clinical studies are needed to confirm whether the combination of FOS and isoflavones would have a beneficial effect in controlling obesity.

In terms of bone, soy treatment alone significantly increased tibial BMD but had no effect on whole body or lumbar BMD. We have previously reported[9] the site specific action of soy on bone which may be similar to other agents known to augment BMD such as estrogen.[26] Kinney et al.,[26] demonstrated that responses to estrogen were weaker in the vertebrae than tibiae. Site specificity of soy isoflavones have also been reported in recent studies using rodent models. [14;15] A study by Mathey et al.[14] reported that a dose of 40 µg isoflavones/g body weight was able to significantly increase diaphyseal BMD of the tibia, but not the metaphyseal BMD, thereby prevent bone loss due to ovariectomy. In the same study, they observed a synergy between isoflavones and FOS. This synergy was credited to FOS's ability to maximize mineral absorption which have been reported by several investigators.[13;27;28]

Although all of the treatments resulted in somewhat improved femoral stiffness, only the Soy group had significantly higher mean stiffness than Ovx control group. This

finding also agrees with that of Mathey and colleagues[14] who reported isoflavone treatment enhanced femoral ultimate load stress?, a structural parameter, without improving material properties such as yield and modulus of elasticity. FOS did not have any synergistic or additive effects on any of the biomechanical properties. We cannot offer an explanation for this observation except speculating that when FOS is given along with isolated isoflavones bone mineralization is affected; however, bone strength is not only dependent on mineral but also on other factors such as bone architecture and matrix protein content.[29-31] Our observations indicate that soy when combined with FOS was effective in reversing the loss of certain trabecular structural parameters in tibia namely trabecular number, thickness and separation. Additionally, the combination of soy and FOS was able to change the rod-like trabeculae to a more favorable plate-like structure as observed from SMI values.

Several studies have reported the beneficial effects of soy and its isoflavone on bone. The findings of the present study indicate that the effects of soy are enhanced by FOS. FOS is known to improve calcium and magnesium absorption in rats.[32] Nonetheless, this may not be the only way by which FOS modulates bone and mineral metabolism. Intestinal calcium malabsoprtion is reported in both postmenopausal women[33] and Ovx rats [34] and is believed to aggravate negative calcium balance and contribute to bone loss.[33;35-37] This, in part, can be abated by FOS as it enhances calcium absorption. Relevant to the present study, FOS also enhances the bioavailabilty of isoflavones as it increases the hydrolysis of glyconated isoflavones to aglycones, the active form. Equol is a metabolite of an isoflavone, diadzein, that is formed by intestinal microflora in humans.[38] Several studies[15;39] have shown that FOS is a prebiotic that

enhances this favorable bacterial proliferation and increases bacterial β-glucosidase activity, an enzyme that cleaves isoflavones to produce its aglycones and metabolites. In summary, FOS enhances the bone modulating effects of soy enough to reverse bone loss due to ovariectomy. Future studies should illustrate the mechanisms by which the beneficial effects of these two components are exerted on bone.

Acknowledgements. This study was funded by the Oklahoma Center for Advancement of Science and Technology, (grant no HR01070). The soy isoflavones used in this study were generously provided by Archer Daniels Midland Company (Decator, IL) and the soy protein preparations used were generously donated by Protein Technologies International (St. Louis, MO).

Table 1

Composition of diets

Ingredient	**Control Diet (g/100g)**	**FOS Diet (g/100g)**	**Soy+FOS Diet (g/100g)**
Soy protein (normal isoflavones)[1]			22.70
Casein[2]	22.70	22.70	-
Corn Starch	20.00	20.00	20.00
Sucrose	41.76	36.76	36.76
FOS[3]	-	5.00	5.00
Cellulose[4]	5.60	5.60	5.60
Corn Oil	5.70	5.70	5.70
Mineral mix, Ca-P deficient (TD 79055)[5]	1.34	1.34	1.34
Vitamin mix (TD 40060)[6]	1.0	1.0	1.0
Calcium carbonate $CaCO_3$	1.16	1.16	1.03
Sodium phosphate monobasic $NaH_2PO_4.H_2O$	0.388	0.388	0.388

Potassium Phosphate Monobasic KH_2PO_4	0.238	0.238	0.238
Potassium citrate monohydrate	0.090	0.090	0.090

[1] Soy protein isolate obtained from The Solae Company (St. Louis, MO).

[2] Teklad diet #88190 (Harlan Teklad, Madison, WI).

[3] Kirkman Laboratories, Inc (Lake Oswega, OR)

[4]Alphacel obtained from ICN Biochemicals (Costa Mesa, CA).

[5]Mineral mixture (TD 79055) obtained from Harlan Teklad (Madison, WI). This mineral mixture is a modification of AIN 76 lacking calcium, phosphorus, and sucrose as diluent).

[6]Vitamin mixture (g/kg diet; TD 40060) obtained from Harlan Teklad (Madison, WI): p-aminobenzoic acid, 0.1101; ascorbic acid, 1.0166; biotin, 0.00044; vitamin B-12 (0.1% trituration), 0.0297; calcium pantothenate, 0.0661, choline dihydrogen citrate, 3.4969; folic acid, 0.00198; inositol, 0.1101; menadione, 0.0495; niacin, 0.0991; pyridoxine HCl, 0.0220; riboflavin, 0.0220; thiamin HCl, 0.0220; dry retinyl palmitate, 0.0044; dry d,l-α-tocopheryl acetate, 0.2423; corn starch (diluent), 4.6669.

Table 2

Effects of ovariectomy (Ovx), soy with isoflavones (Soy), fructooligosacchrides (FOS) and their combination (Soy+FOS) on food consumption, and body and uterus weights

Measure	**Sham**	**Ovx**	**Soy**	**FOS**	**Soy+FOS**	**P-Value**
Food Intake	15.35±0.30	14.99±0.36	15.10±0.35	14.98±0.35	15.01±0.36	0.9850
Body Weights (g)						
Initial	312 ± 7	314 ± 8	311 ± 7	315 ± 8	311 ± 7	0.9938
Final	320 ± 7^{c}	372 ± 8^{a}	372 ± 6^{a}	362 ± 6^{ab}	346 ± 6^{b}	<0.0001
Final Fat Mass (g)	69±7^{c}	128±8^{a}	113±8^{ab}	105±8^{b}	85±8^{c}	<0.0001
Uterus	0.66±0.03^{a}	0.16±0.04^{b}	0.19 ±0.04^{b}	0.16 ± 0.04^{b}	0.18 ± 0.04^{b}	<0.0001

Values are means ± SEM, Values that do not share the same superscript letters are significantly (P<0.05) different from each other; n = 13 rats per group.

Table 3

Effects of ovariectomy (Ovx), soy with isoflavones (Soy), fructooligosacchrides (FOS) and their combination (Soy+FOS) on bone mineral content (BMC) and density (BMD)

Measure	**Sham**	**Ovx**	**Soy**	**FOS**	**Soy+FOS**	**P-Value**
Whole Body						
Before Treatment						
BMC (g)	10.76 ± 0.20	10.73 ± 0.20	10.87 ± 0.20	10.86 ± 0.20	10.55 ± 0.20	0.7811
BMD (g/cm^2)	0.1704 ± 0.002^{a}	0.1564 ± 0.002^{b}	0.1597 ± 0.002^{b}	0.1605 ± 0.002^{b}	0.1583 ± 0.002^{b}	<0.0001
Final						
BMC (g)	10.90 ± 0.19	10.94 ± 0.20	11.23 ± 0.20	11.39 ± 0.20	10.85 ± 0.19	0.2187
BMD (g/cm^2)	0.1663 ± 0.002^{a}	0.1540 ± 0.002^{c}	0.1568 ± 0.002^{bc}	0.1613 ± 0.002^{b}	0.1590 ± 0.002^{b}	0.0002
Tibia						
BMC (g)	0.3584 ± 0.007^{a}	0.3102 ± 0.008^{c}	0.3423 ± 0.008^{ab}	0.3269 ± 0.008^{bc}	0.3221 ± 0.008^{bc}	0.0005
BMD (g/cm^2)	0.1993 ± 0.002^{a}	0.1825 ± 0.002^{c}	0.1908 ± 0.002^{b}	0.1892 ± 0.002^{b}	0.1887 ± 0.002^{b}	<0.0001
4th Lumbar Vertebra						
BMC (g)	0.1279 ± 0.004^{a}	0.1071 ± 0.004^{c}	0.1135 ± 0.004^{bc}	0.1200 ± 0.003^{ab}	0.1147 ± 0.003^{bc}	0.0013
BMD (g/cm^2)	0.2264 ± 0.003^{a}	0.1986 ± 0.003^{c}	0.2046 ± 0.003^{bc}	0.2100 ± 0.003^{b}	0.2088 ± 0.003^{b}	<0.0001

Values are means ± SEM Values that do not share the same superscript letters are significantly ($P<0.05$) different from each other; n = 13 rats per group.

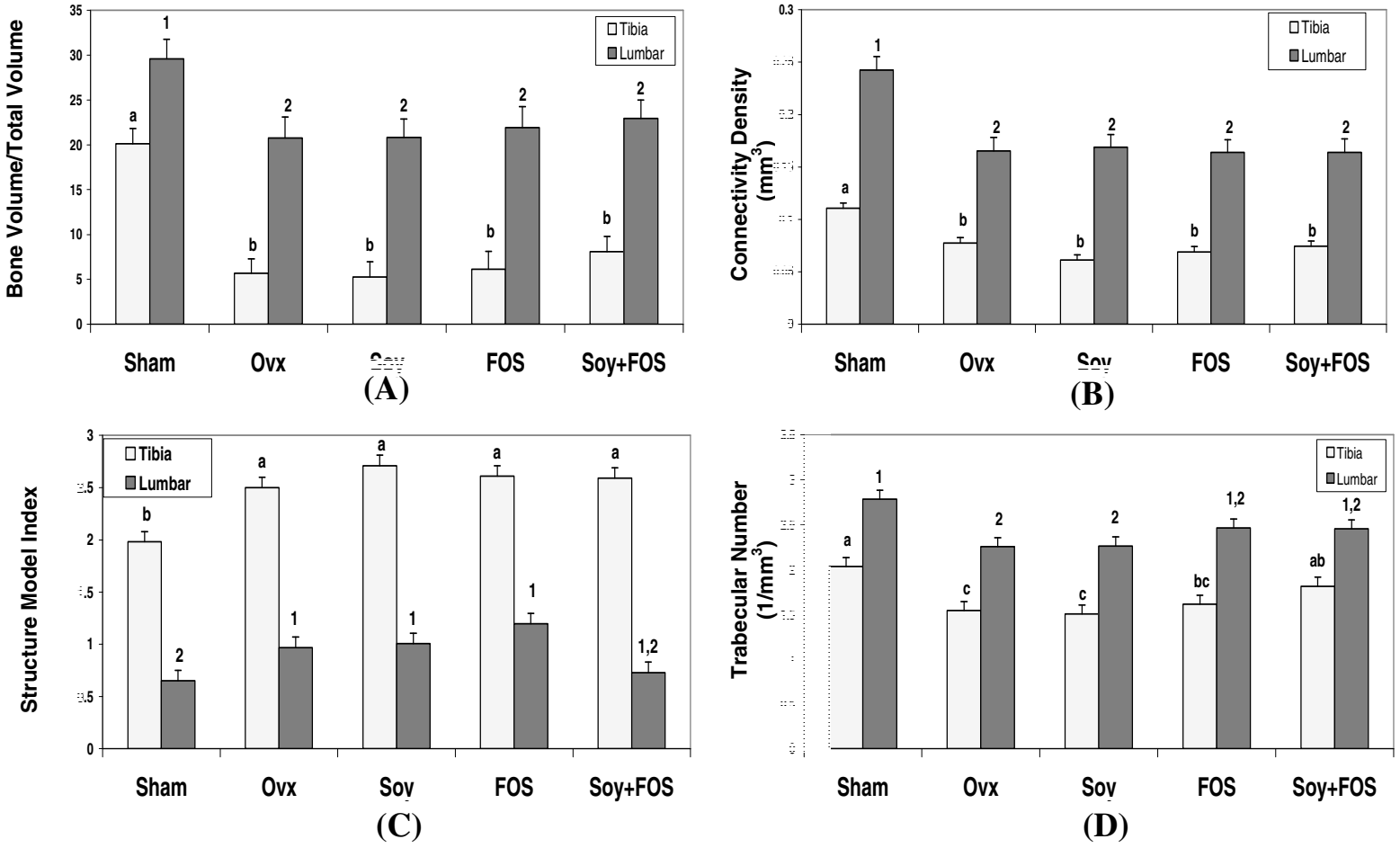
Table 4

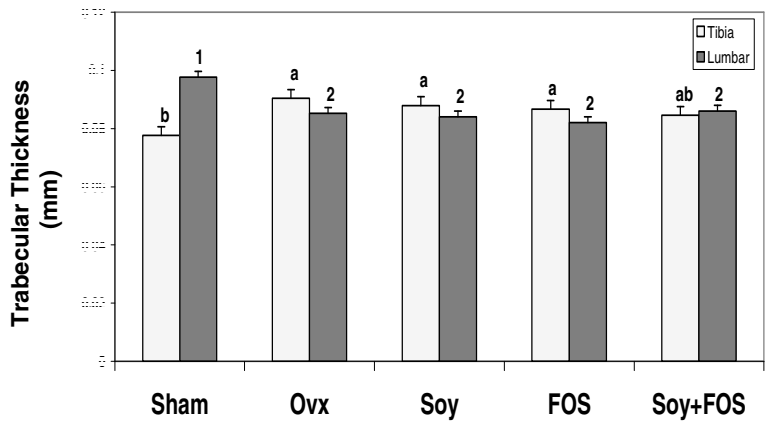
Effects of ovariectomy (Ovx), soy with isoflavones (Soy), fructooligosacchrides (FOS) and their combination (Soy+FOS) on Biomechanical Properties of Femur

Measures	Sham	Ovx	Soy	FOS	Soy+FOS	P-Value
Femur						
Ultimate Load (N)	69.23 ± 0.24	69.37 ± 0.27	69.04 ± 0.26	69.62 ± 0.26	69.90 ± 0.30	0.3255
Yield Load (N)	85.56 ± 5	80.28 ± 6	76.66 ± 6	85.23 ± 5	85.22 ± 5	0.7231
Stiffness (N/mm)	143 ± 3.69^{a}	125 ± 1.46^{c}	138 ± 3.98^{ab}	133 ± 3.98^{abc}	134 ± 3.98^{abc}	0.0370
Ultimate Stress (N/mm^2)	96 ± 6^{b}	122 ± 7^{a}	93 ± 7^{b}	98 ± 6^{b}	101 ± 7^{b}	0.0173
Yield Stress (N/mm^2)	118 ± 10	137 ± 14	106 ± 12	119 ± 9	120 ± 9	0.5384
Modulus Elasticity (N/mm^2)	4034 ± 287	4649 ± 324	3644 ± 315	3767 ± 310	4000 ± 310	0.2315

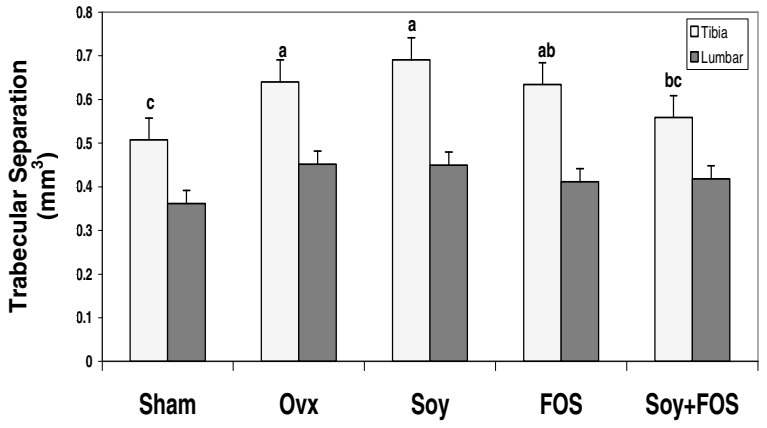
Values are means ± SEM Values that do not share the same superscript letters are significantly ($P<0.05$) different from each other; n = 13 rats per group.

Fig. 1
Effects of ovariectomy (Ovx), soy with isoflavones (Soy), fructooligosacchrides (FOS) and their combination (Soy+FOS) on tibial and lumbar microarchotectural properties





(E)



(F)

Values are means ± SEM, Bars that do not share the same superscript letters are significantly (P<0.05) different from each other; n = 13 rats per group

Reference List

1. America's Bone Health: The State of Osteoporosis and Low Bone Mass in Our Nation. NOF, 2002. NOF 2002.
2. Tosteson AN, Grove MR, Hammond CS et al. Early discontinuation of treatment for osteoporosis. Am J Med 2003;115:209-16.
3. Arjmandi BH, Birnbaum R, Goyal NV et al. Bone-sparing effect of soy protein in ovarian hormone deficient rats is related to its isoflavone content. Am J Clin Nutr 1998; 68: 1364S-8S.
4. Arjmandi BH, Alekel L, Hollis BW et al. Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis. J Nutr 1996;126:161-67.
5. Picherit C, Chanteranne B, Bennetau-Pelissero C et al. Dose-dependent bone-sparing effects of dietary isoflavones in the ovariectomised rat. Br J Nutr 2001;85:307-16.
6. Alekel DL, Germain AS, Peterson CT, Hanson KB, Stewart JW, Toda T. Isoflavone-rich soy protein isolate attenuates bone loss in the lumbar spine of perimenopausal women. Am J Clin Nutr 2000;72:844-52.
7. Potter SM, Baum JA, Teng H, Stillman RJ, Shay NF, Erdman JWJ. Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. Am J Clin Nutr 1998;68 Supp:1375S-9S.
8. Arjmandi BH, Lucas EA, Khalil DA et al. One year soy protein supplementation has positive effects on bone formation markers but not bone density in postmenopausal women. Nutr J 2005;4:8.
9. Arjmandi BH, Getlinger MJ, Goyal NV et al. The role of soy protein with normal or reduced isoflavone content in reversing bone loss induced by ovarian hormone deficiency in rats. Am J Clin Nutr 1998;68:1358S-63S.
10. Picherit C, Bennetau-Pelissero C, Chanteranne B et al. Soybean isoflavones dose-dependently reduce bone turnover but do not reverse established osteopenia in adult ovariectomized rats. J Nutr 2001;131:723-28.
11. Xu X, Harris KS, Wang HJ, Murphy PA, Hendrich S. Bioavailability of soybean isoflavones depends upon gut microflora in women. J Nutr 1995;125:2307-15.
12. Tuohy KM, Rouzaud GC, Bruck WM, Gibson GR. Modulation of the human gut microflora towards improved health using prebiotics--assessment of efficacy. Curr Pharm Des 2005;11:75-90.
13. Ohta A, Ohtsuki M, Baba S, Adachi T, Sakata T, Sakaguchi E. Calcium and magnesium absorption from the colon and rectum are increased in rats fed fructooligosaccharides. J Nutr 1995;125:2417-24.

14. Mathey J, Puel C, Kati-Coulibaly S et al. Fructooligosaccharides maximize bone-sparing effects of soy isoflavone-enriched diet in the ovariectomized rat. Calcif Tissue Int 2004;75:169-79.

15. Ohta A, Uehara M, Sakai K et al. A combination of dietary fructooligosaccharides and isoflavone conjugates increases femoral bone mineral density and equol production in ovariectomized mice. J Nutr 2002;132:2048-54.

16. Arjmandi BH, Lucas EA, Juma S et al. Dried plums prevent ovariectomy-induced bone loss in rats. JANA 2001;4:50-56.

17. Gowen M, Lazner F, Dodds R et al. Cathepsin K knockout mice develop osteopetrosis due to a deficit in matrix degradation but not demineralization. J Bone Miner Res 1999;14:1654-63.

18. Arjmandi BH, Juma S, Beharka AA, Bapna MS, Akhter M, Meydani SN. Vitamin E improves bone quality in the aged but not in young adult male mice. J Nutr Biochem 2002;13:543-49.

19. Akhter MP, Iwaniec UT, Haynatzki GR, Fung YK, Cullen DM, Recker RR. Effects of nicotine on bone mass and strength in aged female rats. J Orthop Res 2003;21:14-19.

20. Baumeister T, Avallone EA. Mark's standard handbook for mechanical engineers, 9th edition. New York: McGraw-Hill, 1999.

21. D.A.KhalilL, L.J.Hammond, L.Devareddy, D.Y.Soung, D.Bellmer, and B.H.Arjmandi. Soy Protein-Based Diet Improves Bone Mineral Density in Osteopenic Female Rats. J Bone Mineral Research . 2003.

22. Lissner L, Lindroos AK, Sjostrom L. Swedish obese subjects (SOS): an obesity intervention study with a nutritional perspective. Eur J Clin Nutr 1998;52:316-22.

23. Slavin JL. Dietary fiber and body weight. Nutrition 2005;21:411-18.

24. Nogowski L, Mackowiak P, Kandulska K, Szkudelski T, Nowak KW. Genistein-induced changes in lipid metabolism of ovariectomized rats. Ann Nutr Metab 1998;42:360-366.

25. Goodman-Gruen D, Kritz-Silverstein D. Usual dietary isoflavone intake and body composition in postmenopausal women. Menopause 2003;10:427-32.

26. Kinney JH, Haupt DL, Balooch M, Ladd AJ, Ryaby JT, Lane NE. Three-dimensional morphometry of the L6 vertebra in the ovariectomized rat model of osteoporosis: biomechanical implications. J Bone Miner Res 2000;15:1981-91.

27. Ohta A, Ohtsuki M, Hosono A, Adachi T, Hara H, Sakata T. Dietary fructooligosaccharides prevent osteopenia after gastrectomy in rats. J Nutr 1998;128:106-10.

28. Morohashi T, Sano T, Ohta A, Yamada S. True calcium absorption in the intestine is enhanced by fructooligosaccharide feeding in rats. J Nutr 1998;128:1815-18.

29. Barengolts EI, Curry DJ, Bapna MS, Kukreja SC. Effects of two non-endurance exercise protocols on established bone loss in ovariectomized adult rats. Calcif Tissue Int 1993;52:239-43.

30. Schaffler MB, Burr DB. Stiffness of compact bone: effects of porosity and density.

    J Biomech 1988;21:13-16.

31. Currey JD. The effect of porosity and mineral content on the Young's modulus of elasticity of compact bone. J Biomech 1988;21:131-39.

32. Ohta A, Ohtuki M, Takizawa T, Inaba H, Adachi T, Kimura S. Effects of fructooligosaccharides on the absorption of magnesium and calcium by cecectomized rats. Int J Vitam Nutr Res 1994;64:316-23.

33. Heaney RP, Recker RR, Saville PD. Menopausal changes in calcium balance performance. J Lab Clin Med 1979;92:953-63.

34. Arjmandi BH, Hollis BW, Kalu DN. In vivo effect of 17 beta-estradiol on intestinal calcium absorption in rats. Bone Miner 1994;26:181-89.

35. Gallagher JC, Riggs BL, DeLuca HF. Effect of estrogen on calcium absorption and serum vitamin D metabolites in postmenopausal osteoporosis. J Clin Endocrinol Metab 1980;51:1359-64.

36. Gallagher JC, Riggs BL, Eisman J, Hamstra A, Arnaud SB, DeLuca HF. Intestinal calcium absorption and serum vitamin D metabolites in normal subjects and osteoporotic patients: effect of age and dietary calcium. J Clin Invest 1979;64:729-36.

37. Gallagher JC. The pathogenesis of osteoporosis. Bone Miner 1990;9:215-27.

38. Setchell KDR, Brown NM, Lydeking-Olsen E. The clinical importance of the Metabolite Equol-A clue to the effectiveness of soy and its isoflavones. J Nutr 2002;132:3577-84.

39. Uehara M, Ohta A, Sakai K, Suzuki K, Watanabe S, Adlercreutz H. Dietary fructooligosaccharides modify intestinal bioavailability of a single dose of genistein and daidzein and affect their urinary excretion and kinetics in blood of rats. J Nutr 2001;131:787-95.

CHAPTER V

SUMMARY AND CONCLUSIONS

Summary

The objective of this research was to evaluate the dose-dependent effects of soy isoflavones in the reversal of bone loss as assessed by bone mineral content, bone mineral density, biomechanical and microarchitectural properties of bone in an ovariectomized rat model of postmenopausal osteoporosis. Additionally, the synergistic role of the combination of soy isoflavones and fructooligosacharides FOS in the reversal of bone loss was also evaluated by assessing BMC, BMD, bone biomechanical and microarchitectural properties.

The research objectives, hypotheses and the specific aims of the study are listed in Chapter I of this dissertation. The *central hypothesis* of this study was that soy protein with its isoflavones reverses bone loss in an ovariectomized (Ovx) rat model of postmenopausal osteoporosis. The *ancillary hypothesis* of this study was that soy isoflavones and FOS exert synergistic effects in the reversal of bone loss in osteopenic ovx rats.

The review of literature that is pertinent to the objectives of this study, which includes review on general bone structure and biology; systemic and local factors of bone

metabolism; estrogen and bone; prevalence; etiology; therapies for postmenopausal osteoporosis; soy and bone health; and FOS health benefits.

Chapter III includes methods, results and discussion addressing the central hypothesis. Although there have been numerous studies examining the role of soy isoflavones in enhancing bone health, to our knowledge there are no studies examining the effects of soy isoflavones in reversing the loss of microarchitectural properties.

In terms of body weight gain, as expected ovariectomy resulted in a significant ($P<0.05$) increase in body weight and this was completely prevented by estrogen. Soy with added isoflavones modestly prevented the body weight gain, however, Soy and Soy- had no such effects on body weight.

Ovariectomy resulted in significant loss of whole body, tibial and lumbar BMD. While none of the dietary treatments had any effect on whole body BMD, Soy was able to increase the tibial BMC and BMD by 10.3% and 4.5% when compared to Ovx controls. Soy-, Soy+ and E_2 did not reverse the loss of tibial BMD. The soy treatments increased the lumbar BMD values numerically, but not significantly.

In the present study, microCT data indicated that Ovx caused a deterioration of trabecular microarchitecture as seen from decreased BV/TV, Tb.N., CD, and increased Tb.Th. Ovariectomy also resulted in change of trabecular morphology from plate- to more inferior rod-like structures. The dietary treatments were not able to improve BV/TV, CD, SMI, or Tb.Th. of both the tibia and the vertebra. Soy with added isoflavones was able to restore the Tb.N. and decrease Tb.Sp. in the tibia, however, the effects of soy were less pronounced in the lumbar vertebra.

The findings of this study indicate that soy protein with normal isoflavone levels has a biphasic and site specific effect on the reversal of bone loss, whereas higher doses of isoflavones may be required to reverse the loss of microarchitectural properties.

Chapter IV addressed the ancillary hypothesis of this study. The objectives of the study were to determine the extent to which soy, FOS and their combination reverses ovarian hormone deficiency-induced bone loss. Additionally, the effects if these dietary treatments of microarchitectural and biomechanical properties were evaluated.

Soy+FOS treatment was somewhat able to prevent ovariectomy-induced gain in body weight. These lower body weight gains in this two treatment groups, in part, can be explained by reduced body fat. The low uterine weight in the Ovx groups indicated the success of ovariectomy and that neither Soy nor FOS diets imparted estrogen-like effects on uterus.

In terms of bone, FOS and Soy+FOS treatments had the most pronounced effects on whole body BMD as the means of these two groups were significantly ($P<0.05$) higher that Ovx controls. No effects on whole body BMC were observed with any of the treatments. Though soy protein with normal levels of isoflavones was able to restore the loss of tibial BMC, FOS and Soy+FOS treatment were unable to reverse the loss of tibial BMC. Soy, FOS and Soy+FOS increased the tibial BMD by 4.5, 3.7 and 3.4%, respectively in comparison with Ovx controls, albeit the values were lower than those of rats in the Sham group. Although, all the dietary treatments were able to improve tibial BMD, soy treatment had the most pronounced effects. The Ovx-induced loss of lumbar BMC was ameliorated only by FOS. Ovariectomy resulted in significant losses of lumbar

BMC and BMD and FOS alone was able to ameliorate these losses. Soy and Soy+FOS had intermediary effects on the reversal of lumbar BMD.

Ovariectomy did not alter ultimate and yield load, yield stress, or modulus of elasticity, however, femoral stiffness was reduced by Ovx rats. Soy, FOS, and Soy+FOS treatments were all able to increase the stiffness by varying degrees with soy treatment reaching a statistical significance. Ultimate stress was increased as a result of Ovx , and all of the dietary interventions (FOS, Soy, and Soy+FOS) brought this parameter to the Sham level.

The combination of soy and FOS had the most pronounced effects in reversing the loss of tibial and lumbar microarchitectural properties. Soy+FOS group was able to somewhat restore the ratio of rods and plates of the trabecular bone to an increased proportion of plates than the Ovx control group in the vertebra, however, this change was not observed in the tibia. Similarly, Soy+FOS treatment significantly increased Tb.N. in the tibia and vertebra. FOS also caused an increase in Tb.N., but only lumbar Tb.N. was up to the level of Sham. Tibial Tb.Th. in Soy+FOS was neither different from that of Sham nor from Ovx control group, whereas in the vertebra, dietary treatments had no effect on lumbar Tb.Th. or Tb.Sp. Tibial Tb.Sp. was reduced by FOS and Soy+FOS treatments with Soy+FOS having a more pronounced effect than FOS alone. In summary, the overall results of the microarchitectural properties indicated that soy protein in combination with FOS may be more efficacious in reversing the loss of trabeculae in ovarian hormone deficiency.

Conclusions

Based on the results of the experiment 1, soy protein and its isoflavones appear to have a modest beneficial effect in established osteoporosis as evident by improvements in tibial BMC and BMD and certain structural parameters. Interestingly, in the present study we found that while too little or too much isoflavones may not have any effect on tibial BMD, higher doses of isoflavones may be required to reverse the loss of tibial microstructural properties. Nonetheless, it is too early to conclude that soy protein or its isoflavones should be supplemented to prevent or reverse bone loss induced by ovarian hormone deficiency.

In experiment 2, the results indicated that the positive bone modulating effects of soy are further augmented by the addition of FOS as seen from the improved BMD and microarchitectural properties. Soy when combined with FOS was effective in reversing the loss of certain trabecular structural parameters in tibia namely trabecular number, thickness, and separation. The combination of soy and FOS was also able to change the rod-like trabeculae to a more favorable plate-like structures as observed from SMI values. The mechanisms by which the combination of Soy and FOS enhance bone quality need to be elucidated.

References

Adachi, J. D., Adami, S., Miller, P. D., Olszynski, W. P., Kendler, D. L., Silverman, S. L., Licata, A. A., Li, Z. & Gomez-Panzani, E. (2001) Tolerability of risedronate in postmenopausal women intolerant of alendronate. *Aging (Milano.)*, **13,** 347-354.

Akesson, K. (2003) New approaches to pharmacological treatment of osteoporosis. *Bull.World Health Organ*, **81,** 657-664.

Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S.I., Itoh, N., Shibuya, M. & Fukami, Y. (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J.Biol.Chem.*, **262,** 5592-5595.

Albright, F. & Reifenstein, E. C. (1948) The parathyroid glands and metabolic bone disease. Williams & Wilkins, Co, Baltimore.

Albright, F., Smith, P. H. & Richardson, A. M. (1941) Postmenopausal osteoporosis: Its clinical features. *J.A.M.A.*, **116,** 2465-2474.

Alekel, D. L., Germain, A. S., Peterson, C. T., Hanson, K. B., Stewart, J. W. & Toda, T. (2000) Isoflavone-rich soy protein isolate attenuates bone loss in the lumbar spine of perimenopausal women. *Am.J.Clin.Nutr.*, **72,** 844-852.

America's Bone Health: The state of osteoporosis and low bone mass in our nation. NOF, 2002. *NOF*

Anderson, J. J., Ambrose, W. W. & Garner, S. C. (1998) Biphasic effects of genistein on bone tissue in the ovariectomized, lactating rat model. *Proc.Soc.Exp.Biol.Med.*, **217,** 345-350.

Anderson, J. W., Johnstone, B. M. & Cook-Newell, M. E. (1995) Meta-analysis of the effects of soy protein intake on serum lipids. *N. Engl. J. Med.*, **333,** 276-282.

Arjmandi B.H., Khalil, D. A., Smith, B. J., Lucas, E. A., Juma, S., Payton, M. E. & Wild, R. A. (2003) Soy protein has a greater effect on bone in postmenopausal women not on hormone replacement therapy, as evidenced by reducing bone resorption and urinary calcium excretion. *J.Clin.Endocrinol.Metab.*, **88,** 0000 (1)-0000 (6).

Arjmandi, B. H., Alekel, L., Hollis, B. W., Amin, D., Stacewicz-Sapuntzakis, M., Guo, P. & Kukreja, S. C. (1996) Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis. *J.Nutr.*, **126,** 161-167.

Arjmandi, B. H., Birnbaum, R., Goyal, N. V., Getlinger, M. J., Juma, S., Alekel, L., Hasler, C. M., Hollis, B. W., Drum, M. L. & Kukreja, S. C. (1998) Bone-sparing effect of soy protein in ovarian hormone deficient rats is related to its isoflavone content. *Am.J.Clin.Nutr.*, **68,** 1364S-1368S.

Arjmandi, B. H., Getlinger, M. J., Goyal, N. V., Alekel, L., Hasler, C. M., Juma, S., Drum, M. L. & Kukreja, S. (1998b) The role of soy protein with normal or reduced isoflavone content in reversing bone loss induced by ovarian hormone deficiency in rats. *Am.J.Clin.Nutr.*, **68,** 1358S-1363S.

Arjmandi, B. H., Hollis, B. W. & Kalu, D. N. (1994) In vivo effect of 17 beta-estradiol on intestinal calcium absorption in rats. *Bone Miner.*, **26,** 181-189.

Arjmandi, B. H., Khalil, D. A. & Hollis, B. W. (2002) Soy protein: Its effects on intestinal calcium transport, serum vitamin D, and insulin-like growth factor-I in ovariectomized rats. *Calc.Tissue Int.*, **30,** epub ahead of print.

Arjmandi, B. H., Khalil, D. A., Lucas, E. A., Juma, S., Sinichi, N., Hodges, S. B., Payton, M. E., Hammond, L., Munson, M. E. & Wild, R. Soy protein with its isoflavones improves bone markers in middle-aged and elderly women. *FASEB J.* 15, A728. 2001.

Arjmandi, B. H., Lucas, E. A., Khalil, D. A., Devareddy, L., Smith, B. J., McDonald, J., Arquitt, A. B., Payton, M. E. & Mason, C. (2005) One year soy protein supplementation has positive effects on bone formation markers but not bone density in postmenopausal women. *Nutr. J.*, **4,** 8.

Arjmandi, B. H., Salih, M. A., Herbert, D. C., Sims, S. H. & Kalu, D. N. (1993) Evidence for estrogen receptor-linked calcium transport in the intestine. *Bone Miner*, **21,** 63-74.

Bain, S. D., Bailey, M. C., Celino, D. L., Lantry, M. M. & Edwards, M. W. (1993) High-dose estrogen inhibits bone resorption and stimulates bone formation in the ovariectomized mouse. *J Bone Miner.Res*, **8,** 435-442.

Baron, R. (1993) Anatomy and Ultrastructure of Bone. Raven Press, New York.

Baron, R. (2003) General Principles od Bone Biology. *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism* (ed M. Favus), pp. 1-8. American Society for Bone and Mineral Research, Washington DC.

Barrett-Connor, E. (1993) Epidemiology and the menopause: A global overview. *Int.J.Fertil.*, **38,** 6-14.

Basu, S., Michaelsson, K., Olofsson, H., Johansson, S. & Melhus, H. (2001) Association between oxidative stress and bone mineral density. *Biochem.Biophys.Res.Commun.*, **288,** 275-279.

Bauer, D. C., Rosen, C. J., Cauley, J. & Cummings, S. R. (1997) Low serum IGF-I but not IGFBP-3 predicts hip and spine fractures. Bone Supplement , 561.

Berg, R. D. (1996) The indigenous gastrointestinal microflora. *Trends Microbiol.*, **4,** 430-435.

Biskobing, D. M., Novy, A. M. & Downs, R., Jr. (2002) Novel therapeutic options for osteoporosis. *Curr.Opin.Rheumatol.*, **14,** 447-452.

Blair, H. C., Jordan, S. E., Peterson, T. G. & Barnes, S. (1996) Variable effects of tyrosine kinase inhibitors on avian osteoclastic activity and reduction of bone loss in ovariectomized rats. *J.Cell.Biochem.*, **61,** 629-637.

Blum, S. C., Heaton, S. N., Bowman, B. M., Hegsted, M. & Miller, S. C. (2003) Dietary soy protein maintains some indices of bone mineral density and bone formation in aged ovariectomized rats. *J.Nutr.*, **133,** 1244-1249.

Boring, C. C., Squires, T. S., Tong, T. & Montgomery, S. (1994) Cancer statistics, 1994 3. *CA Cancer J Clin.*, **44,** 7-26.

Brezinski, A. & Debi, A. (1999) Phytoestrogens: the "natural" selective estrogen receptor modulators. *Eur.J.Ob.& Gyn.& Rep.Bio* 85, 47-51..

Burke, G. L., Vitolins, M. Z. & Bland, D. (2000) Soybean isoflavones as an alternative to traditional hormone replacement therapy: are we there yet? *J.Nutr.*, **130,** 664S-665S.

Burns, A. J. & Rowland, I. R. (2004) Antigenotoxicity of probiotics and prebiotics on faecal water-induced DNA damage in human colon adenocarcinoma cells. *Mutat.Res*, **551,** 233-243.

Cebra, J. J. (1999) Influences of microbiota on intestinal immune system development 1. *Am.J Clin.Nutr.*, **69,** 1046S-1051S.

Center, J. & Eisman, J. (1997) The epidemiology and pathogenesis of osteoporosis 10. *Baillieres Clin.Endocrinol.Metab.*, **11,** 23-62.

Census 2000 PHC-T-9: (2000) Female population by age, race and hispanic or latino origin for the United States. United States Bureau of Census.

Chen, Y. M., Ho, S. C., Lam, S. S., Ho, S. S. & Woo, J. L. (2003) Soy isoflavones have a favorable effect on bone loss in Chinese postmenopausal women with lower bone mass: a double-blind, randomized, controlled trial. *J.Clin.Endocrinol.Metab.*, **88,** 4740-4747.

Chow, J., Tobias, J. H., Colston, K. W. & Chambers, T. J. (1992) Estrogen maintains trabecular bone volume in rats not only by suppression of bone resorption but also by stimulation of bone formation. *J Clin.Invest.*, **89,** 74-78.

Clarkson, T. B. (2000) Soy phytoestrogens: what will be their role in postmenopausal hormone replacement therapy?. *Menopause.*, **7,** 71-75.
Cummings, J. H. & Macfarlane, G. T. (1991) The control and consequences of bacterial fermentation in the human colon. *J Appl.Bacteriol.*, **70,** 443-459.

Cunningham-Rundles, S. & Lin, D. H. (1998) Nutrition and the immune system of the gut. *Nutrition*, **14,** 573-579.

Dalais, F. S., Rice, G. E., Wahlqvist, M. L., Grehan, M., Murkies, A. L., Medley, G. Ayton, R. & Strauss, B.J.G. (1998) Effects of dietary phytoestrogens in postemenopausal women. *Climacteric*, **1,** 124-129.

Dang, Z. C., Van Bezooijen, R. L., Karperien, M., Papapoulos, S. E. & Lowik, C. W. (2002) Exposure of KS483 cells to estrogen enhances osteogenesis and inhibits adipogenesis. *J Bone Miner.Res.*, **17,** 394-405.

Deitcher, S. R. & Gomes, M. P. (2004) The risk of venous thromboembolic disease associated with adjuvant hormone therapy for breast carcinoma: a systematic review. *Cancer*, **101,** 439-449.

DeLuca, H. F. & Cantorna, M. T. (2001) Vitamin D: its role and uses in immunology. *FASEB J.*, **15,** 2579-2585.

Di Gregorio, G. B., Yamamoto, M., Ali, A. A., Abe, E., Roberson, P., Manolagas, S. C. & Jilka, R. L. (2001) Attenuation of the self-renewal of transit-amplifying osteoblast progenitors in the murine bone marrow by 17 beta-estradiol. *J Clin.Invest*, **107,** 803-812.

Doggrell, S. A. (2004) Recent important clinical trials of drugs in osteoporosis. *Expert.Opin.Pharmacother.*, **5,** 1635-1638.

Eghbali-Fatourechi, G., Khosla, S., Sanyal, A., Boyle, W. J., Lacey, D. L. & Riggs, B. L. (2003) Role of RANK ligand in mediating increased bone resorption in early postmenopausal women. *J Clin.Invest.*, **111,** 1221-1230.

Eriksen, E. F. & Robins, D. A. (2004) Teriparatide: A bone formation treatment for osteoporosis. *Drugs Today (Barc.)*, **40,** 935-948.

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

Gallagher, J. C., Rafferty, K., Haynatzka, V. & Wilson, M. (2000) Effects of soy protein on bone metabolism. *J.Nutr.,* 130, 667S.

Gennari, C., Agnusdei, d., Nardi, P. & Civitelli, R. (1990) Estrogen preserves a normal intestinal responsiveness to 1,25-dihydroxyvitamin D_3 in oophorectomized women. *J.Clin.Endocrinol.Metab.*, **71,** 121-129.

Gibson, G. R. & Roberfroid, M. B. (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nutr.*, **125,** 1401-1412.

Grady, D., Herrington, D., Bittner, V., Blumenthal, R., Davidson, M., Hlatky, M., Hsia, J., Hulley, S., Herd, A., Khan, S., Newby, L. K., Waters, D., Vittinghoff, E. & Wenger, N. (2002) Cardiovascular disease outcomes during 6.8 years of hormone replacement therapy: heart and estrogen/progestin replacement study follow-up (HERS II). *JAMA*, **288,** 49-57.

Hart, D. M., Farish, E., Fletcher, C. D., Barnes, J. F., Hart, H., Nolan, D. & Spowart, K. (1998) Long-term effects of continuous combined HRT on bone turnover and lipid metabolism in postmenopausal women. *Osteoporos..Int.*, **8,** 326-332.

Heaney, R. P. (2000) Calcium, dairy products and osteoporosis. *J Am.Coll.Nutr*, **19,** 83S-99S.

Ho, S. C., Chan, S. G., Yi, Q., Wong, E. & Leung, P. C. (2001) Soy intake and the maintenance of peak bone mass in Hong Kong Chinese women. *J. Bone Miner.Res.*, **16,** 1363-1369.

Hofbauer, L. C., Khosla, S., Dunstan, C. R., Lacey, D. L., Spelsberg, T. C. & Riggs, B. L. (1999) Estrogen stimulates gene expression and protein production of osteoprotegerin in human osteoblastic cells. *Endocrinology*, **140,** 4367-4370.

Hsu, H., Lacey, D. L., Dunstan, C. R., Solovyev, I., Colombero, A., Timms, E., Tan, H. L., Elliott, G., Kelley, M. J., Sarosi, I., Wang, L., Xia, X. Z., Elliott, R., Chiu, L., Black, T., Scully, S., Capparelli, C., Morony, S., Shimamoto, G., Bass, M. B. & Boyle, W. J. (1999) Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc. Natl.Acad.Sci U.S.A*, **96,** 3540-3545.

Hughes, D. E., Dai, A., Tiffee, J. C., Li, H. H., Mundy, G. R. & Boyce, B. F. (1996) Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-β. *Nat.Med.*, **2,** 1132-1136.

Hughes, D. E., Wright, K. R., Uy, H. L., Sasaki, A., Yoneda, T., Roodman, G. D., Mundy, G. R. & Boyce, B. F. (1995) Bisphosphonates promote apoptosis in murine osteoclasts *in vitro* and *in vivo*. *J.Bone Miner.Res.*, **10,** 1478-1487.

Jee W.S.S. (1983) Histology: Cell and Tissue Biology. (eds L. Weiss & Lansing), Elseviar Biomedical, New York.

Jee, W. S. & Yao, W. (2001) Overview: animal models of osteopenia and osteoporosis. *J. Musculoskelet.Neuronal.Interact.*, **1,** 193-207.

Jiang, Y., Zhao, J. J., Mitlak, B. H., Wang, O., Genant, H. K. & Eriksen, E. F. (2003) Recombinant human parathyroid hormone (1-34) [teriparatide] improves both cortical and cancellous bone structure. *J Bone Miner.Res.*, **18,** 1932-1941.

Jilka, R. L., Takahashi, K., Munshi, M., Williams, D. C., Roberson, P. K. & Manolagas, S. C. (1998) Loss of estrogen upregulates osteoblastogenesis in the murine bone marrow. Evidence for autonomy from factors released during bone resorption. *J Clin.Invest.*, **101,** 1942-1950.

Jordan, V. C. (2001) The past, present, and future of selective estrogen receptor modulation. *Ann.N.Y.Acad.Sci.*, **949,** 72-79.

Kalu, D. N. (1991) The ovariectomized rat model of postmenopausal bone loss. *Bone and Min.*, **15,** 175-192.

Kalu, D. N. (1997) The ovariectomized rat model of postmenopausal bone loss. *Bone Miner*, **15,** 175-192.

Kaskani, E., Lyritis, G. P., Kosmidis, C., Galanos, A., Andypas, G., Chorianopoulos, K., Giagiosis, A., Iliadou, K., Karagianis, A., Katsimichas, K., Koskinas, A. & Matsouka, K. (2005) Effect of intermittent administration of 200 IU intranasal salmon calcitonin and low doses of 1alpha(OH) vitamin D3 on bone mineral density of the lumbar spine and hip region and biochemical bone markers in women with postmenopausal osteoporosis: a pilot study. *Clin.Rheumatol.*, **24,** 232-238.

Kimmel, D. (2002) Animal Models in Osteoporosis Research. *Principles of Bone Biology* (eds J. P. Bilezikian, L. G. Raisz & G. A. Rodan), pp. 1635-1556. Academic Press.

Knight, D. C. & Eden, J. A. (1996) A review of the clinical effects of phytoestrogens. *Obstet.Gynecol.*, **87,** 897-904.

Komulainen, M. H., Kroger, H., Tuppurainen, M. T., Heikkinen, A. M., Alhava, E., Honkanen, R. & Saarikoski, S. (1998) HRT and Vit D in prevention of non-vertebral fractures in postmenopausal women; a 5 year randomized trial. *Maturitas*, **31,** 45-54.

Kotzan, J. A., Martin, B. C. & Wade, W. E. (1999) Persistence with estrogen therapy in a postmenopausal Medicaid population. *Pharmacotherapy*, **19,** 363-369.

Kousteni, S., Bellido, T., Plotkin, L. I., O'Brien, C. A., Bodenner, D. L., Han, L., Han, K., DiGregorio, G. B., Katzenellenbogen, J. A., Katzenellenbogen, B. S., Roberson, P. K., Weinstein, R. S., Jilka, R. L. & Manolagas, S. C. (2001) Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell*, **104,** 719-730.

Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S. & Gustafsson, J. A. (1997) Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology*, **138,** 863-870.

Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der, B. B. & Gustafsson, J. A. (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology*, **139,** 4252-4263.

Lacey, D. L., Timms, E., Tan, H.-L., Kelly, M. J., Dunstan, C. R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E., Capparelli, C., Eli, A., Qian, Y.-X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., Guo, J., Delaney, J. & Boyle, W. J. (1998) Osteoprotogerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell*, **93,** 165-176.

Laib, A., Kumer, J. L., Majumdar, S. & Lane, N. E. (2001) The temporal changes of trabecular architecture in ovariectomized rats assessed by MicroCT. *Osteoporos.Int.*, **12,** 936-941.

Langlois, J. A., Rosen, C. J., Visser, M., Hannan, M. T., Harris, T., Wilson, P. W. & Kiel, D. P. (1998) Association between insulin-like growth factor I and bone mineral density in older women and men: the Framingham Heart Study. *J.Clin.Endocrinol.Metab.*, **83,** 4257-4262.

Lees, C. J. & Ginn, T. A. (1998) Soy protein isolate diet does not prevent increased cortical bone turnover in ovariectomized macaques. *Calcif.Tissue Int.*, **62,** 557-558.

Liberman, U. A., Weiss, S. R., Broll, J., Minne, H. W., Quan, H., Bell, N. H., Rodriguez-Portales, J., Downs, R. W., Jr., Dequeker, J. & Favus, M. (1995) Effect of oral alendronate on bone mineral density and the incidence of fractures in postmenopausal osteoporosis. The Alendronate Phase III Osteoporosis Treatment Study Group. *N.Engl. J. Med.*, **333,** 1437-1443.

Lyritis, G. P., Magiasis, B. & Tsakalakos, N. (1995) Prevention of bone loss in early nonsurgical and nonosteoporotic high turnover patients with salmon calcitonin: the role of biochemical bone markers in monitoring high turnover patients under calcitonin treatment. *Calcif.Tissue Int.*, **56,** 38-41.

Manolagas, S. C. (2000) Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *End.Rev.*, **21,** 115-137.

Manolagas, S. C. & Jilka, R. L. (1995) Bone marrow, cytokines, and bone remodeling: emerging insights into the pathophysiology of osteoporosis. *N.Engl.J.Med.*, **332,** 305-311.

Marfarlane, G. T. & McBain, A. J. (1999) The human colonic microbiota. *Colonic Microbiota, Nutrition and Health* (eds G. R. Gibson & M. B. Roberfroid), pp. 3-28. Kluwer Scientific Publishers.

Marks, S. C. & Odgren, P. R. (2002) Structure and Development of Skeleton. *Principles of Bone Biology* (eds J. P. Bilezikian, L. G. Raisz & G. A. Rodan), pp. 3-15. Academic Press.

Martin, R. B., Burr, D. B. & Sharkey, N. A. (1998) Skeletal Tissue Mechanics.pp. 29-49. Springer-Verlag New York Inc, New York.

Mathey, J., Puel, C., Kati-Coulibaly, S., Bennetau-Pelissero, C., Davicco, M. J., Lebecque, P., Horcajada, M. N. & Coxam, V. (2004) Fructooligosaccharides maximize bone-sparing effects of soy isoflavone-enriched diet in the ovariectomized rat. *Calcif.Tissue Int.*, **75,** 169-179.

McBride J . (1999) Can foods forestall aging?. *Agri. Res.* 47[2], 14-17.

McCombs, J. S., Thiebaud, P., McLaughlin-Miley, C. & Shi, J. (2004) Compliance with drug therapies for the treatment and prevention of osteoporosis. *Maturitas*, **48,** 271-287.

McKane, W. R., Khosla, S., Burritt, M. F., Kao, P. C., Wilson, D. M., Ory, S. J. & Riggs, B. L. (1995) Mechanism of renal calcium conservation with estrogen replacement therapy in women in early postmenopause--a clinical research center study. *J.Clin. Endocrinol.Metab.*, **80,** 3458-3464.

Melton, L. J., III (2003) Adverse outcomes of osteoporotic fractures in the general population. *J. Bone Miner.Res.*, **18,** 1139-1141.

Melton, L. J., III, Gabriel, S. E., Crowson, C. S., Tosteson, A. N., Johnell, O. & Kanis, J. A. (2003) Cost-equivalence of different osteoporotic fractures. *Osteoporos.Int.*, **14,** 383-388.

Miksicek, R. J. (1993) Commonly occurring plant flavonoids have estrogenic activity. *Mol.Pharmacol.*, **44,** 37-43.

Morito, K., Hirose, T., Kinjo, J., Hirakawa, T., Okawa, M., Nohara, T., Ogawa, S., Inoue, S., Muramatsu, M. & Masamune, Y. (2001) Interaction of phytoestrogens with estrogen receptors alpha and beta. *Biol.Pharm.Bull.*, **24,** 351-356.

Murakami, H., Takahashi, N., Sasaki, T., Udagawa, N., Tanaka, S., Nakamura, I., Zhang, D., Barbier, A. & Suda, T. (1995) A possible mechanism of the specific action of bisphosphonates on osteoclasts: tiludronate preferentially affects polarized osteoclasts having ruffled borders. *Bone*, **17,** 137-144.

Murkies, A. L., Wilcox, G. & Davis, S. R. (1998) Clinical review 92: Phytoestrogens 1. *J. Clin.Endocrinol.Metab.*, **83,** 297-303.

NIH Consensus Statement: Osteoporosis prevention, diagnosis, and therapy. (2000) National Institutes of Health Consensus Development Conference Statement. 17(2), 1-36..

Neer, R. M., Arnaud, C. D., Zanchetta, J. R., Prince, R., Gaich, G. A., Reginster, J. Y., Hodsman, A. B., Eriksen, E. F., Ish-Shalom, S., Genant, H. K., Wang, O. & Mitlak, B. H. (2001) Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. *N.Engl.J. Med.*, **344,** 1434-1441.

Nettleton, J. A., Greany, K. A., Thomas, W., Wangen, K. E., Adlercreutz, H. & Kurzer, M. S. (2005) The effect of soy consumption on the urinary 2:16-hydroxyestrone ratio in postmenopausal women depends on equol production status but is not influenced by probiotic consumption. *J. Nutr.*, **135,** 603-608.

Ohta, A., Ohtuki, M., Takizawa, T., Inaba, H., Adachi, T. & Kimura, S. (1994) Effects of fructooligosaccharides on the absorption of magnesium and calcium by cecectomized rats. *Int. J. Vitam.Nutr. Res.*, **64,** 316-323.

Oursler, M. J., Cortese, C., Keeting, P., Anderson, M. A., Bonde, S. K., Riggs, B. L. & Spelsberg, T. C. (1991) Modulation of transforming growth factor-beta production in normal human osteoblast-like cells by 17 beta-estradiol and parathyroid hormone. *Endocrinology*, **129,** 3313-3320.

Parfitt, A. M. (1994) Osteonal and hemi-osteonal remodeling: the spatial and temporal framework for signal traffic in adult human bone. *J. Cell. Biochem.,* **55,** 273-286.

Picherit, C., Bennetau-Pelissero, C., Chanteranne, B., Lebecque, P., Davicco, M. J., Barlet, J. P. & Coxam, V. (2001) Soybean isoflavones dose-dependently reduce bone turnover but do not reverse established osteopenia in adult ovariectomized rats. *J. Nutr.*, **131,** 723-728.

Picherit, C., Chanteranne, B., Bennetau-Pelissero, C., Davicco, M. J., Lebecque, P., Barlet, J. P. & Coxam, V. (2001) Dose-dependent bone-sparing effects of dietary isoflavones in the ovariectomized rat. *Br.J.Nutr.*, **85,** 307-316.

Pitsillides, A. A., Rawlinson, S. C., Suswillo, R. F., Bourrin, S., Zaman, G. & Lanyon, L. E. (1995) Mechanical strain-induced NO production by bone cells: a possible role in adaptive bone (re)modeling? *FASEB J.*, **9,** 1614-1622.

Potter, S. M., Baum, J. A., Teng, H., Stillman, R. J., Shay, N. F. & Erdman, J. W. J. (1998) Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. *Am.J.Clin.Nutr.*, **68 Supp,** 1375S-1379S.

Quandt, S. A., Thompson, D. E., Schneider, D. L., Nevitt, M. C. & Black, D. M. (2005) Effect of alendronate on vertebral fracture risk in women with bone mineral density T scores of-1.6 to -2.5 at the femoral neck: the Fracture Intervention Trial
1. *Mayo Clin.Proc.*, **80,** 343-349.

Raisz, L. (2004) Bone physiology. *Nutrition and Bone Health* (eds M. F. Holick & B. Dawson-Hughes), pp. 43-62. Humana Press, New Jersey.

Raisz, L. G., Kream, B. E. & Lorenzo, J. A. (1998) Metabolic bone disease. *Williams Textbook of Endocrinology* (eds Willson, Foster, Kronenberg & Larsen), Sauders Company, Philadelphia.

Reeve, J., Hesp, R., Williams, D., Hulme, P., Klenerman, L., Zanelli, J. M., Darby, A. J., Tregear, G. W. & Parsons, J. A. (1976) Anabolic effect of low doses of a fragment of human parathyroid hormone on the skeleton in postmenopausal osteoporosis. *Lancet*, **1,** 1035-1038.

Register, T. C., Jayo, M. J. & Anthony, M. S. (2003) Soy phytoestrogens do not prevent bone loss in postmenopausal monkeys. *J.Clin.Endocrinol.Metab.*, **88,** 4362-4370.

Reid, G. & Burton, J. (2002) Use of Lactobacillus to prevent infection by pathogenic bacteria. *Microbes.Infect.*, **4,** 319-324.

Resnick, D., Manolagas, S. C., Niwayama, G. & Fallon, M. D. (1995) Histogenesis, anatomy, and physiology of bone. *Diagnosis of Bone and Joint Disorders* (ed D. Resnick), Saunders Company, Philadelphia.

Riggs, B. L., Khosla, S. & Melton, L. J., III (1998) A unitary model for involutional osteoporosis: estrogen deficiency causes both type I and type II osteoporosis in postmenopausal women and contributes to bone loss in aging men. *J. Bone. Miner.Res.*, **13,** 763-773.

Riggs, B. L., Wahner, H. W., Seeman, E., Offord, K. P., Dunn, W. L., Mazess, R. B., Johnson, K. A. & Melton, L. J., III (1982) Changes in bone mineral density of the proximal femur and spine with aging. Differences between the postmenopausal and senile osteoporosis syndromes. *J. Clin.Invest.*, **70,** 716-723.

Rosen, V., Thies, R. S. & Lyons, K. (1996) Signaling pathways in skeletal formation: a role for BMP receptors. *Ann.N.Y.Acad.Sci.*, **785,** 59-69.

Rossouw, J. E., Anderson, G. L., Prentice, R. L., LaCroix, A. Z., Kooperberg, C., Stefanick, M. L., Jackson, R. D., Beresford, S. A., Howard, B. V., Johnson, K. C., Kotchen, J. M. & Ockene, J. (2002) Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *JAMA*, **288,** 321-333.

Russell, R. G. G. & Rogers, M. J. (1999) Bisphosphonates: from the laboratory to the clinic and back again. *Bone*, **25,** 106.

Schinke T & Karsenty G (2002) Cells of Bone: Osteoclast Generation. *Principles of Bone Biology* (eds J. P. Bilezikian, L. G. Raisz & G. A. Rodan), pp. 109-126. Academic Press.

Schmidt, A., Rutledge, S. J., Endo, N., Opas, E. E., Tanaka, H., Wesolowski, G., Leu, C. T., Huang, Z., Ramachandaran, C., Rodan, S. B. & Rodan, G. A. (1996) Protein-tyrosine

phosphatase activity regulates osteoclast formation and function: inhibition by alendronate. *Proc.Natl.Acad.Sci.U.S.A*, **93,** 3068-3073.

Setchell, K. (1998) Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. *Am.J.Clin.Nutr.* 68[suppl], 1333S-1346S..

Setchell, K. D. (2000) Absorption and metabolism of soy isoflavones-from food to dietary supplements and adults to infants. *J. Nutr.*, **130,** 654S-655S.

Setchell, K. D. (2001) Soy isoflavones--benefits and risks from nature's selective estrogen receptor modulators (SERMs). *J. Am.Coll.Nutr.*, **20,** 354S-362S.

Shutt, D. A. & Cox, R. I. (1972) Steroid and phyto-oestrogen binding to sheep uterine receptors in vitro. *J. Endocrinol.*, **52,** 299-310.

Simonet, W. S., Lacey, D. L., Dunstan, C. R., Kelley, M., Chang, M.-S., Nguyen, H. Q., wooden, S., Bennett, L. & et al (1997) Osteoprotogerin: a novel secreted protein involved in the regulation of bone density. *Cell*, **89,** 309-319.

Storm, T., Thamsborg, G., Steiniche, T., Genant, H. K. & Sorensen, O. H. (1990) Effect of intermittent cyclical etidronate therapy on bone mass and fracture rate in women with postmenopausal osteoporosis. *N.Engl.J. Med.*, **322,** 1265-1271.

Thamsborg, G., Storm, T. L., Sykulski, R., Brinch, E., Nielsen, H. K. & Sorensen, O. H. (1991) Effect of different doses of nasal salmon calcitonin on bone mass. *Calcif.Tissue Int.*, **48,** 302-307.

Thompson, D. D., Simmons, H. A., Pirie, C. M. & Ke, H. Z. (1995) FDA Guidelines and animal models for osteoporosis. *Bone*, **17,** 125S-133S.

Tikkanen, M. J., Wahala, K., Ojala, S., Vihma, V. & Adlercreutz, H. (1998) Effect of soybean phytoestrogen intake on low density lipoprotein oxidation resistance. *Proc. Natl.Acad.Sci.U.S.A*, **95,** 3106-3110.

Tokunaga, T. (2004) Novel physiological function of fructooligosaccharides. *Biofactors*, **21,** 89-94.

Tomkinson, A., Reeve, J., Shaw, R. W. & Noble, B. S. (1997) The death of osteocytes via apoptosis accompanies estrogen withdrawal in human bone.*J. Clin. Endocrinol. Metab.*, **82,** 3128-3135.

Tucci, J. R. (1998) Osteoporosis update. *Med.Health. R.I.*, **81,** 169-173.

Turner, A. S. (2001) Animal models of osteoporosis--necessity and limitations. *Eur.Cell Mater.*, **1,** 66-81.

Vogelvang, T. E., van der Mooren, M. J. & Mijatovic, V. (2004) Hormone replacement therapy, selective estrogen receptor modulators, and tissue-specific compounds: cardiovascular effects and clinical implications. *Treat.Endocrinol.*, **3,** 105-115.

Wangen, K. E., Duncan, A. M., Merz-Demlow, B. E., Xu, X., Marcus, R., Phipps, W. R. & Kurzer, M. S. (2000) Effects of soy isoflavones on markers of bone turnover in premenopausal and postmenopausal women. *J.Clin.Endocrinol.Metab.*, **85,** 3043-3048.

Watts, N. B., Harris, S. T., Genant, H. K., Wasnich, R. D., Miller, P. D., Jackson, R. D., Licata, A. A., Ross, P., Woodson, G. C., III, Yanover, M. J. & . (1990) Intermittent cyclical etidronate treatment of postmenopausal osteoporosis. *N.Engl.J. Med.*, **323,** 73-79.

Weaver, C. M. & Cheong, J. M. (2005) Soy isoflavones and bone health: the relationship is still unclear. *J. Nutr.*, **135,** 1243-1247.

Wei, H., Bowen, R., Cai, Q., Barnes, S. & Wang, Y. (1995) Antioxidant and antipromotional effects of the soybean isoflavone genistein. *Proc.Soc.Exp.Biol.Med.*, **208,** 124-130.

Westerlind, K. C., Wronski, T. J., Ritman E.L., Luttges, M. W., An, K. N., Bell, N. H. & Tuner, R. T. (1997) Estrogen regulates the rate of bone turnover but bone balance in ovariectomized rats is modulated by prevailing mechanical strain. *Proc.Natl.Acad.Sci USA*, **8,** 4199-4204.

Wiechmann, A. F. (1996) Recoverin in cultured human retinoblastoma cells: enhanced expression during morphological differentiation. *J. Neurochem.*, **67,** 105-110.

World health Organization: Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. Report of the WHO Study Group. 843, 1-129. 1994. World Health Organ.Tech.Rep.Ser.

Wronski, T.J., Lowry, P.L., Walsh, C.C. & Ignaszewski, L.A. (1985) Skeletal alterations in ovariectomized rats. *Calcif.Tissue Int.*, **37,** 324-328.

Wronski, T.J. & Yen, C.F. (1991) The ovariectomized rat as an animal model for postmenopausal bone loss. *Cells and Materials*, **1 (suppl),** 69-74.

Xu, X., Harris, K. S., Wang, H. J., Murphy, P. A. & Hendrich, S. (1995) Bioavailability of soybean isoflavones depends upon gut microflora in women. *J. Nutr.*, **125,** 2307-2315.

Yanovski, J. A., Sovik, K. N., Nguyen, T. T. & Sebring, N. G. (2000) Insulin-like growth factors and bone mineral density in African American and White girls. *J. Pediatr.*, **137,** 826-832.

Yildirim, Z. & Johnson, M. G. (1998) Characterization and antimicrobial spectrum of bifidocin B, a bacteriocin produced by Bifidobacterium bifidum NCFB 1454 *J. Food Prot.*, **61,** 47-51.

VITA

Latha Devareddy

Candidate for the Degree of

Doctor of Philosophy

Thesis: SOY ISOFLAVONES MAY REVERSE BONE LOSS IN AN OVARIECTOMIZED RAT MODEL OF POSTMENOPAUSAL OSTEOPOROSIS

Major Field: Human Environmental Sciences-Nutritional Sciences

Biographical:

Education:

2002-2005: Doctor of Philosophy, Department of Nutritional Sciences, College of Human Environmental Sciences, Oklahoma State University, Stillwater, OK.
1999- 2002: Master of Science in Human Nutrition, Nutritional Sciences Department College of Human Environmental Sciences, Oklahoma State University, OK.
1996 – 1998: Master of Science in Foods and Nutrition, Queen Mary's College, Madras, India
1993 – 1996: Bachelor of Science in Nutrition, Dietetics and Food Service Management, Madras University, Madras, India.

Experience:

2003-2005: *Study Coordinator*, Native American Women's Cholesterol Study, Department of Nutritional Sciences, College of Human Environmental Sciences, Oklahoma State University, Stillwater, OK.
1999 – Present: *Graduate Research Assistant*, Department of Nutritional Sciences, College of Human Environmental Sciences, Oklahoma State University, Stillwater, OK.
1998 – 1999: *Dietician,* Voluntary Health Sciences, Madras, India
1998 – 1999: *Research Assistant,* Diabetes Department, Voluntary Health Sciences, Madras, India

Professional Memberships:

Kappa Omicron Nu

Name: Latha Devareddy Date of Degree: July, 2005

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: SOY ISOFLAVONES MAY REVERSE BONE LOSS IN AN OVARIECTOMIZED RAT MODEL OF POSTMENOPAUSAL OSTEOPOROSIS

Pages in Study: 99 Candidate for the Degree of Doctor of Philosophy

Major Field: Human Environmental Sciences-Nutritional Sciences

Scope and Method of Study: Soy protein is reported to prevent bone loss in both women and rat models of osteoporosis. However, the role of soy isoflavones on the trabecular microarchitectural properties needs to be explored. In the present study, we examined whether soy protein with graded doses of isoflavones reverses loss of bone mineral density (BMD), bone mineral content (BMC), and trabecular microstructure in an ovariectomized (Ovx) osteopenic rat model. Seventy-eight 9-m old female Sprague-Dawley rats were either sham-operated (Sham; 1 group) or Ovx (5 groups) and fed a semi-purified casein-based diet. After 90 days the occurrence of bone loss was confirmed using dual energy x-ray absorptiometry. Thereafter, rats were assigned to the following treatments: Sham, Ovx (control), Ovx+17β-estradiol (E_2; 10 μg/kg body wt. twice per wk), Ovx + soy protein depleted of isoflavones (Soy-; 0.06 mg isoflavones/g protein), Ovx + soy protein with normal isoflavone content (Soy; 3.55 mg isoflavones/g protein), and Ovx + soy protein enriched isoflavones (Soy+; 7.10 mg isoflavones/g protein).

Findings and Conclusions: After 125 days of treatment, rats were euthanized and tissues were collected for the assessment of BMD and BMC, and tibial and 4^{th} lumbar trabecular micro-architectural properties via x-ray microcomputed tomography. Soy significantly increased tibial BMC and BMD by 10 % and 4.5% in comparison with Ovx control, whereas the effects of Soy-, Soy+ and E_2 were less pronounced. All of the soy-based diets, irrespective of their isoflavone content, had a modest effect on the lumbar BMD. However, only the Soy+ diet positively affected the tibial architectural properties including trabecular thickness, separation, and number. None of the treatments had any effect on trabecular microarchitectural properties of the fourth lumbar vertebra. In summary, our findings suggest that isoflavones may exert a biphasic effect on tibial BMD, and higher doses of isoflavones may be required to reverse the loss of tibial microstructural properties.

ADVISER'S APPROVAL: _____ (Dr. Bahram H Arjmandi)