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The Effects of Soy Protein on Bone Mineral Density on Ovariectomized Rats

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A Thesis

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ABBREVIATIONS

AIN	American Institute of Nutrition
AIN-93M	American Institute of Nutrition Modified
ANOVA	Analysis of variance
BGP	Bone glutamic protein
BMC	Bone mineral content
BMD	Bone mineral density
BUD	Broadband ultrasound
°C	Celsius
cm	Centimeter
d	Day
DEXA	Dual energy x-ray absorptiometry
e.g.	exempli gratia (for example)
ER	Estrogen receptors
ERT	Estrogen replacement therapy
FDA	Food and Drug Administration
FSH	Follicle-stimulating hormone
g	Gram
HDL	High density lipoprotein cholesterol
HOPE	Health, Osteoporosis, Progestin, Estrogen trial
HRT	Hormone replacement therapy
i.e.	id est (that is)
kg	Kilogram

KHz	Kilohertz
L	Liter
LH	Luteinizing hormone
LSU	Louisiana State University and Agricultural & Mechanical College
MGP	Matrix glutamic protein
mg	Milligram
ml	Milliliter
NORA	National Osteoporosis Risk Assessment
OC	Ovariectomized, Casein diet
O5S	OVX, 5% soy diet
O10S	OVX, 10% soy diet
O15S	OVX, 15% soy diet
OH	OVX, High casein and high fat diet
pDEXA	Peripheral dual energy x-ray absorptiometry
PEPI	Postmenopausal Estrogen/Progestin Intervention
OCT	Quantitative computed tomography
QUS	Quantitative ultrasound scans
OVX	Ovariectomy, ovariectomized
SD	Standard deviation
SERM	Selective estrogen receptor modulator
ShC	Sham-operated
SPA	Single photon absorptiometry
SXA	Single energy x-ray absorptiometry

µ	Micro
µl	Microliter
WHO	World Health Organization

DEFINITIONS

ad libitum - freely

bone mineral content - a measure of bone mass expressed as the content of mineral per centimeter of bone; used to assess the amount of bone accumulated prior to cessation of growth

bone mineral density- a measure of bone density expressed in grams per centimeter squared (area density); used to assess the amount of bone after the developmental period is complete

DEXA - instrument used to measure bone density expressed as grams of bone mineral per square centimeter and bone mineral content expressed as grams of bone mineral per cubic centimeter; considered the gold standard for bone densitometry

hyperphagia - overeating

isoflavone - a subclass of phenol phytochemicals, found in beans and other legumes (especially soybeans), that may have bone-protection properties

menopause - the permanent cessation of menstruation due to loss of ovarian follicular function clinically diagnosed after twelve months of amenorrhea

osteopenia - low bone mass

osteoporosis - loss of bone tissue to the point that the specific skeletal site is unable to sustain ordinary strains

ovariectomy - surgical procedure to remove the ovaries

perimenopause - a period of changing ovarian function preceding final menses by between two and eight years

phytoestrogen - estrogen-like molecules derived from soybeans, clover, and other plant sources; act on estrogen receptors more like selective estrogen receptor modulators than true estrogens

ABSTRACT

The accelerated rate of bone loss that occurs after menopause can be ameliorated by estrogen replacement therapy or possibly by consuming soy protein, which contains phytoestrogens. A dose-response to three levels of soy protein (5%, 10%, or 15%) was evaluated in a rat model of postmenopausal women. Retired Sprague-Dawley breeder rats were stratified by weight and assigned randomly to one of five groups for an thirteen week study. Rats were either ovariectomized (OVX, n=40) or sham-operated (ShC, n=10) and assigned to control diets (OC, n=10 or ShC, n=10) or one of three levels of soy (O5S, n=10; O10S, n=10; O15S, n=10). Bone mineral density (BMD) of the vertebrae, humerus, femur, and tibia were measured by peripheral dual energy x-ray absorptiometry (DEXA). Increasing levels of soy protein caused increasing BMD in a dose dependent fashion. Vertebral BMD values (g/cm^2) were: ShC=0.1798 $\pm$ 0.0116; C=0.1755 $\pm$ 0.0194; 5S=0.1803 $\pm$ 0.0183; 10S=0.2013 $\pm$ 0.0254; 15S=0.2016 $\pm$ 0.0293. Bone mineral density for the O10S and O15S groups was significantly greater than both the ShC or OC groups ($p<0.05$). Food intake, total body weight, abdominal fat, heart, spleen, and kidneys were not affected by diet or surgery; however, differences were observed in the liver and uterus. Soy protein, fed as a sole source of protein or as 2/3 of the dietary protein, prevented vertebral bone loss in OVX rats suggesting there may be beneficial effects in humans as well.

CHAPTER 1

INTRODUCTION

Overview

In past decades, menopause was not the health concern that it is today. At the turn of the 20th century, life expectancy for women in the United States was approximately 48 years. As of 1999, women can expect to live to 79.4 years (1). The average age for the onset of menopause is about 51 years (2). Although there has been an increase in life expectancy over the years (1), the age of menopause has not changed over the past few centuries (2). In previous centuries, few women lived beyond menopause; whereas, today, most women in the United States will spend one-third of their life post-menopausal (2). The North American Menopause Society reported that as of July 2000, there were over 40 million U.S. women who had reached natural menopause (2). Surgical menopause, which refers to induced menopause following surgical removal of both ovaries in a woman who is still menstruating, accounts for 481,000 cases of menopause (2). This is the first time in human history where postmenopause existence has been witnessed on such a large scale (3).

Many deleterious health effects are associated with menopause. One of these effects is a decrease in bone mass leading to osteoporosis (4). Osteoporosis is defined as a decrease in the amount of bone, leading to fractures after minimal trauma (4). Osteoporosis is an enormous public health problem and is responsible for at least 1.2 million fractures in the U.S. each year. Hip fractures, one of the most common and catastrophic types of fracture, is fatal in 12% to 20% of cases (5) and results in long-term

nursing home care for half of those who survive (4). The direct and indirect costs of osteoporosis are estimated at $6.1 billion annually in the U.S (6).

The sharp withdrawal of ovarian estrogen production is the predominant cause of rapid bone loss during menopause and increases a woman's risk for osteoporosis (7, 8). Studies have shown that estrogen replacement therapy (ERT) is the most effective way to reduce the rate of postmenopausal bone loss; however, ERT may be accompanied by severe side effects, notably endometrial and breast cancer (8, 9, 10). It is important to find a treatment that will prevent or minimize bone loss in menopausal women without these adverse effects.

Interest in soy isoflavones has exploded in the past five years as studies have suggested that phytoestrogens found in soybeans may have beneficial health effects (11, 12, 13, 14), including improvement of bone health (11, 15, 16). Much of this interest has been directed to women's health and is stimulated by promising data from clinical studies showing that phytoestrogen-rich soy protein-containing foods are effective in a variety of hormone-related conditions (14). Animal studies and in vitro bone cell studies have suggested that isoflavones have significant direct and indirect hormonal and non-hormonal effects, such as reducing risk of osteoporosis; ameliorating symptoms of menopause, such as hot flashes; lowering serum total cholesterol levels; and preventing cancer (17, 18, 19, 20).

Ovariectomy (OVX) reduces the circulating estrogen levels in rats in a manner similar to the way menopause reduces circulating estrogen levels in humans (21). Since the life span of rats is much shorter than that of humans, health effects of reduced estrogen levels can be observed within weeks after OVX compared to months or years

after menopause in humans (21). The OVX rat is an excellent and established model for studying the effects of hormonal changes on bone associated with menopause (22).

Justification

As life expectancy continues to increase, there is a pressing need to reduce the risk of osteoporosis that accompanies menopause. Preventing bone loss using naturally occurring estrogenic isoflavones found in soybeans may be effective in reducing bone loss due to estrogen deficiency without the undesirable side effects of other treatments.

Study Objective

The objective of this study was to use retired OVX female breeder rats as a model of postmenopausal women to measure the effects of increasing levels of soy protein on bone density.

Hypotheses

The hypotheses of this study were that:

1. OVX will contribute to an increase in food intake, body weight, and abdominal fat.
2. OVX or the soy protein diet will not affect mean weights of the liver, heart, kidney, spleen, or abdominal fat.
3. OVX will decrease mean uterine weight.
4. Soy protein will not affect mean weight of the uteri in OVX rats.
5. OVX in Sprague-Dawley rats will contribute to bone loss in the vertebrae, humerus, femur, and tibia.

6. Bone mass in the vertebrae, humerus, femur, and tibia of the OVX rats will be preserved with in a dose-dependent fashion with dietary soy protein.

Assumptions

- OVX rats are an appropriate model for human menopause.
- OVX in rats produces similar hormonal changes as natural menopause does in women, subsequently resulting in parallel or equivalent changes in bone mineral density (BMD).
- The sample size was adequate to detect changes in bone density.
- Peripheral dual energy x-ray absorptiometry (pDEXA) is an accurate and precise method for determining bone mineral density.
- Uterine atrophy could be used to confirm OVX.

Limitations

- Food spillage had to be estimated, which may have contributed to inaccurate food intake measurements.
- The study may not have been long enough to examine fully the effect of soy isoflavones on bone density in rats.
- Unavoidably, the pDEXA machine had to be changed after the first set of scans; although, each machine was standardized rigorously, it is known that changes in machinery can influence bone scan data.
- Placement on of the rats and individual bones on the pDEXA platform for scanning may have been inconsistent due to multiple laboratory assistants performing scans on the rats.

Biochemical markers to confirm changes in BMD were not performed.

CHAPTER 2

REVIEW OF LITERATURE

Laboratory animals as models for menopause

An animal model of postmenopausal bone loss can be defined as a living animal in which spontaneous or induced bone loss due to ovarian hormone deficiency can be studied, and in which the characteristics of the bone loss resemble those found in postmenopausal women in many ways (21). Ovariectomized nonprimate animals are often used as models of menopause because they develop pathophysiologic changes similar to those seen in postmenopausal women (23). Rats, for example, can be ovariectomized (OVX) to make them sex-hormone deficient and to stimulate the accelerated bone loss that occurs in women after menopause (21). Bone loss in OVX rats and postmenopausal bone loss has similar characteristics, including increased rate of bone turnover with resorption exceeding formation; a rapid phase of bone loss initially followed by a much slower phase; greater loss of trabecular than cortical bone; decreased intestinal absorption of calcium; some protection against loss by obesity; and similar responses to hormonal therapies (22, 24). This broad range of similarities provides powerful evidence that the OVX rat bone loss model is appropriate for studying health problems associated with postmenopausal bone loss (21).

Bone composition

Bone is both an organ and a living tissue. Bones consist of a matrix of organic materials, primarily collagen fibers. Calcium and phosphate salts combine with hydroxyl ions to form hydroxyapatite crystals. Collagen and hydroxyapatite combine to give bones their strength. Other components of the bone matrix include osteocalcin,

osteopontin, and several other matrix proteins (25). Most bones of the human body are composed of two layers: the outer layer, or cortical bone, surrounds the inner trabecular bone. Other bones also contain a cavity for bone marrow. The cortical bone is compact and comprises approximately 75% of the total bone in the body. It consists of layers of mineralized collagen and is found mainly in the shafts of the long bones of the limbs. Trabecular bone has a sponge-like appearance and constitutes about 25% of the skeleton in the human body (26). It is found primarily in the knobby ends of the long bones, the vertebrae, the wrists, and the pelvic area (4). The occurrence of fractures late in life is due to the loss of trabecular bone (25). Bone tissues are subjected to bone modeling during vertical growth and bone remodeling after growth ceases (25).

Bone remodeling

Throughout its lifetime, bone is continuously subjected to a process of resorption and formation known as remodeling, or turnover; this required for the maintenance and overall health of bone (27). The two types of bone cells primarily involved in bone remodeling are osteoblasts and osteoclasts. Osteoblasts are responsible for the formation of bone tissue, while osteoclasts aid in resorption or breakdown of bone. Normally, osteoblastic activity and osteoclastic activity balance each other; therefore, there are no obvious changes in bone (28). Bone mass increases until about the age of thirty. Afterwards, a period of stability occurs for five to ten years where osteoblast and osteoclast activity remain in balance until the onset of age-related bone loss (29, 30). The decrease in bone mass is due to accelerated osteoclastic activity resulting in a gradual loss of bone mineral mass (28).

Bone mineralization

Mineralization, involving primarily calcium, phosphorus, fluoride, sodium, and magnesium, is required for all bones. Minerals constitute approximately 63% of bone weight; about 37% of bone weight is due to water and proteins, such as collagen, osteonectin, osteopontin, osteocalcin or bone glutamic acid (BGP), and matrix glutamic acid protein (MGP). Bone glutamic acid and MGP require vitamin K for proper calcium binding. Calcitriol, the active form of vitamin D, stimulates biosynthesis of osteocalcin. Bone glutamic acid, the major noncollagen protein found in bone, is secreted by osteoclasts during bone formation. It appears in the bone at the onset of hydroxyapatite deposition and may be involved in bone remodeling and calcium mobilization. The exact biological function of osteocalcin is uncertain at present (25, 26). Some osteocalcin, however, is released into the blood and has been used as a marker of bone formation. Levels of osteocalcin in the blood are directly related to bone formation (26).

Physiology of perimenopause

Perimenopause is defined as a period of changing ovarian function (31, 32). It precedes the final menses, or postmenopause, by two to eight years. The physiology and the clinical manifestations of perimenopause are not well understood, but it is suspected to occur in stages (31).

In early perimenopause, the neurohormonal systems that control ovulation begin to become dysregulated without obvious changes in menstrual cycle length. Irregular menses with increased periods of amenorrhea may characterize middle to late perimenopause. The beginning of perimenopause is characterized by three to eleven months of amenorrhea or for those without amenorrhea, an increase in menstrual

irregularity. While menopause has a clear and accepted definition, perimenopause does not (31, 32).

During perimenopause, follicle-stimulating hormone (FSH) concentrations may increase to a postmenopausal range during some cycles but decline to premenopausal concentrations during subsequent cycles (33). In addition to irregular menstrual cycles during perimenopause, some women experience common postmenopause symptoms, such as hot flashes and unfavorable lipid profiles. Moreover, a decrease in bone mineral density (BMD) may occur during the years before menopause (34, 35, 36). The timing of onset of bone loss in relation to menopause is not known.

The decrease of BMD during perimenopause is controversial, which could be related to the type of study, i.e. cross-sectional or longitudinal, to the short duration, or to small, inadequate sample size. In a cohort study of 272 premenopausal and perimenopausal women ages 31-89, BMD decreased rapidly in trabecular and cortical bones (i.e. femur neck, trochanter, and lumbar spine) at the end of three years in the perimenopausal women (37). The decrease of BMD in the perimenopause women was attributed to a decrease in estrogen secretion. There was no significant bone loss in the premenopausal women (37).

The physiology of menopause

The literal meaning of the term menopause is "pause in the menses" and refers to the permanent cessation of the menstrual cycle (3, 31, 32). Menopause occurs in women at about age 51. In a newborn female, the ovaries contain about 2 million oocytes or immature ovum. Oocytes are contained in ovarian follicles. By puberty, the number of oocytes and follicles decreases to about 350,000. During a woman's reproductive years,

only about 400 oocytes will be released from the follicles in a process known as ovulation. The anterior pituitary gland produces and secretes two hormones involved in the structure and function of the follicle: follicle stimulating hormone (FSH) and luteinizing hormone (LH) (38). Under stimulation of FSH, the ovarian follicle forms a bulge on the surface of the ovary. This growth is accompanied by a rapid rate of estrogen secretion. The increased production of estrogen triggers LH, which forces the follicle to rupture and releases its oocyte into the uterine tube; thus, ovulation occurs due to the sequential effects of FSH and LH on the ovarian follicles (39).

After ovulation, LH stimulates the empty follicle to undergo structural and biochemical changes to become a corpus luteum. The corpus luteum secretes both estrogen and progesterone. These high levels of estrogen and progesterone result in negative feedback inhibition of FSH and LH secretion, which serves to stop the development of new follicles and prevents multiple ovulations during the same cycle. Levels of estrogen and progesterone fall to very low levels when the corpus luteum regresses and stops functioning. Inhibin, another hormone secreted by the corpus luteum, also surpresses FSH secretion. This withdrawal of ovarian hormones causes menstruation and allows a new cycle of follicle development to occur (39).

Menopause, also referred to as postmenopause, is clinically defined as the cessation of menses for at least 12 months; however, some researchers consider a lack of menses for 6 months as an indication of menopause onset (3, 40). Once menopause occurs, the ovaries contain very few follicles and production of estrogen diminishes rapidly to extremely low levels. An increase in the secretion of FSH and LH by the pituitary gland results from a lack of negative feedback. A plasma FSH level >30-50

International Units per liter is used to confirm menopause in addition to a lack of menses for a set period of time (3, 40). It is the reduction of estrogen secretion from the ovaries that causes the many symptoms of menopause. Although most women experience menopause as a normal physiologic process with no obvious physical or psychological symptoms, some women experience a variety of symptoms associated with menopause (3).

Effects of menopause

The decrease in estrogen results in symptoms of menopause, which include "hot flashes" or extreme flushing of the skin, irritability, fatigue, loss of memory, insomnia, urogenital atrophy, anxiety, and occasional psychotic state. There is also increase risk of heart disease and increase progression of osteoporosis (3, 40).

Central Nervous System

Estrogen receptors are widely distributed throughout many parts of the central nervous systems (41). Estrogen regulates the synthesis and secretion of neuropeptides, neurotransmitters, and noradrenergic transmission in the brain. Estrogen enhances sensory perception, locomotor activity, limb coordination, and balance (42). An estrogen deficiency reduces serotonin synthesis, which may cause insomnia (43).

Vasomotor System

One of the most obvious symptoms of menopause is the instability of body temperature resulting in hot flashes and flushing of the skin (31). Hot flashes result from vasodilation of the skin capillaries and usually effect the chest and facial areas. Skin temperature increases 1-2° Celsius (C) (3). Episodes generally last from 30 seconds to 5 minutes (31) and can vary in frequency and severity (3). Hot flashes usually regress

spontaneously. About 25-50% of women will continue to suffer from symptoms for up to 5 years following menopause (3).

Urogenial atrophy

The loss of estrogen affects the reproductive tract, resulting in vaginal dryness, atrophy of the endometrial lining, cervix, and vagina. The bladder and urethra are also affected by the reduction of estrogen leading to incontinence, increase in frequency of cystitis, and dysuria (40).

Coronary artery disease

Coronary artery disease is the leading cause of death in American women (3). The risk of coronary artery disease increases significantly after menopause, suggesting that estrogen may play a protective role. Other factors contributing to increased risk of coronary heart disease due to estrogen deficiency after menopause includes hypertension, a rise in total cholesterol and triglyceride levels, and increased coagulation factors (3). A full description of this is beyond the scope of this thesis.

Osteoporosis

Definition of osteoporosis

Osteoporosis, a term that literally means "porous bone," is usually defined as a skeletal disease resulting from a combination of low bone mass, or osteopenia, and low impact fractures (44-49). The decrease in bone mass results in the inability of the skeleton to sustain ordinary strains, causing a high incidence of low trauma fractures (3, 50).

The Consensus Development Conference defined osteoporosis as "a systemic skeletal disease characterized by low bone mass and microarchitectual deterioration of

bone tissue with a consequent increase in bone fragility and susceptibility to fracture" (50-53). This internationally agreed upon definition considers that low bone mineral density is an important factor of fracture risk but recognizes that other abnormalities in the skeleton contribute to skeletal fragility (45, 46, 49, 52). Bone mineral density measurements are used to diagnose osteoporosis, as well as to assess risk factors for fractures (52).

Pathophysiology of osteoporosis

Bone mineral density is correlated with peak bone mass and subsequent bone loss. Bone continuously cycles through resorption and reformation throughout life. From birth, bone mass builds wherein bone formation exceeds resorption. Peak bone mass is achieved by about age 30 years followed by a plateau for 5 to 10 years. In older adults, the rate of resorption and formation, or bone remodeling, increases. Osteoporosis occurs when the rate of resorption exceeds the rate of formation, resulting in a reduction in bone mass or too little bone (47, 50, 51, 53-55). Bone composition is unchanged, but the amount of bone per unit volume is reduced (48, 50, 54).

Bone remodeling occurs at distinct skeletal sites, particularly cortical and trabecular bone (55). Bone resorption always follows bone formation, a phenomenon known as coupling. The process of bone resorption followed by synthesis of bone matrix and mineralization, takes up to eight months (47, 51). Remodeling imbalance occurs when bone resorption does not match bone formation, resulting in bone loss (47, 50, 53, 54).

Osteoporotic fractures tend to occur at sites composed of more than 50% of the more metabolically active trabecular bone (47). High incidences of fractures occur in the

vertebrae, wrist, and hip (44, 47, 52, 53). Osteoporosis in women is particularly associated with menopause, since the loss of estrogen accelerates bone loss, especially trabecular bone (50). Age-related bone loss accelerates to about 0.5% per year (3, 4, 54). Immediately following menopause, a period of increased bone loss of about 4% per year has been recognized, effecting mainly trabecular bone. This lasts for approximately four years (3, 4, 48). Afterwards, the yearly rate of bone loss slows to 1%. Without hormone replacement therapy, A woman will have lost 30-50% of her bone mass by age 80 (3, 45).

Prevalence of osteoporosis

Osteoporosis affects 25 million Americans and is the cause of 1.2 million fractures per year of the hip, spine, and wrist (31, 48, 51). Of the 1.2 million fractures, 250,000 are hip fractures, 240,000 are wrist fractures, and 500,000 are spinal fractures. Hip fractures cause the most serious medical complications of osteoporosis (48).

After age 65, 1 in 2 women and 1 in 5 men develop osteoporosis-related fractures (51). Osteoporotic fractures are an important cause of disability (31). A hip fracture increases the likelihood by 5 to 20% that an older person will die within one year (48, 51, 56). Among survivors, 15 to 25% of those who had lived independently prior to the fracture will reside in a long-term care facility for one year after a hip fracture. Up to 20% of hip fracture cases in those 80 years old or older lead to death (48).

In 1995 in the United States alone, the cost of managing osteoporosis fractures was approximately $13.8 billion (48, 57). Costs are expected to increase due to an increase in the size of the population age 50 years or older (aging of the baby boomers) and an increase in longevity (48, 57).

Types of osteoporosis

There are two primary types of osteoporosis. The types are classified by the gender of the individual, the age at which fractures occur, and the kinds of bone involved (48). Type 1, or postmenopausal osteoporosis, affects women 15 to 20 years after menopause. It involves primarily loss of trabecular bone tissue due to the cessation of estrogen production. Men rarely develop type 1 osteoporosis if they have a significant decline in androgen production (48). Type 1 osteoporosis is characterized by fractures of the distal radius (Colles' fracture) and crush fractures of the lumbar vertebrae. The rapid rate of bone loss that occurs in women after menopause is directly related to the lack of estrogen (47). Bone with high trabecular bone content, such as the pelvis, and the proximal femur are also affected by postmenopausal osteoporosis (47).

Type 2, or senile osteoporosis, occurs initially at age 70 years for women and 80 years for men. Senile osteoporosis is related to age and is characterized by both cortical and trabecular bone loss. In the elderly, the processes of bone resorption and bone formation become unbalanced; formation is decreased resulting in resorption exceeding formation (47). Fractures occur in the hip and vertebrae with the incidence of vertebral fractures continuing to increase with age. Vertebral fractures lead to back pain, loss of height, spinal deformities, and kyphosis or "dowager's hump." Many women lose several inches in height between 50 and 80 years of age (3, 51). A loss of more than about 1.5 inches in height is typical of osteoporosis (51). Fractures may occur during ordinary activities, such as raising a window or even turning over in bed (47, 51). A large percentage of hip fractures result from a fall (47).

Senile osteoporosis affects both genders, but women are more severely affected because they have a smaller skeletal mass than men do and they live longer than men. Hip fractures affect nearly 20% of postmenopausal women up to age 80 and almost 50% of those beyond that age (65).

Secondary osteoporosis results from drugs or diseases that cause bone loss. It accounts for about 20% of cases in women and 40% of cases in men (65).

Diagnosis of osteoporosis

Bone mineral density measurements are useful tools for diagnosing osteoporosis and in decisions about starting therapy (53). Since bone mass is nearly normally distributed in a given healthy population (51, 53), peak bone mass of healthy young adults has become the standard by which BMD is compared in terms of standard deviation (SD) units around the mean. World Health Organization (WHO) criteria for low BMD are based on BMD measurements at the forearm of healthy, white young adult women aged 20 to 29 years old. T scores are calculated as follows:

$$\text{T score} = \frac{\text{BMD of participant - mean BMD of reference population}}{\text{SD of BMD of reference population}}$$

A T score of zero means that the measured BMD is equivalent to the mean peak BMD of the reference population. Osteopenia is defined as a T score between -1.0 to -2.49 SD units below the average for the reference population. A T score of -2.5 SD units or more below that of young healthy adults indicates osteoporosis (3, 51-53, 58). The relationship between decreasing BMD and fracture risk is exponential, so that fracture risk more than doubles (increases by 2.5) for every SD that BMD is below normal. By the same standard, a person whose BMD is four SD below the normal mean has a 10-fold increase in fracture risk (51).

Risk factors for osteoporosis

There are many risk factors associated with bone loss. Female gender, positive family history, petite or thin frame, and early menopause (before the age of 45 years) increases the risk of osteoporosis (51, 53, 58). African Americans have a higher peak bone mass than those of Caucasian and Asian races. Thus, the incidence of osteoporosis and related fractures in African American women is half that of Caucasian women. (51).

Estrogen deficiency contributes to osteoporosis in women. Oophorectomy or use of gonadotropin-releasing hormones that suppress ovarian function is associated with accelerated bone loss (59). Use of HRT decreases the rate of vertebral, hip, and wrist fractures (60, 61).

Decreased calcium absorption can also promote osteoporosis. A decrease in serum levels of calcitriol can decline by as much as 50% with age because of decreased renal enzyme activity, decreased sunlight exposure, and decreased dietary intake (44, 51, 53). A negative calcium balance as small as 30 milligrams per deciliter (mg/dl) can lead to a 30% loss of bone mass over 30 years (51).

Lifestyle factors that contribute to bone loss by decreasing osteoblastic activity or bone formation include sedentary lifestyle and alcohol intake. Smoking may increase hepatic metabolism of estrogens (51, 53, 58).

Some drugs can also increase bone loss. Glucocorticoids, antiepileptic drugs, excessive substitution therapy (i.e., thyroxine, hydrocortisone), and anticoagulant drugs (i.e., warfin, heparin) can cause calcium to leach out of bones, resulting in bone loss. Endocrine, hematologic, rheumatologic, and some gastrointestinal diseases are also risk factors for osteoporosis (48, 53).

The largest study of postmenopausal osteoporosis conducted in the United States was the 19-month longitudinal observational study conducted by the National Osteoporosis Risk Assessment (NORA) of 200,160 ambulatory postmenopausal women aged 50 years or older who had no previous diagnosis of osteoporosis (53, 58). The objectives of the study were to determine the occurrence of low BMD in postmenopausal women, its risk factors, and fracture incidence during the 12-month follow up. The study concluded that low body weight (<58 kilograms), current smoking, first degree relative with low trauma fracture and personal history of low trauma fracture could be used to identify women at risk for fracture (53, 58).

Prevention of osteoporosis

Prevention of osteoporosis should be given high priority because current therapy for osteoporosis is insufficient and osteoporotic bone cannot be restored to normal. Osteoporosis is a public health problem (62). Preventative measures are needed throughout life. Some risk factors may be present in childhood or adolescence. For example, optimal peak bone mass may not be reached as a result of low calcium intake during growing years (62. Many factors for bone loss may occur later in life (63, 64). For most individuals, preventative lifestyle factors and HRT in postmenopausal years are appropriate preventative measures. The small number of individuals whose osteoporosis is secondary to drug therapy or disease require guidance on an individual basis specific to their condition (48).

The goal in prevention of osteoporosis should be to increase peak bone mass and to reduce the rate of bone loss (47). Hormone replacement therapy is the most effective preventative treatment against osteoporosis. Therapy against bone loss should be

targeted to postmenopausal women whose BMD at the lumbar spine or hip is more than one SD below the mean for their age. Women with BMD above the mean for young adults probably do not require HRT for prevention of osteoporosis. Women with intermediate bone densities may benefit from HRT if they lose bone at a faster rate than average. This can be determined from repeat measurements of BMD after two years (47).

Bone densitometry

Measuring bone density, or bone densitometry, is useful in predicting sites of high fracture risk and monitoring response to treatment (66). Bone density techniques measure bone mineral composition (BMC) as either surface density in grams per centimeter squared (g/cm^2) or as volumetric density in grams per centimeter cubed (g/cm^3). Reduced bone mass is an important predictor of fracture risk even though it is not the only indicator of bone strength (67).

Bone densitometry measurements should be accurate and precise. Accuracy refers to the difference in the measurement from a known standard. Precision is defined as the observed deviation of a sequence of measurements over time (66). Regardless of the bone densitometry technique chosen, it is necessary that measurements be made using the same method, the same instrument, and the same testing site (67).

Measurement of bone mass

Osteopenia, or low bone mineral density, is a precursor to osteoporosis. Preventing osteoporosis involves detecting the asymptomatic disease in its early stages. (68). Techniques used to measure bone density include quantitative ultrasound scans (QUS), single photon and single energy x-ray absorptiometry (SPA, SXA), quantitative

computed tomography (QCT), and dual energy x-ray absorbtiometry (DEXA) (67, 69, 70).

Quantitative ultrasound scans (QUS)

Quantitative ultrasonography measures the velocity of sound through bone and the attenuation of the sound beam. The velocity and attenuation of sound waves are related to the biomechanical properties of the bone; therefore, ultrasound can provide information about the quality of the bone structure. When sound waves are transmitted through bone at frequencies of 200 to 600 kilohertz (KHz), the difference between the levels of transmissions at different frequencies through the bone provides information about bone structure. This is referred to as broadband ultrasound attenuation (BUA) (71) and is measured in decibels per megahertz (67). Normal bone has a higher attenuation than osteoporotic bone; both bone volume and hardness are closely correlated with attenuation. Additionally, the velocity of the sound wave through normal bone will be higher than through osteoporotic bone (67, 71).

Broadband attenuation measurements require a peripheral bone with little overlying soft tissue, such as a heel bone or patella. Results of broadband attenuation correlate with vertebral and femoral neck density measured by DEXA, single-photon absorptiometry of the distal forearm, and QCT of the lumbar spine (67). Quantitative ultrasound is limited to screening to predict the risk of osteoporotic fractures; it may not be suitable for the follow-up assessment of response to treatment because a 9% difference in BUA is needed to detect a change in bone density (70, 72, 73). Some researchers have suggested using ultrasound as a selective prescreening device for DEXA referrals (74).

The QUS method is beneficial to patients because it is quick, inexpensive, noninvasive, and does not use radiation (70).

Single photon and single energy x-ray absorptiometry

Single photon absorptiometry was developed in the 1960's and uses iodine-125 as a gamma ray source and a gamma counter or detector to measure bone density (67, 70). The size and shape of the beam is restricted due to collimation of the source and detector. The total bone mineral content in the scan path is calculated from the difference in absorption between bone and soft tissue. Bone mineral content is expressed as g/cm^2 (70). This method is limited to areas with minimal soft tissues, which include the radius and the calcaneus (heel or os calcis). To correct for overlying soft tissues, the area studied is placed in a water bath or surrounded by a material that can be molded around the extremity, such as a gel-filled bag (67, 70). Accuracy error for measurements of the radius range from 3% to 5% and 1% for precision error. The accuracy and precision errors of calcaneus measurements are less than 3% (70).

Single energy x-ray absorptiometry (SXA) is the x-ray equivalent of SPA. Similar to SPA, SXA uses a water bath to correct for overlying soft tissues (67, 70). Unlike SPA, SXA does not use a radioactive isotope; SXA is also less expensive than SPA (70). The distal radius is usually studied; however, the calcaneus can also be studied with SXA (67, 70).

Quantitative computed tomography

Before the introduction of DEXA, QCT was used to measure BMD (67). Quantitative computed tomography measures the amount of absorption of ionizing radiation by calcified tissue (44). Quantitative computed tomography measurements are

obtained using a general-purpose computed tomography scanner with a specialized software package and calibration phantom. A detector rotates around the body, making a series of measurements at a given point along a line. The point on the line is viewed from up to 1000 different directions (44). This process produces a map as a cross-sectional slice of the region of interest. Scans are usually performed on the spine and hip but computed tomography has been used to measure peripheral sites, which include the radius and proximal tibia. Computed tomography slices taken at the midvertebral area requires the patient to lie on a phantom containing tubes of varying concentrations of potassium hydrogen phosphate, which act as a reference standard. After scanning the vertebral area, the mean computed tomography number is measured in Hounsfield units (70). The mean is compared to the calibration tubes to produce a true volumetric measurement of BMD in g/cm^3 (44, 67, 70). Quantitative computed tomography produces a three dimensional analysis, representing a true density measurement (44, 67).

The use of an automated slice selection system (QCT) increases precision and decreases operator variance (70). Consistency is important because a one-millimeter change in the position of the selected scan site can cause a 1% difference in BMD (75). Software automatically defines the outer edges of the selected site and calculates the size of the region of interest, thus producing a more precise measurement (70). The accuracy of QCT is affected by marrow fat in the vertebra, which causes QCT to underestimate BMD (70). The accuracy of measurements decreases as the age of the patient increases because marrow fat increases with age. Calculations can correct for marrow fat errors and accuracy can be improved (70). Accuracy error of QCT measurements ranges from 5% to 15% (70).

Quantitative computed tomography is not as accurate as DEXA in measuring BMD, but it is extremely sensitive because only trabecular bone is measured (67, 75). Because trabecular bone is more metabolically active than cortical bone, detection of small changes in BMD can be detected with QCT. Early detection in small changes in BMD is especially important in postmenopausal women who have bone loss (44).

Dual Energy X-ray Absorptiometry

Since its introduction into clinical practice in 1987, dual energy x-ray absorptiometry (DEXA) has largely replaced other methods used in measuring bone density (67). Dual energy x-ray absorptiometry has become the "gold standard" for measuring bone density (51, 70, 76, 77). Generally, DEXA is used to measure BMD at the lumbar spine, proximal femur, and distal radius (67, 70). The DEXA uses a low radiation dosage of 1 μSv per site scanned. The dosage is slightly higher than background radiation, which enables the technologist to remain in the exam room without shielding (76). Dual energy x-ray absorptiometry calculates bone mass using photons produced by a radionuclide source or x-ray tube, and produces two energy levels of radiation that allow for different absorption by bone and soft tissue (51, 70, 77, 78). As the patient lies on the DEXA table, two x-ray beams pass through the body from below. Overhead, a scintillation counter detects and calculates the amount of radiation that passes through the body (78). Results of BMD measurements are expressed as grams of minerals per centimeter bone (g/cm). Bone mineral density is expressed as grams per centimeters squared (g/cm^2) and is calculated from bone mineral content (BMC) divided by the width of the bone at the measurement site (70, 77). DEXA printouts provide BMD values, as well as scores representing the number of standard deviations above or below

the mean in order to assess fracture risks (51). For each area examined, the BMD is compared with values for sex- and age-matched control subjects (Z score) and to normal, healthy young control subjects at peak bone mass (T score). Although the Z score analysis is important in evaluating the younger patient to determine the presence of osteopenia, the T score is the value used for clinical decision-making in the older population. If the Z score were used in the postmenopausal population, a patient might appear relatively normal when compared with their peers, and the incidence of osteoporosis would not rise with increasing age, despite decreasing bone mass and an increased incidence of fractures (67, 70).

Standard spine DEXA analysis includes values obtained in the anteroposterior lumbar spine for each vertebra from L1 to L4 and a total value for the four sites combined (67). For each site and for the total, the area (expressed in square centimeters), BMC (expressed in grams), and BMD (expressed in grams per square centimeters) are reported.

Scanning of the hip by DEXA evaluates BMD of the femoral neck, greater trochanter, intertrochanter region, and the total femur. Additionally, a BMD value is reported for Ward's triangle, which reflects the cancellous bone in the femoral neck. Until recently, the femoral neck was used for reporting the BMD of the hip. Consensus among manufacturers of the equipment that produces DEXA has now led to the use for the total femur, based on the National Health and Nutritional Examination Survey hip reference database (79).

In addition to providing information regarding BMD, DEXA may be used to determine total body composition including lean body mass, fat body mass, and total

body bone mineral (80). These determinations are now primarily used for research purposes only. Although it is possible to derive BMD measurements of specific bone sites from whole body analysis, these measurements have been shown to be inaccurate and should not replace site-specific measurements for documentation of regional osteopenia (67).

Advantages to this non-invasive technique include high accuracy and precision within 0.4%, better special resolution, reduced radiation exposure, and minimum scanning time (51, 66, 67). Clinically, the precision of DEXA is important because the BMD measurements representing bone loss and treatment of osteoporosis can be tracked over time (51).

A study comparing three-dimensional micro-computed tomography to DEXA and bone histomorphometry of bone mass in a rat model of disuse osteoporosis showed that bone mass and parameters measured with the three techniques were similar. Further, DEXA detected bone loss earlier than bone histomorphometry, indicating that DEXA is a sensitive and accurate measurement technique for bone mass (80).

Effects of hormone replacement therapy

The goal in preventing osteoporosis should be to increase peak bone mass and to reduce further rate of bone loss (47). Estrogen protects against bone loss and fractures and is considered to be the best therapy for both prevention and treatment of postmenopausal osteoporosis (47, 82, 83). For those women who have risk factors for osteoporosis, the Food and Drug Administration (FDA) has approved agents for prevention. The antiresorptive agents approved for prevention are estrogens, selective

estrogen-receptor modulators (SERMs), bisphosphates, and calcitonin. The FDA may soon approve bone-stimulating agents, such as fluoride and parathyroid hormone (82).

Estrogen has been the most-studied agent for the prevention of osteoporosis (82); however, the effect of estrogen on BMD is unknown (83). Further studies are needed to determine whether estrogen slows the loss of bone associated with menopause and aging, maintains BMD, or increases BMD. Assuming estrogen increases BMD, it is important to know whether the increase is for a short duration (i.e. 1 year) or continues beyond that time. Also unresolved is whether the addition of a progestin enhances the bone-sparing effects of estrogen (83).

Both behavioral and physiological factors, including smoking, alcohol use, calcium intake, physical activity, ethnicity, body mass, parity, time since menopause, and early oophorectomy have been shown to influence BMD. It is not known whether these factors modify the effect of estrogen on bone (83).

Studies indicate that estrogen replacement therapy (ERT) prevents bone loss when begun at menopause and significantly increases bone mass if started at a later age (61, 84, 85). A randomized, placebo-controlled study of women with similar BMC showed that estrogen effectively protected against bone loss for those participants who initially started on estrogen treatment. Additionally, participants who started on estrogen therapy at a later date showed an increase in BMD. The study also showed the importance of continuing estrogen therapy over time as the participants who were on estrogen for two years and then switched to a placebo demonstrated a significant decline in bone mass once treatment was withdrawn (84). This supports the fact that estrogen treatment can actually increase BMD and that women must stay on estrogen treatment for the protective

effects to continue. In fact, after stopping estrogen, a woman will lose bone mass at the same rate as if she had never been on hormones before (82, 84).

A five year randomized, double-blind, placebo controlled trial of 67 women 75 years old and older, showed nine months of hormone replacement therapy (HRT) significantly increased BMD compared with placebo, of the lumbar spine and femoral neck. HRT resulted in a significant decrease in serum alkaline phosphatase levels, a marker of bone formation, and urine N-telopeptide levels, a marker of bone resorption (86).

Estrogen is commonly used in combination with a progestin to reduce the risk of uterine cancer; however, the effect that progestin has on BMD is still being debated (83). The Postmenopausal Estrogen/Progestin Intervention (PEPI) trial was a three year, multicenter, randomized, double-blind, placebo controlled clinical trial that examined the effects of hormone therapy on BMD in the spine and hip of postmenopausal women. A total of 875 healthy women aged 45 to 64 years old were recruited for the study. Participants were assigned to one of the five following 28-day cycle regiments: placebo, estrogen, or a combination of estrogen with one of three increasing levels of progesterone. The BMD was measured at the lumbar spine and hip at baseline, 12 months, and 36 months. The study concluded that estrogen use increased BMD in the spine and hip during three years of therapy. The combined estrogen-progestin treatment maintained or increased BMD at both sites measured, but the combination treatment was not superior to unopposed estrogen. Participants taking placebo lost BMD at both the spine and the hip (83).

Drugs commonly used in hormone replacement therapy

Tibolone

Tibolone is a synthetic steroid that exhibits a combination of weak estrogenic, weak progestrogenic, and weak androgenic actions (3). Tibolone may be used alone or as a supplement for postmenopausal women to reduce vasomotor symptoms and to decrease postmenopausal bone loss. The rate of increase in BMD is estimated at about 1.5-2.0% over two years of use; therefore, it is less effective than estrogen. It may help to alleviate vasomotor symptoms without causing mastalgia but may cause bloating, fluid retention, and psychological symptoms (3, 31). Tibolone also has an amenorrheic effect in most users; it may decrease total cholesterol but also reduce high density lipoprotein (HDL) cholesterol as well. It is prescribed to women who have passed menopause, who do not wish to bleed, or in those with contraindications to estrogen use (3, 31).

Raloxifene

Raloxifene is a non-steroidal benzothiaphene that is classified as a selective estrogen receptor modulator (SERM). They exert estrogenic effects on specific body tissues but not others. Raloxifene binds to estrogen receptors and inhibits bone resorption similar to estrogen (86).

Raloxifene acts as an agonist and antagonist in different tissues that respond to estrogen. Unlike estrogen, the drug acts as an estrogen antagonist on the uterus and breast (87-90). Complications related to raloxifene use include an increase in the incidence of hot flashes and increase risk of thromobombolism. Treatment with raloxifene, therefore, is appropriate for postmenopausal women who do not complain of hot flashes and who are at increased risk for osteoporosis (88)

Compliance with hormone replacement therapy

Typically, women struggle to maintain long-term compliance with HRT (82, 91). No more than 50-60% of women will continue to take HRT beyond one year. Reasons given for discontinuation of treatment include uterine bleeding, fear of breast cancer, and the need for long-term use. Unscheduled vaginal bleeding was the most common reason for discontinuation of HRT (82, 91).

A substudy of the Health, Osteoporosis, Progestin, Estrogen (HOPE) trial of 822 postmenopausal women was conducted to determine the safety and efficacy of low-dose HRT on BMD. Participants underwent scanning at the spine, femoral neck, and trochanter. The subjects were randomly assigned either low-dose or high-dose estrogen, with or without added progesterone. A two-year follow-up of the participants showed that BMD had increased significantly from baseline at all sites measured in the active treatment groups but had decreased in women in the placebo group. All doses of HRT produced an average increase of 4% in BMD compared with placebo. Serum osteocalcin levels, a marker of bone formation, increased to the same degree in all dose levels. Urinary N-telopeptide, a marker of bone resorption, decreased to the same degree at all dose levels. The study concluded that it is possible to prevent bone loss with lower doses of HRT than has previously been recommended (92).

Further analysis of data from the HOPE trial indicates that lower doses of estrogen plus progestin were even more effective at stopping bleeding than the commonly prescribed levels and took effect more quickly (92). Investigators concluded that low-dose HRT regimens are an appropriate choice for patients who are newly menopausal (92).

A two-year, double-blind, placebo-controlled study was conducted to compare the effect of estrogen alone or estrogen plus progestin (93). Participants included 1265 women over 40 years old who had undergone spontaneous menopause within the last 5 years and who had an intact uterus. The study found that high dosages of estrogen increased bone density significantly, but also increased the incidence of endometrial hyperplasia, which is a risk factor for endometrial cancer. No endometrial hyperplasia was seen in women who took estrogen plus progestin; therefore, estrogen-only therapy, therefore, can be given safely only to women who have had a hysterectomy (93).

In the past, progestins have been prescribed to prevent estrogen-induced endometrial stimulation. A progestin is taken cyclically for 12-14 days every month, followed by menstrual discharge. As an alternative method of therapy, progestin and estrogen may be used concurrently to induce amenorrhea. The main adverse effects associated with progesterone use are unscheduled bleeding, fluid retention, bloating, breast tenderness, weight gain, acne, and seborrhea (93).

Isoflavones and soy protein

Phytoestrogens are plant-derived compounds including isoflavones, coumestans, and lignans (94, 95). Phytoestrogens are found in grains, beans, fruits, and nuts. Soybeans and foods made from soy are especially rich sources of isoflavones (94-96). Major isoflavones found in soy are genistein (4',5,7-trihydroxyisoflavone) (~60%), diadzein (4',7-dihydroxyisoflavone) (~25%), and glycetein (7,4'-dihydroxy-6-methoxyisoflavone) (~15%) (12, 15). The chemical structures of the isoflavones genistein and daidzein differ from each other by only the absence of a hydroxyl group on

the 5 position of the A ring of the generic chemical structure (Figure 1). The difference, however, has a significant effect on the functions of the two molecules (12).

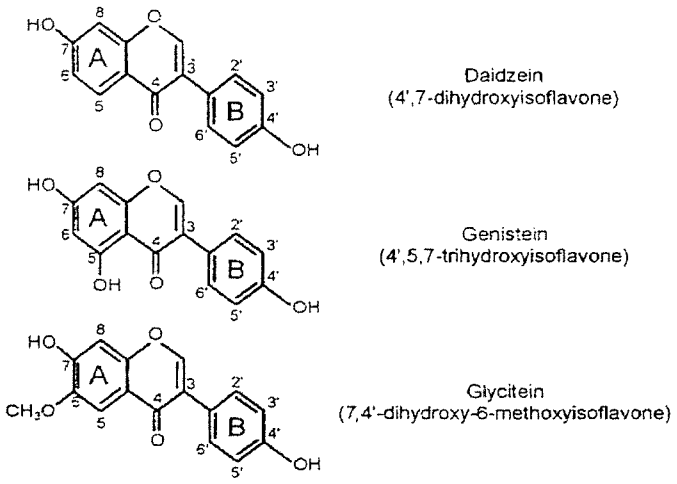

Figure 1. Isoflavones present in soybeans.

In foods, isoflavones are conjugated with a glucose molecule to form glycosides or glycones (50, 12). The primary isoflavones, or aglycones, in soybeans are genistein and daizdein and their β-glycosides genistin and daidzin, respectively. Much lower amounts of glycitein and its glycoside, glycitin, are also present in soybeans (12, 15, 50).

Sugars are attached at the 7 position of the A ring of the aglycone to form the glycoside. Soy isoflavones naturally exist almost entirely as glycosides (12). Genistein and daizdein can also be derived from their precursors, biochanin A and formononetin, respectively (95).

Metabolic transformations of the isoflavones occur that converts them to functional molecules (12). Major metabolic changes transform genistin to genistein and daidzin to daidzein. Genistin and daidzin are referred to as glycones because they contain a glucose molecule attached to the A ring of the structure. Genistein and daidzein

are specified as aglycones because the glucose molecules are enzymatically removed. Gut bacteria (12, 50) form several derivatives, such as equol and p-Ethyl phenol.

High concentrations of isoflavones in soybeans have been recognized since 1931. Genistein glycoside was isolated from soybeans 10 years later (98). Hormonal properties of isoflavones were first suspected in the mid 1940's in Australia after observing widespread infertility in sheep. Sheep were grazing on a particular type of clover containing high levels of the isoflavones biochanin A and formononetin (99); these are metabolized to genistein, and daidzein, respectively in sheep rumen (50). Daidzein is further metabolized by the intestinal bacteria to equol, which is a unique isoflavone with a high affinity for estrogen receptors (100). The infertility problem in sheep was traced to these non-steroidal weak estrogens (99, 101). Further evidence of a reproductive effect was seen following the use of soybeans in the feed of captive cheetah in the Cincinnati zoo. An infertility syndrome developed among the animals which was reversed when the soybean was removed from the feed (102). Equol, which far exceeded levels of endogenous estrogens, was later found in human urine and blood following soy consumption. This lead to the hypothesis that isoflavones could be biologically active in humans (102, 103).

Isoflavones are structurally similar to 17β-estradiol (Figure 2), a naturally occurring estrogen (98). Isoflavones can act as estrogen agonists and antagonists, depending upon the tissue in which they act (11, 16, 50, 97, 102).

Since they are structurally similar to17β-estradiol, phytoestrogens can occupy estrogen receptors (ER); however, the occupancy time or affinity for the receptor is

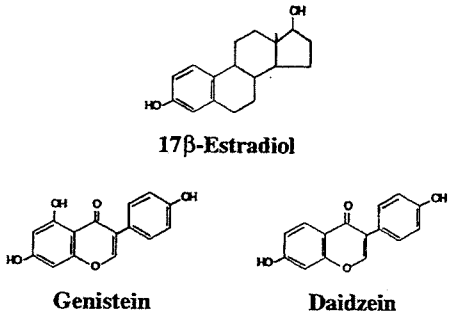

Figure 2. Comparison of the chemical structures of 17β-estradiol to genistein, and daizdzein.

significantly lower compared with 17β-estradiol (Table1) (95). The ability to bind to the ER is influenced by the concentration dependency, receptor status, presence or absence of endogenous estrogens, and the type of target organ or cell.

Table 1. Affinities of selected phytoestrogens for the human ER in comparison to 17β-estradiol.

Molecule	Relative Binding Affinity
17β-estradiol	1.0
Genistein	0.0125
Daidzein	0.0010
Biochanin A	0.00005

Digestion, absorption, and utilization of isoflavones

Digestion of the glycones to aglycones and glucose is thought to occur within the gut lumen (12). Bacterial enzymes, or glycosidases, cleave glucose from glycone in the fluid environment of the illeum and large intestine. Free aglycones solubilize in the micelles formed from bile (12).

Once the lipid-soluble aglycones become incorporated into micelles in the gut lumen, they migrate to and are absorbed by the brush border of the small intestine. Micelles permit their contents of fat-soluble molecules, including aglycones, to enter the cells by passive diffusion. The molecules are either used for cellular needs or they are repackaged into newly forming chylomicrons for subsequent release into the lymphatic

system and distribution to the body. Isoflavones may leave the chylomicrons when they reach any extrahepatic tissue, such as reproductive organs and bones, for use by cells in that tissue. After taken up by cells, isoflavones can lower blood lipids, improve vascular epithelial cells, impede the proliferation of cells that might become malignant, or enhance bone forming activities of osteoblasts (12).

The metabolic fat of these bioactive isoflavones is illustrated by equol, a derivative of daidzein generated by bacteria in the gut lumen. This substance is apparently made in large amounts within the gut lumen and is recycled in the enterohepatic circulation many times before excreted in the stool or urine. The largest amount of any isoflavone found in urine is equol. Blood concentrations of equol in soy consumers are typically higher than the concentration of any other isoflavone. Equol, therefore, serves as a good marker of soy consumption because of its relatively high circulating blood concentration and its steady levels in blood over a 24-hour period (12).

Food sources of isoflavones

Of the many plants that constitute the legume family, only soy and foods derived from soy provide significant amounts of genistein, daidzein, and glystein in the human diet (12). Soybean contains approximately 0.2 to 1.6 mg of isoflavones per gram of dry weight (Table 2) (105).

Table 2. Major dietary soy sources of isoflavones (mg/100g).

	Major Soy Sources of Isoflavones	
	Daidzein (mg/100g)*	**Genistein (mg/100g)***
Soybeans	84	111
Soybeans (roasted)	56	87
Soy flour	23	81
Tempeh	27	32
Tofu	15	16

*On per weight basis.

Chickpeas and other legumes, such as mung beans; mushrooms; and clover also contain isoflavones but in lower amounts. Most beans typically contain approximately 10-100 fold less phytoestrogen than do soybeans. Urinary phytoestrogen excretion studies indicate that soybean consumption is only significant in populations in the Far East. The average daily isoflavone intake in Asian populations has been estimated as approximately 30 mg per day (106). In the United States, the average amount of soy consumed by a person is estimated to be approximately 5 g a day (11). Beans and peas, tea and coffee, nuts, grains, rice, and cereals are main dietary sources of isoflavones in Western populations (106, 107).

Cellular mechanism of isoflavones

Phytoestrogens act on cells in a similar way as estradiol (11). When taken orally by animals or humans, phytoestrogens do not have the same potency as estrogen because the soy food material containing the isoflavones has to be digested, modified by gut bacteria, absorbed, and distributed around the body via chylomicrons. Further, many of the isoflavone molecules recirculate in the enterohepatic circulation many times before steady-state blood concentrations are achieved (11).

The isoflavones are referred to as partial estrogen agonists or antagonists. In bone tissue, which contains more β-ERs than α-ERs, the estrogens appear to be more agonistic than in other tissues because they help to maintain bone tissue, or at least keep a balance between osteoblastic activities and osteoclastic activities. The result of the action of isoflavones is that little or no net bone loss occurs, which is comparable to the treatment with estradiol or the equivalent of conjugated estrogen. The actual dose of isoflavone may be 100 to1000 folds higher than that of estradiol. In addition, isoflavones may act as antagonist on α-ERs, which are found in much greater numbers in reproductive tissues, like the uterus. In these tissues, isoflavones may occupy ERs and block the stimulation of DNA and protein synthesis by estradiol, the normal action of this hormone. Isoflavones, especially genistein, may act as selective ER modulators (SERMs). SERMs

represent a new class of drugs, such as tamoxifen and raloxifene, used to prevent osteoporosis, and isoflavones may be considered to act typically as SERMS. The benefit of the isoflavones in the diet is they help maintain a balance between osteoblasts and osteoclasts that result in better bone conservation and the potential prevention of osteoporosis (12).

Effects of isoflavones on human skeleton

Some health benefits, notably skeletal benefits, of isoflavones in bone have been identified (12). The use of isoflavones to prevent osteoporosis is currently under study; however, there are a limited number of completed human trials that looked at the effects of isoflavones on bone (12, 94, 103).

Despite encouraging results from rodent model studies (108), humans may require higher doses of isoflavones before an improvement of BMD can be observed. In a six-month study of 66 postmenopausal women (108), BMD and bone mineral content (BMC) was determined after feeding 40 g soy protein supplement enriched with ~56 mg or ~90 mg of isoflavones per day or a control diet with 40 g casein and non-fat dry milk per day. Significant increases (2%) were shown in BMD and BMC of the lumbar spine, but not of the proximal femur, in the group of women who consumed ~ 90 mg of the soy protein supplement, when they were compared with the control group. The group who consumed ~56 mg of the enriched soy protein supplement also showed gains in the lumbar vertebrae, but the gains were not significant (108). These findings suggest that a threshold dose of ~50-90 mg per day must be reached before a benefit to humans is observed (12).

Alekel and co-workers (96) published the first study designed specifically to examine bone in perimenopausal women. A 24 week study evaluated the lumbar spine of 69 perimenopausal women; participants who consumed 80.4 mg per day of soy isoflavones had a significantly higher BMD and BMC of the lumbar spine than did those who consumed 4.4 mg per day of soy-containing isoflavones or the control group (whey

protein). It was concluded that isoflavones attenuated bone loss from the lumbar spine in estrogen-deficient perimenopausal women, who may otherwise be expected to lose 2% to 3% of bone per year (96).

In a controlled study of pre- and postmenopausal women, Wangen and associates (7) failed to detect significant changes in the markers of bone turnover following high dose isoflavone treatment. The study evaluated the effects of soy protein isolates containing increasing concentrations of isoflavones (8 to 130 mg/day) consumed for 3 months by pre- and postmenopausal women. This study did not support any beneficial effect on bone turnover in estrogen-deficient women (7); however, biochemical markers were used as indicators of changes in bone–not bone density measures.

Studies in postmenopausal women in Asian nations suggest that the consumption of soy products in plentiful amounts over a lifetime may be beneficial to bone retention and subsequently, reduce the risk of hip fractures (11). For instance, soy and soy products provide about 15% of total calcium consumed by the Japanese, compared with about 1% in the U.S. These products also provide significant amount of isoflavones (109, 110). Studies of Asian women suggest average intakes of soy as high as 30 mg per day; this provides almost 40 mg of isoflavones a day. Asians consume 10-100 times more isoflavones than Western people (111). Correlational evidence, based on the low prevalence of hip fractures in Japanese women (112) and the high soy intake of the Japanese, lead to the hypothesis that the high consumption of isoflavones from soy products may be protective against fractures, especially hip fractures. (11, 113).

Recently, daily intake of isoflavones was assessed in 478 postmenopausal Japanese women by Somekawa and associates (114). Bone mineral density was measured at the lumbar spine using DEXA. Women were assigned to two groups according to years since menopause (early and late postmenopausal groups). Each main group then was divided into four sub-groups according to dietary isoflavone intake. After adjusting for years since menopause, a significant difference was seen in BMD in

participants who consumed the highest amounts of isoflavones compared with those who consumed the lowest amount in the early and late menopausal groups (114).

Another Japanese study by Horiuchi and associates (115) evaluated the effects of soy protein on BMD and biochemical markers in 85 postmenopausal women. Bone mineral density of the lumbar spine was measured with DEXA: serum alkaline phosphatase and serum osteocalcin, bone formation markers, and urinary pyridinoline and urinary deoxypyridinoline, bone resorption markers, were also measured. Results from the study suggest that high soy protein intake is associated with higher BMD and a lower level of bone resorption (115).

Dietary studies in humans that last only a short period of time (several weeks) may not be sufficient to see changes in BMD or BMC because the remodeling cycle ranges from 30 to 80 weeks (96). It would be premature to assume that soy isoflavones has a significant long-term bone-sparing effect or that soy reduces bone fractures of the spine. Studies of two or three years in length are necessary to test for long-term bone-sparing effects. It would be a great benefit to peri- and postmenopausal women if soy with isoflavones, alone or with other therapies were shown to prevent bone loss in the spine. This would provide these women with an alternative approach to HRT for maintaining bone in the vertebra (96, 114). This is especially true with the negative health reports appearing about HRT (116).

Soy isoflavones and rodent studies

Experimental animal models, especially rats, are often used to determine dose-response effects and changes in tissues after treatment with isoflavones (116, 117). Published reports of studies that used ovariectomized (OVX) rat models of osteoporosis, show animals fed feed containing isoflavones improved bone mass compared with animals fed diets that did not contain isoflavones (117, 118).

A study by Arjmandi and co-workers (119) examined whether soybean protein isolate prevents bone loss in rats with an ovarian hormone deficiency. Thirty-two 95-day

old Sprague Dawley rats were assigned randomly to four treatment groups: sham-operated (SHAM); OVX; OVX + soybean; or OVX + 17β-estradiol. Rats in the OVX group had significantly lower BMD in the right femur and fourth lumbar vertebra than rats in the SHAM group. Lower bone densities were not observed in animals receiving 17β-estradiol or those fed soybeans. It was concluded that dietary soybean protein is effective in preventing bone loss due to ovarian hormone deficiency (119).

Another study by the same group of researchers evaluated whether isoflavones in soy protein are responsible for bone-protective effects (120). Forty-eight 95-day old Sprague Dawley rats were divided into four groups: sham-operated fed a casein-based diet (SHAM); OVX fed a casein-based diet (OVX + casein); OVX fed soy protein with normal isoflavone content (OVX + soy); and OVX fed soy protein with reduced isoflavone content (OVX + soy-). The OVX groups had a significant reduction in femoral bone density (4.8%) during the 35-day period after OVX compared with the SHAM group. The soy, but not the soy-, prevented the loss of BMD in the femur. Congruent with results from other studies, data from this study suggest that isoflavones do not slow the enhanced rate of bone turnover induced by OVX (120). Arjmandi and associates found the rate of bone formation was higher in the OVX group than in the SHAM group; bone formation rates were not significantly different in the OVX + soy or OVX + soy- groups. Data suggest that the positive effect on bone mass is due to formation exceeding resorption.

Arjmandi and co-workers also investigated whether a soy protein diet fed to rats immediately after OVX prevented bone loss (121). The study found that a slight reversal of cortical bone loss may occur partially due to higher femoral insulin-like growth factor resulting from both soy and reduced soy diet. The study suggested that the OVX induced increases in indices of bone turnover were not ameliorated by either of the soy diets, which, in turn, suggest that any positive effect of soy, was achieved through bone formation rather than slowed bone resorption. The results of this short-term study

suggest that for soy protein to reverse bone loss, long-term consumption may be necessary (121).

Picherit et al found that daily soy protein isoflavones did not reverse established osteopenia in adult OVX rats (122). They also demonstrated that a daily dose of soybean isoflavones fed to adult OVX rats reduced bone turnover at the highest doses of 40 and 80 milligrams per kilogram of body weight per day (mg/kg/d), depressing OVX-induced increase in bone turnover (specifically bone resorption) than the lowest dose (20 mg/kg/d) (122).

Studies have also been conducted to determine the exact component in soy that prevents bone loss. For example, Ishida and co-workers (8) examined effects of daidzin and genistin on bone loss in OVX rats fed a calcium-deficient diet. Daidzin or genistin were orally administered to two groups OVX rats for four weeks at a dose of 50 mg/kg/d each. Another group of OVX rats was fed a calcium-deficient diet. Animals receiving subcutaneous estrone (7.5 μg/kg/d) served as a control. OVX induced uterine atrophy. The femurs of OVX rats on the calcium-deficient diet showed significantly lower density, strength (breaking forces), ash weight, and calcium and phosphorus content compared with those of sham-operated rats. The animals treated with daidzin, genistin, and estrone had significantly higher femur density and strength than rats in the OVX group, but had similar or lower values compared with the sham-operated group. Results suggest that daidzin treatment appeared to prevent bone loss in the femurs in a dose-dependent fashion of 50 mg/kg/d. Results suggest that atrophy of the uterus was prevented by estrone and daidzin but not genistin. Daidzin appeared to have a dose-dependent effect on uterine tissue at 50 mg/kg/d. Genistin treatment was also effective in preventing bone loss in the femur similarly to daidzin at the same dose of 50 mg/kg/d. Daidzin and genistin administered for four weeks can prevent a decrease in breaking strength and mineral content in cortical bone of OVX rats (8).

A similar study investigated the effects of daidzin, genistin, as well as glycitin on bone loss in 36 eleven-week old female Sprague-Dawley rats (123). Results from this study show that 50 mg/kg/d of glycitin, as well as 50 mg/kg/d of daidzin prevented OVX-induced uterine atrophy similar to that of estrone. Genistin prevented OVX-induced uterine atrophy only at a dose of 100 mg/kg/d. The findings of this study suggest that daidzin, genistin, and glycitin are effective in preventing bone loss. The preventative effect of diadzin or glycitin on bone loss in OVX rats may be due to suppression of bone turnover, as is the case of estrone (123).

Phytoestrogens show promise for the prevention and treatment of osteoporosis, but results of more human studies are needed before making conclusive statements about the efficacy of these plant components in human subjects. Worldwide use of soybeans and soy products make this approach, as opposed to drug treatment, such as HRT, more attractive to many women in Western nations. It is generally assumed that the consumption of soy products corresponds with intake of isoflavones. Nevertheless, the positive effects of phytoestrogens on bone observed so far in humans are small and limited to the lumbar vertebrae. In addition to the improved intakes of phytoestrogens, other nutritional benefits may result from the consumption of soy products, such as an increased intake of calcium or magnesium from tofu, vitamin K, and protease inhibitors. A remaining concern about the use of phytoestrogen-enriched preparations is excessive consumption and potential adverse effects to cells of bone and other tissues (11).

CHAPTER 3

ANIMALS AND METHODS

The Louisiana State University Institutional Animal Care and Use Committee (IACUC) approved this study on May 14, 2001 (Appendix A).

Animals and Treatment Assignment

Eighty-seven 9 month-old, non-pregnant retired female breeder Sprague-Dawley rats (Harlan, Co.; Indianapolis, IN) were used in this study. Rats were housed individually in 24 centimeters (cm) x 28 centimeters (cm) x 18 centimeters (cm) hanging stainless steel wire cages. Animals were kept in Room 654 of the Life Sciences' animal care facility of Louisiana State University and Agricultural and Mechanical College (LSU) at 22° Celsius (C) with a humidity level of 60% and a 12-hour light/dark schedule (0700 light/1900 dark). A timeline of events throughout the study is presented in Table 3.

Table 3. Timeline of study events.

Weeks 1-2	Rats received and randomly assigned to novel diets
Week 3	Body composition and bone density analyzed using pDEXA
Week 4	Surgeries performed on rats
Week 8	Body composition and bone density analyzed using pDEXA
Week 9	Rats placed in metabolism cages to collect urine samples
Week 10	Routine feeding and weighing rats continued
Week 11	Rats placed in metabolism cages to collect urine samples
Week 12	Routine feeding and weighing rats continued
Week 13	Body composition and bone density analyzed using pDEXA
Week 14	Rats sacrificed

Initially, the rats were used to test the effects of novel diets on recovery from travel to LSU A&M and from their previous pregnancy/lactation cycle. Upon arrival, each rat was weighed and placed randomly into a cage. Fifty-one rats were placed in

cages containing the same commercial rat chow (Harlan Co.; Madison, WI) used in the Harlan breeding facility. Seventeen rats were placed in cages containing the American Institute of Nutrition (AIN)-93M diet; the remaining seventeen rats were housed in cages containing AIN-93M diet and chow diet. The two "extras" were placed in cages containing only chow. Food and water were provided *ad libitum*.

Average weight of the rats in each diet group was calculated on day one. Rats were redistributed to achieve approximately the same average weight among diet groups. The lightest and heaviest rats (246g and 437g, respectively) were removed from the group and maintained as "extras".

At the end of the fifth week on AIN-93M and chow diets, the primary study examining the effects of soy protein isolate on bone mineral density of ovariectomized rats began. Fifty rats were redistributed to obtain approximately the same average weight among diet groups. Rats were randomly assigned to five surgery/diet treatment groups (n=10). Forty-seven rats were used in another study not addressed by this thesis. The five treatment groups were identified as sham-operated (ShC); ovariectomized, control (OC); ovariectomized, 5% soy (O5S); ovariectomized, 10% soy (O10S); ovariectomized, 15% soy (O15S). Rats in the ShC and OC treatment groups were fed a casein-based diet; rats in the O5S, O10S, and O15S treatment groups were fed a diet containing either 5%, 10%, or 15% soy, respectively. Food and water were provided *ad libitum*.

Diets

For the initial study, AIN-93M diet was prepared as described below (Table 4). Macronutrients were added to a 20-quart stainless steel mixing bowl and mixed at a low speed (#1 on the mixer) using a Hobart mixer (model no. A-200-FD; Hobart Mfg. Co.;

Troy, OH) with a paddle attachment. After mixing for 10 minutes, the bowl was scraped; the mixture was mixed for another 5 minutes. Micronutrients (Table 2) were sieved sequentially into a medium mixing bowl using a sieve with a small mesh. Crystals remaining in the bottom of the sieve were ground finely using a mortar and pestle and added to the other micronutrients. Using a spoon, the micronutrients were stirred by hand to achieve a uniform distribution. Micronutrients were added to the macronutrients in the 20-quart mixing bowl; the mixture was mixed on low speed for 10 minutes. The bowl was scraped and mixed for another 5 minutes. Soybean oil containing butylated hydroxytouluene (BHT), a preservative, was added to the dry ingredients in the 20-quart mixing bowl. The mixture was mixed for 5 minutes. The bowl and paddle were scraped, and the mixture was mixed for another 10 minutes.

Table 4. American Institute of Nutrition (AIN)-93M Diet for maintenance of adult rodents.

INGREDIENTS	AIN-93M g/kg diet
Macronutrients	
Casein	108
Sucrose	600
Cornstarch	255.4
Dextrinized cornstarch	930
Cellulose[a]	300
Soybean oil[b]	240
Micronutrients	
Mineral mix AIN-93M[c]	210
Vitamin mix AIN-93M	60
Choline bitartrate[c]	15
L-cystine	10.8

[a]fiber

[b]added 0.717 grams BHT as an anti-oxidant

[c]crush with mortar and pestle

Diets were stored in tightly sealed Ziploc bags from which all excess air was removed. The bags were labeled with diet type, date, batch number, and initials of the preparer. A small sample was taken from each batch and stored in a small plastic bag labeled with diet type, date, batch number, and preparer's initials. The samples were placed in a larger bag and stored in the freezer for later analysis.

Diet for the primary study was prepared as described above. Table 5 shows the ingredients for the five surgery/diet treatment groups for the primary study.

Table 5. Diet composition of five surgery/diet treatment groups (g/kg batch).

Ingredient	Sham	Control	5% Soy	10% Soy	15% Soy
Macronutrients					
Casein	150	150	100	50	-----
Soy isolate	-----	-----	50	100	150
Sucrose	100	100	100	100	100
Cornstarch	455.70	455.70	455.70	455.70	455.70
Dextrinized cornstarch	155	155	155	155	155
Corn oil, stripped	40	40	40	40	40
Cellulose[a]	50	50	50	50	50
Micronutrients					
Mineral mix AIN-93M[b]	35	35	35	35	35
Vitamin mix AIN-93M	10	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5	2.5
L-cystine	1.8	1.8	1.8	1.8	1.8

[a]fiber

[b]crush with mortar and pestle

Measurements of food and water intake and body weight

Rats were weighed to the nearest gram, and food intake was quantified by weighing the empty food cup and estimating spillage to the nearest gram three times per week (Monday/Wednesday/Friday). Intake was calculated as full food cup - [empty food

cup + estimated spillage]. All weights were measured using a 500-gram analog scale (Toledo Scale Co.; Toledo, OH) and recorded.

Ovariectomy and sham surgeries

Faculty and students from the LSU School of Veterinary Medicine performed ovariectomy (OVX) and sham (Sh) operations two weeks after placing the rats on soy protein diets. Animals were anesthetized with a mixture of gaseous Isoflurane (Abbott Labs, N. Chicago, IL) and oxygen via an induction chamber. Buprenorphine hydrochloride (Buprenex® Reckitt & Colman Products; Hull, England) (0.05 mg/kg subcutaneous) was administered after induction of the gaseous Isoflurane-oxygen mixture to provide postoperative analgesia. Lubricant ophthalmic ointment (Akwa Tears; Buffalo Grove, IL) was applied to the eyes to prevent drying out while the animal was under anesthesia. Hair covering the mid to dorsal areas of the abdomen was clipped. The skin was disinfected with Betadyne, then 70% isopropyl alcohol to prepare for aseptic surgery. Isoflurane inhalation was continued during surgery via a nose cone (Figure 3).

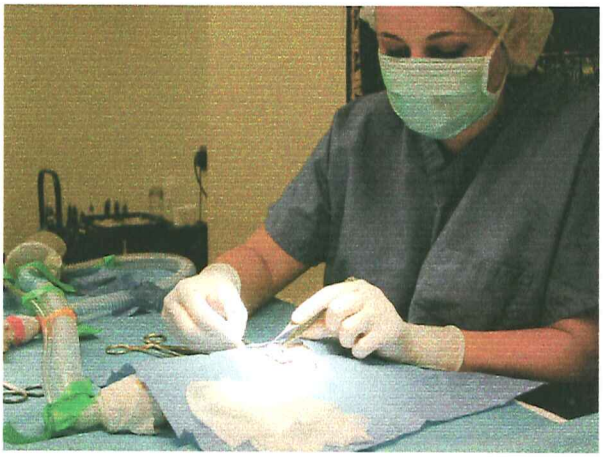

Figure 3. Isoflurene administered via nose cone during surgery.

Entry into the peritoneal cavity was gained through 1-cm bilateral incisions made halfway between the middle of the back and the base of the tail approximately 1 1/2-2 cm off the dorsal midline. Ovaries were removed from all rats (OVX) except those in the Sh group; for those rats, the horns of the uterus were returned to the peritoneal cavity (sham-operated). One suture was placed in the muscle layers with 5-0 PDS II nylon suture (Ethicon, Inc.™; Somerville, NJ). Skin was closed with Instant Krazy Glue (Elmer's Products, Inc.; Columbus, OH). Subcutaneous injections of Lactated Ringer's solution (10% of body weight) (Abbott Lab; Chicago, IL) were used for hydration following surgery. Animals were placed in a holding tank where they were allowed to recover from anesthesia. While in the holding tank, rats were placed on heating pads under a heat lamp to minimize heat loss; they were returned to their cages after recovery.

Peripheral dual energy x-ray absorptiometer

A peripheral dual energy x-ray absorptiometer (pDEXA) was used to measure bone density and body composition of the rats 3 times during the study: 3 weeks prior to surgery, 4 and 8 weeks post-surgery. Each rat was anesthetized using Isofluorene via a nose cone before placing the rat on the platform to be scanned. Lubricant ophthalmic ointment (Akwa Tears; Buffalo Grove, IL) was applied to the eyes to prevent drying out while the animal was under anesthesia.

The rat was measured with a ruler in centimeters (cm) from the back of the ear to the base of the tail (Figure 4). The animal's weight and measurement were recorded in a laboratory manual and entered in the pDEXA computer to be used for later analysis.

While under anesthesia, the rat was placed on the pDEXA platform and scanned from the upper torso to the base of the tail (Figure 5). This area was chosen to obtain an estimate of total body fat, lean tissue, and bone density of the vertebrae.

Figure 4. Rat measured with a ruler from the back of the ear to the base of the tail.

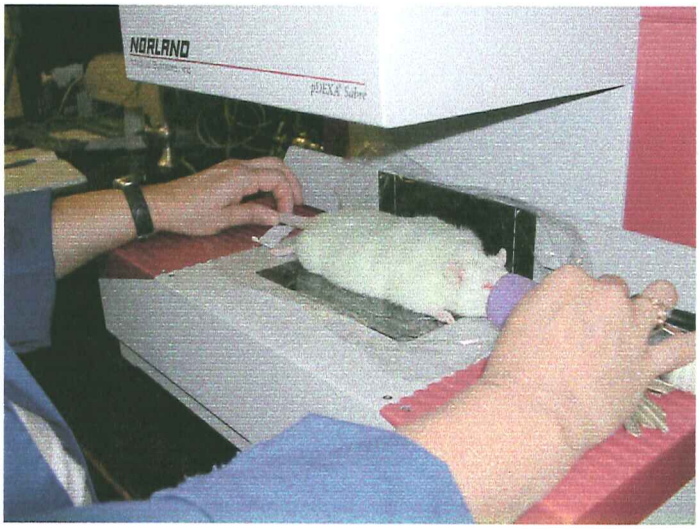

Figure 5. Rat placed on the pDEXA platform to be scanned.

Table 6 shows the pDEXA scan parameters used to scan the rats.

Table 6. Peripheral dual energy x-ray absorptiometry scan parameters for rat scans.

	pDEXA scan parameters
Resolution	1.0 x 1.0 mm
Scan width	11.40 cm
Scan length	15.00 cm
Speed	40 mm/sec

After each scan, the nose cone was removed; the rat was returned to the cage to recover from the anesthesia.

Analysis of whole body scans

Whole body pDEXA scans obtained three weeks prior to ovariectomy and sham operation, four weeks post-surgery, and eight weeks post-surgery were analyzed to determine BMD in the vertebra and abdominal fat mass of each rat. The rat's length obtained prior to each scanning session was used to calculate the portion of the vertebrae to be analyzed. To ensure consistency of measurement, 25% of the rat's length was used to obtain BMD of the vertebrae. To analyze BMD of the vertebrae, a region was selected beginning from the point on the scan image at which the pelvis joined the spine and extended up the vertebrae to the calculated length. For example, if the length of the rat from the back of the ears to the base of the tail was 16.0 cm, then the following calculation would be made:

$$16 \times 0.25 = 4.0 \text{ cm}$$

The selected region would begin at the point where the pelvis joined the spine and extended 4.0 cm up the spine. The width of the region included only the width of the vertebrae.

To determine abdominal fat mass, a second region was selected beginning from the base of the tail and extending to the end of the first region, including the vertebral region. The width extended outward to the rat's widest point. After the regions were formed, the scans were analyzed for BMD and fat mass. See Appendix B for a sample of a bone scan image of the vertebrae.

Individual bone scans

Individual bones collected from each rat at sacrifice were used to determine final BMD for comparison among treatment groups. Bones used for analysis included the right humerus, right femur, and right tibia. Soft tissue was removed from each bone using dissecting scissors. Each bone was carefully placed on the pDEXA platform and scanned. Table 7 shows the following scan parameters used to scan individual bones.

Table 7. Scan parameters for scanning the humerus, femur, and tibia of each rat.

	pDEXA scan parameters
Resolution	0.2 x 0.2 mm
Scan width	2.0 cm
Scan length	4.0 cm
Speed	10 mm/sec

Each scan was analyzed to determine BMD of the humerus, femur, and tibia. Analysis of the humerus included the total humerus, elbow, and shoulder. Regions selected for analysis of the tibia included the total tibia and tibrial midshaft; the total femur, hip, knee, and femoral midshaft were analyzed for BMD. To maintain consistency of measurement for each analysis, a standard length and width was used for each region was selected. Table 8 shows the length and width measurements (cm) for each region selected for analysis for BMD. See Appendices C, D, and E for samples of bone scan images of the humerus, femur, and tibia.

Table 8. Length and width measurements (cm) for each region selected for analysis for BMD.

Bone	Length (cm)	Width (cm)
Humerus		
Elbow	0.60	0.86
Shoulder	0.40	0.60
Femur		
Hip	0.46	0.40
Knee	0.60	0.66
Midshaft	0.60	0.60
Tibia		
Midshaft	0.60	0.60

Statistical analysis

Data from this study was analyzed using SPSS® Student Version 9.0 for Windows® (©SPSS, Inc., 1999; Chicago, IL). Means and standard deviations (SD) for food intake, body weight, organ weights, abdominal fat, bone density of the vertebrae among treatment groups and within treatment groups, right humerus, right femur, and right tibia were calculated. Mean values for food intake, body weight, organ weights, abdominal fat, bone density of the vertebrae among treatment groups and within treatment groups, right humerus, right femur, and right tibia were compared using analysis of variance (ANOVA).

Independent t-tests were used to determine differences of mean total food intake, mean total body weight, mean total organ weight, mean abdominal weight, mean BMD of the vertebrae among and between treatment groups, and mean BMD of the right humerus, right femur, and right tibia between treatment groups. In all cases, a p value ≤ 0.05 was considered significant.

CHAPTER 4

RESULTS

Food intake

Table 9 shows mean food intake per day (grams per day) throughout the study for rats in the sham-operated (ShC); ovariectomized, control (OC); ovariectomized, 5% soy; ovariectomized, 10% soy; and ovariectomized, 15% soy treatment groups.

Treatment Group	Food Intake (g/d ± SD)
ShC	17.699 ± 1.143
OC	17.002 ± 1.065
O5S	17.058 ± 1.845
O10S	16.794 ± 1.051
O15S	18.103 ± 2.064

Table 9. Mean food intake (grams per day ± standard deviation) for rats in the sham-operated (ShC); OVX, control (OC); OVX, 5% soy (O5S); OVX, 10% soy (O10S); and OVX, 15% soy (O15S) treatment groups.

No significant differences in food intake were found among treatment groups at the end of the study ($p=0.269$).

Table 10 shows the total amount of isoflavones per 100g of diet consumed per day (milligrams per day) by rats in the ShC, OC, O5S, O10S, and O15S treatment groups.

Table 10. Total amount of isoflavones per 100g of diet consumed per day (milligrams/day) by rats in the ShC, OC, O5S, O10S, and O15S treatment groups.

Treatment Group	Total amount isoflavones per 100g of diet consumed per day (mg/d)
ShC	0
OC	0
O5S	3.3
O10S	6.6
O15S	10.4

Weight gain

Figure 6 illustrates the mean weekly weight (g) of rats in the ShC, OC, O5S, O10S, and O15S treatment groups over the course of the 13-week study.

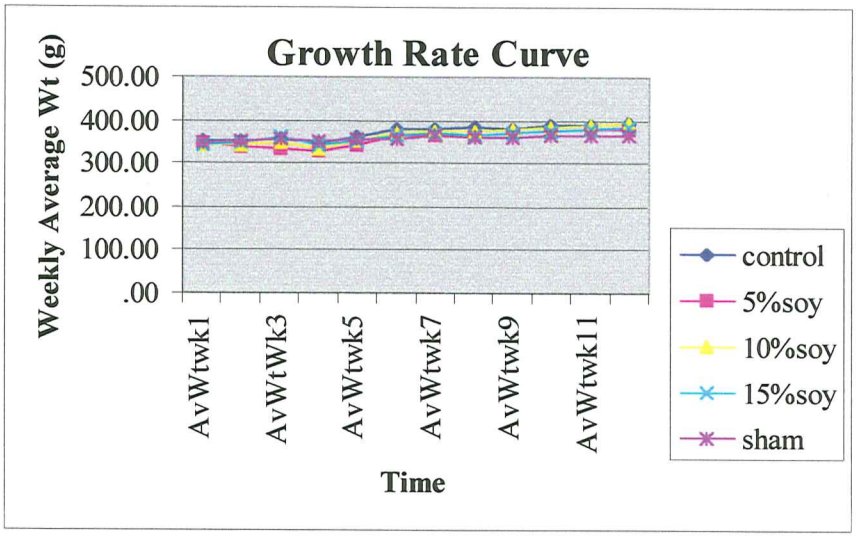

Figure 6. Mean weekly weight (g) of rats in the sham-operated (ShC); OVX, control (OC), OVX, 5% soy (O5S); OVX, 10% soy (O10S); and OVX, 15% soy (O15S) treatment groups.

Table 11 shows mean weight of rats in each treatment group at the end of the study. No significant differences in mean body weight were seen among any of the treatment groups (p=0.538). Numerically, mean weight of the rats in the ShC group (366.80 ± 29.78) was higher than those in other groups but not significantly higher.

Table 11. Mean final body weight (g ± SD) of rats in ShC, OC, O5S, O10S, and O15S treatment groups at the end of the study.

	ShC	OC	O5S	O10S	O15S
Final body weight	366.80±29.78	388.60±41.65	382.90±38.89	394.60±23.82	385.70±46.33

Table 12 shows the mean weights (g ± SD) of the liver, heart, spleen, kidneys, uterus, and abdominal fat for rats in the ShC, OC, O5S, O10S, and O15S treatment groups.

Table 12. Mean weights (g ± SD) of the liver, heart, spleen, kidneys, uterus, and abdominal fat of rats in the ShC, OC, O5S, O10S, and O15S treatment groups.

	ShC	OC	O5S	O10S	O15S
Liver	10.332±0.951	9.941±0.942	9.865±1.006	9.507±0.751	9.055±1.032
Heart	1.225±0.107	1.178±0.135	1.223±0.098	1.151±0.081	1.194±0.141
Spleen	0.733±0.078	0.775±0.127	0.767±0.137	0.752±0.110	0.698±0.099
Kidneys	1.879±0.215	1.845±0.199	1.706±0.116	1.722±0.112	1.718±0.150
Uterus	0.686±0.229	0.274±0.142	0.214±0.055	0.199±0.031	0.211±0.031
Abdominal Fat	17.904±6.315	21.956±7.813	22.360±7.182	26.261±5.496	23.191±6.685

No statistically significant differences were observed in the mean weights of the heart, spleen, kidneys, or abdominal fat of rats among the treatment groups (p=0.566, p=0.566, p=0.058, p=0.601, respectively). There were, however, significant differences in mean weights of the liver and uterus of the rats in each treatment group. Figure 7 illustrates the mean liver weight (g) of rats in the ShC, OC, O5S, O10S, and O15S treatment groups.

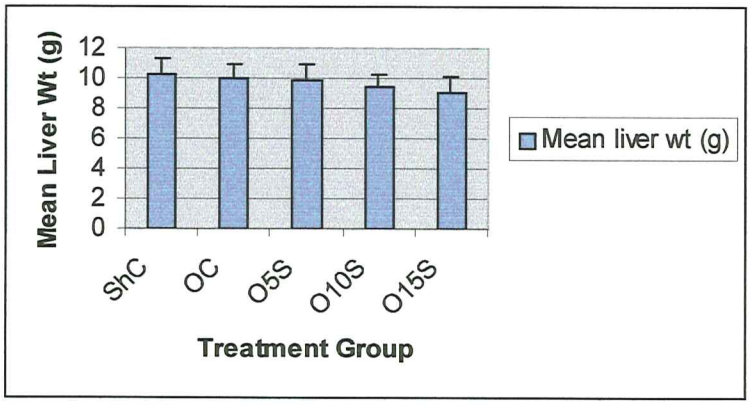

Figure 7. Mean liver weight (g) of rats in the ShC, OC, O5S, O10S, and O15S treatment groups.

Mean weight of the liver in the O15S group (9.055 ± 1.032) was significantly lower than those of the OC (9.941 ± 0.942) and ShC groups (10.332 ± 0.951) (p=0.047). No other significant differences were observed among the other treatment groups. Additionally, significant differences were observed in mean weight (g) of the uterus. Figure 8 illustrates mean uterine weight of rats in the ShC, OC, O5S, O10S, and O15S groups.

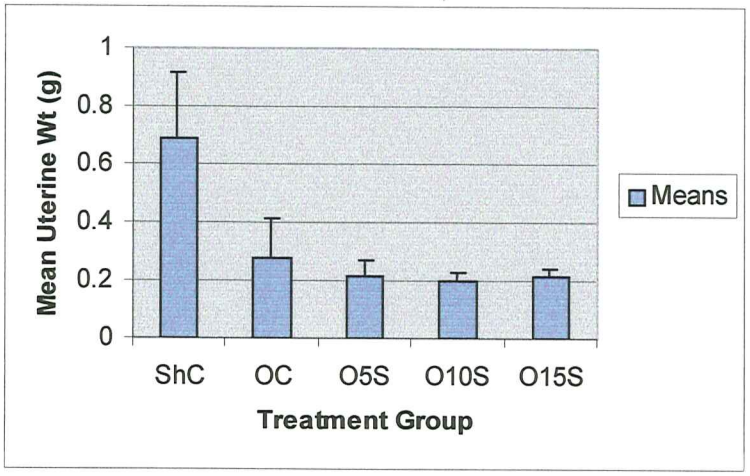

Figure 8. Mean uterine weight (g) of rats in ShC, OC, O5S, O10S, and O15 treatment groups.

The mean weight of the uterus (g) of the rats in the ShC group (0.686 ± 0.229) was significantly higher than the mean weights of the rats in the OC (0.274 ± 0.142), O5S (0.214 ± 0.055), O10S (0.199 ± 0.031), and O15S (0.211 ± 0.031) treatment groups (p=0.000). No other significant differences were observed between treatment groups.

Bone mineral density for specific bones

Humerus

Figure 9 shows mean bone mineral density (BMD) (g/cm^2) at the end of the study for the total humerus, elbow, and shoulder in ShC, OC, O5S, O10S, and O15S.

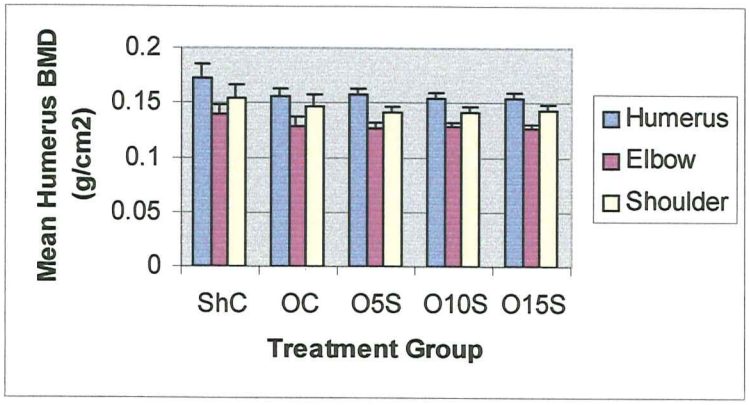

Figure 9. Mean BMD (g/cm^2) of total humerus, elbow, and shoulder for rats in ShC, OC, O5S, O10S, and O15S treatment groups.

Table 13 shows mean BMD (g/cm^2 $\pm$ SD) of the total humerus, elbow, and shoulder of each treatment group at the end of the study. Mean BMD of total humerus in the rats in the ShC group (0.173 $\pm$ 0.012) was significantly higher than those of the OC group (0.156 $\pm$0.008), O5S (0.157 $\pm$ 0.007), O10S (0.155 $\pm$ 0.005), and the O15S group (0.154 $\pm$ 0.006) (p=0.000). No other significant differences were determined.

Mean BMD of the elbow in the ShC group (0.139 $\pm$ 0.009) was significantly higher than those of the OC group (0.128 $\pm$ 0.009), O5S (0.127 $\pm$ 0.005), 010S (0.129 $\pm$ 0.004), and the O15S group (0.127 $\pm$ 0.004) (p=0.001). No other statistically significant differences were observed.

As with the total humerus and elbow, mean BMD of the shoulder of the ShC group (0.155 $\pm$ 0.012) was significantly greater than those of the OC group (0.147 $\pm$ 0.011), O5S (0.141 $\pm$ 0.006), O10S (0.141 $\pm$ 0.006), and the O15S (0.143 $\pm$ 0.006) (p=0.005). Again, no other significant differences were observed.

Table 13. Mean bone mineral density (g/cm^2 ± SD) of the total humerus, elbow, and shoulder for rats in ShC, OC, O5S, O10S, and O15S treatment groups.

	ShC	OC	O5S	O10S	O15S
Total humerus	0.173±0.012	0.156±0.008	0.157±0.007	0.155±0.005	0.154±0.006
Elbow	0.139±0.009	0.128±0.009	0.127±0.005	0.129±0.004	0.127±0.004
Shoulder	0.155±0.012	0.147±0.011	0.141±0.006	0.141±0.006	0.143±0.006

Femur

Figure 10 shows mean BMD (g/cm^2) at the end of the study for the total femur, hip (femoral neck), knee, and femoral midshaft for the ShC, OC, O5S, O10S, and O15S treatment groups.

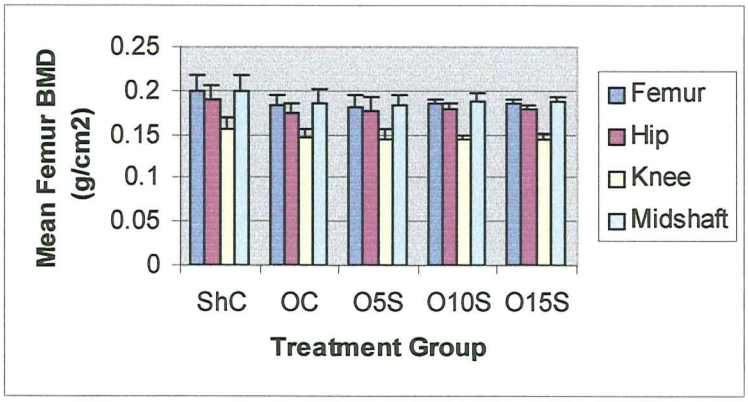

Figure 10. Mean BMD (g/cm^2) of the total femur, the hip (femoral neck), knee, and femoral midshaft for rats in the ShC, OC, O5S, O10S, and O15S treatment groups.

Table 14 shows mean BMD (g/cm^2 ± SD) for the total femur, hip (femoral neck), knee, and femoral midshaft for each treatment group at the end of the study. Mean BMD of the total femur of the ShC group (0.200 ± 0.017) was significantly higher than those of the OC group (0.183 ± 0.012), O5S (0.182 ± 0.012), O10S (0.186 ± 0.005), and the O15S group (0.186 ± 0.005) (p=0.005). No other treatment groups were significantly different.

Mean BMD of the hip of the ShC group (0.191 ± 0.015) was significantly higher than the OC (0.174 ± 0.012), O5S (0.177 ± 0.015), and the O15S (0.179 ± 0.005) groups; however, no significant difference was observed among the ShC and the O10S groups (0.180 ± 0.006) (p=0.035). Mean BMD of the O10S group, however, was not significantly different from any treatment group.

The mean BMD of the knee for the rats in the ShC group (0.156 ± 0.014) was significantly greater than those of the OC (0.147 ± 0.008), O5S (0.145 ± 0.011), O10S (0.145 ± 0.005), and the O15S groups (0.145 ± 0.006) (p=0.041). No significant differences were observed among any other groups.

The mean BMD of the femoral midshaft of the ShC group (0.199 ± 0.020) was significantly higher than those of the OC (0.186 ± 0.015) and O5S groups (0.183 ± 0.011) (p=0.086). No significant differences were observed in the O10S (0.188 ± 0.010) and the O15S groups (0.188 ± 0.005) and the ShC group, nor were there significant differences among the OC, O5S, O10S, and O15S treatment groups.

Table 14. Mean BMD (g/cm^2 ± SD) of the total femur, hip, knee, and midshaft for rats in the ShC, OC, O5S, O10S, and O15S treatment groups.

	ShC	OC	O5S	O10S	O15S
Total femur	0.200±0.017	0.183±0.012	0.182±0.012	0.186±0.005	0.186±0.005
Hip	0.191±0.015	0.174±0.012	0.177±0.015	0.180±0.006	0.179±0.005
Knee	0.156±0.014	0.147±0.008	0.145±0.011	0.145±0.005	0.145±0.006
Midshaft	0.199±0.020	0.186±0.015	0.183±0.011	0.188±0.010	0.188±0.005

Tibia

Mean BMD (g/cm^2) for the total tibia and tibia midshaft for ShC, OC, O5S, O10S, and O15S groups at the end of the study is shown in Figure 11.

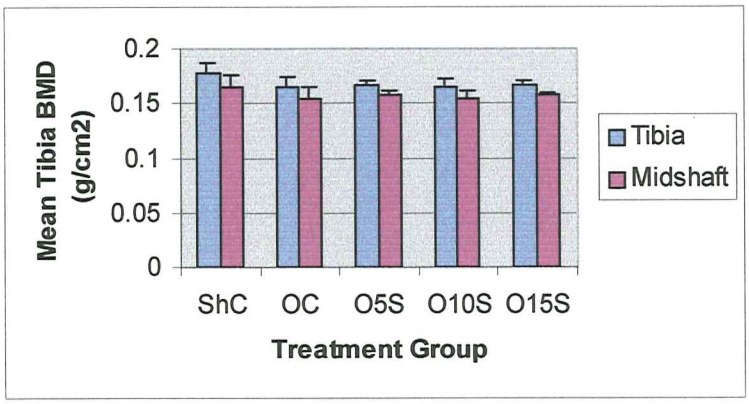

Figure 11. Mean BMD (g/cm^2) of the total tibia and midshaft for rats in ShC, OC, O5S, O10S, and O15S treatment groups.

Table 15 shows mean BMD ($g/cm^2 \pm$ SD) for the total tibia and tibia midshaft for each treatment group at the end of the study. Mean BMD of the total tibia of the ShC group (0.178 ± 0.010) was significantly higher than those of the OC (0.165 ± 0.010), O5S (0.167 ± 0.004), O10S (0.166 ± 0.007), and the O15S groups ($0.167 \pm .004$) ($p=0.003$). No statistically significant differences were noted among any other groups.

Mean BMD of the tibia midshaft of the ShC (0.165 ± 0.011) group was significantly greater than the OC (0.155 ± 0.011), O10S (0.155 ± 0.007), and O15S groups (0.157 ± 0.003) ($p=0.070$). There was no significant difference between ShC and the O5S groups (0.157 ± 0.005). Also, the O5S group was not significantly different from any other treatment group.

Table 15. Mean BMD (g/cm^2 ± SD) of the total tibia and midshaft for rats in ShC, OC, O5S, O10S, and O15S treatment groups.

	ShC	OC	O5S	O10S	O15S
Total tibia	0.178±0.010	0.165±0.010	0.167±0.004	0.166±0.007	0.167±0.004
Midshaft	0.165±0.011	0.155±0.011	0.157±0.005	0.155±0.007	0.157±0.003

Vertebrae

Mean BMD (g/cm^2 ± SD) of the vertebrae of ShC, OC, O5S, O10S, and O15S treatment groups three weeks before the ovariectomy and sham operations, four weeks after surgery, and eight weeks after surgery is shown in Table 16.

Table 16. Mean BMD (g/cm^2 + SD) of the vertebrae of rats in the ShC, OC, O5S, O10S, and O15S treatment groups 3 weeks pre-surgery, 4 weeks post-surgery, and 8 weeks post-surgery.

	ShC	OC	O5S	O10S	O15S
3 weeks pre-surgery	0.178±0.017	0.183±0.020	0.181±0.029	0.190±0.020	0.190±0.014
4 weeks post-surgery	0.176±0.014	0.179±0.015	0.172±0.015	0.176±0.012	0.177±0.013
8 weeks post-surgery	0.180±0.012	0.175±0.019	0.180±0.018	0.201±0.025	0.206±0.029

No significant differences were observed in BMD of the vertebrae among treatment groups three weeks before surgery or four weeks after surgery. Eight weeks post-surgery, however, mean BMD for the vertebrae of rats in the O10S (0.201 + 0.025) and O15S (0.206 + 0.029) groups were significantly higher than those in the ShC (0.180 + 0.012), OC (0.175 + 0.019), and O5S (0.180 + 0.018) treatment groups (p=0.006). Figure 12 illustrates mean BMD (g/cm^2) of the vertebrae of each treatment group eight weeks after surgery. There were no significant differences among the ShC, OC, and O5S treatment groups. Additionally, no significant differences were observed between O10S and O15S groups.

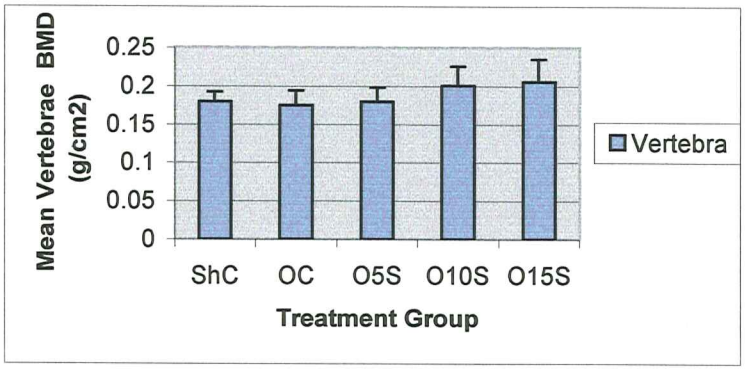

Figure 12. Mean BMD (g/cm^2) of the vertebrae of rats in the ShC, OC, O5S, O10S, and O15S treatment groups eight weeks post-surgery.

Figure 13 illustrates mean BMD (g/cm^2) for the vertebrae within treatment groups three weeks prior to surgery, four weeks after surgery, and eight weeks post surgery.

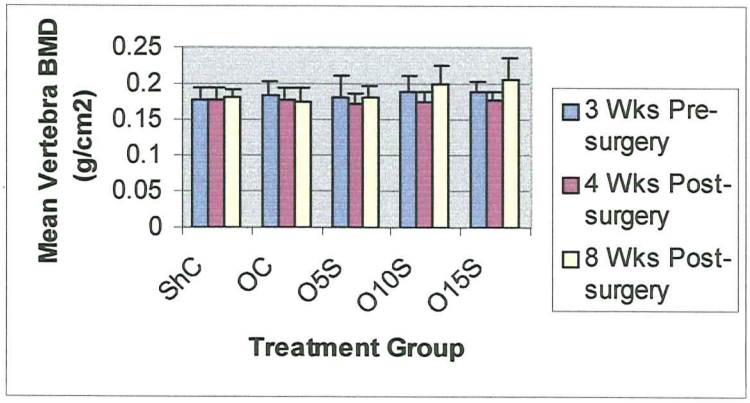

Figure 13. Mean BMD (g/cm^2) of the vertebrae of rats within groups of ShC, OC, O5S, O10S, and O15S treatment groups.

No significant differences were observed within the OC (0.180 $\pm$ 0.012, 0.179 $\pm$ 0.015, 0.175 $\pm$ 0.019, respectively), O5S (0.181 $\pm$ 0.029, 0.172 $\pm$ 0.015, 0.180 $\pm$ 0.018, respectively), and ShC (0.178 $\pm$ 0.017, 0.178 $\pm$ 0.017, 0.180 $\pm$ 0.012, respectively) groups. Data indicate BMD of the vertebrae in the O10S (0.190 $\pm$ 0.020, 0.176 $\pm$ 0.012, 0.201 $\pm$ 0.025, respectively), and O15S (0.190 $\pm$ 0.014, 0.177 $\pm$ 0.013, 0.206 $\pm$ 0.029,

respectively) groups increased significantly between four and eight weeks post surgery ($p=0.013$).

Table 17 shows the BMD for the vertebrae within treatment groups three weeks prior to surgery, four weeks and eight weeks post-surgery.

Table 17. Mean BMD ($g/cm^2 \pm SD$) of the vertebrae of rats within treatment groups 3 weeks pre-surgery, 4 weeks post surgery, and 8 weeks post surgery.

	3 weeks pre-surgery	4 weeks post-surgery	8 weeks post-surgery
ShC	0.178 ± 0.017	0.178 ± 0.017	0.180 ± 0.012
OC	0.183 ± 0.020	0.179 ± 0.015	0.175 ± 0.019
O5S	0.181 ± 0.029	0.172 ± 0.015	0.180 ± 0.018
O10S	0.190 ± 0.020	0.176 ± 0.012	0.201 ± 0.025
O15S	0.190 ± 0.014	0.177 ± 0.013	0.206 ± 0.029

CHAPTER 5

DISCUSSION

This study was designed to evaluate whether soybean protein isolates are effective in preventing bone loss following ovariectomy (OVX) in retired breeder Sprague Dawley rats; and if so, at what level provides the maximum effect. In this study, there were no significant differences in food intake or weight gain among treatment groups. There were no significant differences in the average weight of the heart, spleen, kidneys, or abdominal fat. Mean liver weight was significantly lower in the OVX 15% soy (O15S) group than in the sham-operated (ShC) and the OVX control (OC) group. Uterine weights of the sham-operated (ShC) group were significantly higher than all other groups. Eight weeks post-OVX, mean bone mineral density (BMD) of the vertebrae from OVX, 10% soy (O10S) and O15S groups was significantly higher than rats in the other treatment groups. Finally, BMD increased significantly between four and eight weeks post-OVX in the O10S and O15S groups.

Food intake

The results of our study did not support the hypothesis that OVX in Sprague-Dawley rats contributes to an increase in food intake. Previous studies in OVX rats have shown inconsistent intakes of food following OVX. It has been reported previously that OVX rats experience an increase in food intake in as few as 28 days after OVX (8, 122, 124). McElroy and co-workers (124) examined the short- and long-term effects of OVX on food intake and body weight in 60 female rats. During the first 5 weeks after OVX, rats overate and rapidly gained weight. Five weeks after OVX food intake returned to the level of the controls and body weights stabilized 12-16% above sham-operated control

weights for the duration of the 12-week study (124). Roy and co-workers (125) found at the end a 44-day study, food intake by OVX rats had not increased significantly, suggesting that variables other than OVX-induced hyperphagia contributed to post-OVX weight gain (125).

It is unclear why there are reports of differences in food intake in OVX animals and why we found no increase in food intake by rats in our study. The age of the animal could impact intake; younger rats that are still growing may eat more than rats that are completely grown. In this study, retired breeder rats were used; while they are not completely grown, they are not growing as rapidly as a younger rat would, and thus did not have the energy needs of growing animals.

Food intake may have to do with the timing of the study. In our laboratory, we have noted in previous studies that food intake in rats, including OVX rats, is highest after arrival of the rats from the vendor. This is due, in part to recovery from travel; however, it has been speculated (126) that exposure to a novel diet increases intake. During the first four weeks that the rats used in the present study were in our facility, this theory was tested using a pelleted rat chow and a powdered American Institute of Nutrition (AIN) diet; it was found that rats did prefer a novel diet. In the part of the study presented in this thesis, rats were switched again to a powdered AIN diet, with or without increasing concentrations of soy protein; since intake did not increase, it could be hypothesized that the powdered diet was no longer "novel."

Other reasons for the observed changes in food intake, or lack thereof, could be the time at which the surgery was performed—we have had the surgery done in our facility, as was done in this study, or in the facility of the vendor. Intake goes down

following surgery. Finally, the diet itself could impact intake; for example, the fat content and overall palatability of the diet are important considerations.

Weight gain

The results of our study did not support the hypothesis that OVX would increase weight and abdominal fat in Sprague-Dawley rats. Weight gain in OVX rats has been reported by others following OVX, but not in sham-operated rats (8, 119, 127, 128), suggesting that hormonal changes following removal of the ovaries stimulated weight gain. Lack of significant differences in body weight among treatment groups at the end of our study conflicts with some of these previously published studies. Arjmandi and co-workers suggested that a soy protein diet prevented OVX-induced body weight gain in rats (119). No definitive explanation was provided by the author for his results; however, he suggested it was because genistein and daidzein in the soy act in a manner similar to estrogen in preventing weight gain after OVX (8, 119, 123). This seems unlikely however, since dietary genistein and diadzein are present at much lower concentrations than is endogenous estrogen.

The results seen in this study are consistent with other studies from our laboratory (M. Hegsted, personal communication). There are several reasons that animals might not have gained weight in the present study. The most obvious is that there were no differences in food intake; and the diets used in this study were relatively low fat. Further, although animals were stratified by weight and mean weights of a treatment group were statitistically equivalent, there were still differences in individual animal weights in the groups. This, coupled with small sample size, and the fact that the rats in

our study were still growing, could have masked changes in weight of individual groups. Lack of differences in abdominal fat weight is consistent with this finding.

Uterus weights

Animals in the OVX groups exhibited uterine atrophy, which was not seen in the ShC group; this supported the hypothesis that OVX would decrease mean uterine weight. In the OVX control and soy treatment groups, uterine atrophy confirmed successful ovariectomy. That there was no difference among the OVX treatment groups, confirms other literature reports that soy isoflavones do not have the uterine-protective effect of estradiol (119, 120, 122, 127). The dose of soy protein used in these studies may not be high enough to elicit this effect. In a study by Ishimi and associates (128), genistein, given at a 10-fold higher dose than that needed to exhibit bone-protective activity in OVX mice, did induce uterine atrophy (128).

Heart, spleen, kidney, and liver weights

The results of this study supported the hypothesis that soy protein would not affect the weights of the heart, spleen, or kidneys in OVX rats. The study does not support the hypothesis that soy protein would not affect liver weight. Published data looking at organ weights in OVX rats are limited; however, Harrison and co-workers (127) reported that soy-fed OVX rats in their study had significantly smaller liver weights compared with the sham-operated rats. Estrogen administration resulted in a significant increase in liver weight in OVX rats (127).

Madani and associates (129) showed that liver weights were higher in rats fed a casein diet with added cholesterol when compared with a soy protein diet. They used five-week old male Wister rats. The use of a different strain, age, and gender of rats may

have accounted for this difference in the liver weights (129). Decrease in mean liver weight of the soy-fed rats may be due to decreased hepatic cholesterol and fat storage. This theory is intuitively appealing because of soy protein's known ability to reduce serum cholesterol levels (130).

Bone density

The results of our study did not support the hypothesis that soy protein would exert a positive effect on BMD of the humerus, femur, or tibia. They did, however, support the hypothesis that the vertebrae of soy fed rats would show a significant increase in BMD.

Several studies using rats have confirmed that BMD decreases with OVX (8, 119-122, 131); several methods have been used to assess BMD. Results from a study by Arjmandi and associates (120) reported that rats injected with 17β-estradiol had a decrease in the concentration of biochemical markers associated with bone loss in OVX animals; they used this as evidence that estrogen prevented bone loss in rats. In that study, thirty-two Sprague-Dawley rats were divided into four treatment groups: sham-operated + casein diet; OVX + casein diet; OVX + soybean; OVX + 17β-estradiol + casein diet. Rats in the OVX + casein group had a lower BMD of the right femur and fourth lumbar vertebra than the other groups; this loss was prevented completely by 17β-estradiol. Compared with the three casein diet groups, the soy protein diet was also effective in preventing bone loss in the fourth vertebrae, and was partially effective in the right femur. Results of that study agree with our results in that the soy protein prevented bone loss in the vertebrae; however, this study does not agree with our findings on the femur. Length of the study, age of the rat at OVX, and soy protein dose could explain the

differences observed between that study and the current study. The concentration of soy used in our study may have been too low to see a difference in BMD of the humerus, femur, and tibia. Our study may not have been long enough to see a difference in the varying levels of soy in the humerus, femur, or tibia.

Harrison and associates (127) demonstrated that a 22% soy protein diet could be as effective as daily estrogen administration in suppressing bone loss due to OVX in the tibia and femur of Sprague-Dawley rats. In that study, rats were OVX or sham-operated and fed standard non-purified diet for two weeks to allow for development of moderate osteopenia. After two weeks, the OVX rats were then changed to either a 22% soy protein diet, a casein diet, or casein diet with estrogen injections; sham-operated rats were fed a casein diet. The rats were sacrificed after four weeks on this high protein diet. Wet and dry weights of the femur and tibia of rats in each treatment group were compared. Compared to the Sham group, OVX caused a slight decrease in the wet weight of the tibia in the casein group, which was prevented by the soy diet and estrogen treatment. Compared to our study, that study showed that a higher dose of soy protein for a shorter period of time has a positive effect on bone density of the tibia and femur of OVX rats. Our study measured bone density using the more sensitive method of peripheral dual energy x-ray absorptiometry (pDEXA), whereas Harrison's study measured wet and dry bone weight to determine effectiveness of soy protein on bone loss. Thus, the difference in the results may be due to the method of measurement (127).

Humans may also require higher doses of isoflavones before improved BMD can be observed. In a study of 66 postmenopausal women by Potter and associates (108), BMD and bone mineral content of the lumbar spine improved significantly following

consumption of ~90 mg of a soy protein supplement; whereas, women consuming ~56 mg of soy did not show this improvement. Alekel and co-workers (96) showed similar results in their study of 69 perimenopausal women—consumption of 80.4 mg soy isoflavones per day resulted in significantly higher BMD and BMC in the lumbar spine than did women consuming lower amounts. Length of the study may be especially important. Dietary studies that last only a short period of time (several weeks) may not be sufficient to see changes in BMD or BMC because the bone remodeling cycle, in humans, ranges from 30 to 80 weeks (96). The length of time for the bone remodeling process to occur in rats is unknown.

The finding of increased bone density in the vertebrae in our study is very exciting. They can be explained by the fact that the vertebrae contain more trabecular bone than the femur, humerus, and tibia. Trabecular bone is more sensitive to compounds that affect remodeling, such as soy isoflavones (4). These data also suggest that the OVX rat model is an appropriate one for testing the effect of soy protein on BMD. Osteoporosis is an enormous health problem responsible for at least 1.3 million fractures per year. Of those fractures, 500,000 are vertebral fractures. One-third of all women over the age of 65 will have vertebral fractures. Bone loss in the vertebra also causes back pain and shortening of height. The direct and indirect costs of osteoporosis are estimated to be $6.1 million annually (4).

Parallels to human studies on soy isoflavones can be drawn. Asians consume 10-100 times more isoflavones than Western people (19). Correlational evidence, based on the low prevalence of hip fractures in Japanese women (112) and the high soy intake of

the Japanese, lead to the hypothesis that the high consumption of isoflavones from soy products may be protective against fractures, especially hip fractures. (11, 113).

In conclusion, this study has demonstrated that soy protein can suppress bone loss due to OVX in vertebral bone. The results provide support for soy diet as a potential alternative to estrogen replacement therapy for the maintenance of bone mass, at least vertebral bone, in postmenopausal women. This could confer protection against osteoporosis.

Future Directions

It is important to determine whether soy protein can be effective in maintaining bone mass in the long bones, as well as the spine. Hip fracture, actually a fracture of the head of the femur, is a major cause of morbidity and mortality in those with osteoporosis; thus, finding a treatment that could assuage bone loss in the femur would be important. Studies of variable length, using higher concentrations of soy protein or isolated isoflavones could be conducted. Parallel studies looking at biochemical markers of bone turnover, such as osteocalcin and urinary deoxypyridinoline could confirm the mechanism by which bone metabolism is affected by soy protein diets in OVX rats.

It might also be important to assess the effect of compounds in soy other that the isoflavones. Other components of soybean, such as peptides, are also able to increase the BMD and bone strength of OVX rats (132). Omi and co-workers (132) suggested that the skeletal effects observed in their study were due to the acceleration of intestinal calcium absorption by the peptides in soybean. Although we did not assess intestinal calcium absorption in this study, the enhanced intestinal absorption of calcium may provide a partial explanation for the beneficial effects of soybean protein on bone health.

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APPENDIX

APPENDIX A

LOUISIANA STATE UNIVERSITY

AND AGRICULTURAL AND MECHANICAL COLLEGE
Institutional Animal Care and Use Committee
Division of Laboratory Animal Medicine

Animal Welfare Assurance # A3612-01
License # 72-3
Multiple Assurance # M1128

May 14, 2001

Dr. Maren Hegsted
Human Ecology

Dear Dr. Hegsted:

Protocol #01-033, entitled "The Effects of Soy Protein and Rice Bran Oxyzanol on bone Density in Ovariectomized Retired Breeder Rats" lists you as the Principal Investigator.

I am happy to inform you that your protocol was **approved** by the IACUC during our regularly scheduled meeting held on May 10, 2001. This approval is valid for 3 years and authorizes the use of 85 rats.

In accordance with federal regulations, all personnel conducting animal-based research must receive training in the rules and regulations of animal use, and proper handling methods for the species involved. To meet this requirement all personnel, including yourself, involved with this research project must attend a rules and regulations class. Exemption from participation in the wet-lab, based on previous experience, may be obtained by written request. You have six months to satisfy this requirement. **This is the only reminder you will receive concerning this.**

When ordering animals for this project, please provide a copy of this letter to DLAM along with your order. This will help keep better track of the animals being used by various investigators. Thank you!

Sincerely,

Rustin Moore

Rustin Moore, DVM, PhD, ACVS Dip.
Acting Chairman

jdb

Revised: March 2001

LSU PROTOCOL FOR ANIMAL CARE AND USE

Instructions for Submission: MUST BE TYPED! (Use additional sheets if necessary and attach to this form or use word processor and add lines). **SUBMIT ORIGINAL plus 12 COPIES** to the IACUC Office (Rm. 1502 School of Veterinary Medicine).

PROTOCOL NUMBER: 01-233

APPROVAL DATE: 5-10-01

SECTION 1: Principal Investigator

Name: Maren Hegsted	**Office Phone:** 578-1518 **Home Phone:** 769-3097	**E-mail address:** mhegsted@lsu.edu

SECTION 2: Project Title (Enter the name of your project/course number in the block below)

The effects of soy protein and rice bran oryzanol on bone density in ovariectomized retired breeder rats

SECTION 3:

Animal Species

Species: Rat	**Strain:** Sprague-Dawley

Revised: March 2001

Number of animals needed: Year 1: ~~75~~ 85 Year 2: 0 Year 3: 0 TOTAL: ~~75~~ 85	Maximum number needed at one time: ~~75~~ 85	Number of animals to be placed in each group: 10 10 rats x ~~8~~ groups = ~~80~~ rats 5 extra rats to test pDEXA live animal methodology

Animal housing and veterinary care have been coordinated with DLAM office OR LSU Agricultural Center Unit.

X YES
☐ NO

Name of Animal Housing Representative Contacted (typed): Laurie Henderson

Signature (required): Laurie Henderson

Location of Animals

	DLAM Vivarium	X	Life Sciences Vivarium
	SVM Barns		SVM Fish Building
	LAES (List Site):		Other (List Site):
	Field Study (Do not complete sections 6, 11, and 12		

SECTION 4: Abstract Plan of Research/Teaching

Provide a brief layman's description of the project in the block below.

Revised: March 2001

Osteoporosis affects over 20 million women in the United States and that number will grow in the coming years as our population ages. Estrogen replacement therapy can reduce osteoporosis risk but many women cannot or will not take estrogen because of unwanted side effects. We are examining the potential benefits of natural phytoestrogens found in soy protein and another phytochemical in rice bran which may impact bones. Ovariectomized rats are an animal model used to evaluate treatment/prevention of osteoporosis. We will evaluate the dose-response of 3 levels of soy protein in preventing ovariectomy-induced bone loss. We will also test 2 levels of rice bran oryzanol and a high protein, high fat diet used previously to determine if levels of protein and fat affect bone. We will have 2 control groups and 6 test groups using 85 retired Sprague-Dawley breeder rats with 10 rats assigned to each group and 5 extra rats. 75 rats will be ovariectomized (ovx) and 10 will receive a sham operation by LSU veterinarians. The treatment groups are: ShC, sham-operation+control diet; OC, ovx+control diet; O5S, ovx+5% soy diet; O10S, ovx+10% soy diet; O15S, ovx+15% soy diet; OH, ovx+high protein, high fat diet; O1O, ovx+1% oryzanol diet and O2O, ovx+2% oryzanol diet. The additional 5 rats requested are for testing methodology for measuring bone density in live animals with the pDEXA instrument (peripheral dual energy x-ray absorptiometry) and to replace any rats lost to surgery or recovery. Bone density will be measured in the live animals before ovx, 4 weeks and 8 weeks post-ovx. Markers for bone resorption and formation will be measured in urine and blood samples. Bones will be removed at sacrifice for further measurements of bone parameters.

SECTION 5: Investigator's Statement. Assurances for the Humane Care and Use of Vertebrate Animals.

By signing this form, we agree to abide by the Policy for the Care and Use of Animals of Louisiana State University, or that of the LSU Agricultural Center. This project will be in accordance with the NIH "Guide for the Care and Use of Laboratory Animals" (except as explained in the accompanying protocol), and the Louisiana State University or the LSU Agricultural Center Animal Welfare Assurance on file with the U.S. Public Health Service.

I further assure the Committee that: **1) I will abide by all federal, state, and local laws and regulations governing the use of animals in teaching and research; 2) the investigators and technicians are adequately trained to perform the research techniques required in these studies; and 3) the fewest number of animals required to produce significant results are being used in this study.**

Revised: March 2001

Maren Hegsted	Professor	5/2/01
Principal Investigator Signature	Title/Rank	Date

Maren Hegsted
(Type Name of Principal Investigator)

Carol E. O'Neil	Professor	5/2/01
Principal Investigator Signature	Title/Rank	Date

Carol O'Neil
(Type Name of Principal Investigator)

Michael Keenan	Associate Professor	5/2/01
Co-Investigator Signature	Title/Rank	Date

Mike Keenan
(Type Name of Co-Investigator)

	Instructor	5/2/01
Surgeon (if applicable) Signature	Title/Rank	Date

Rhett Stout
(Type Name of Surgeon)

SECTION 6: Special Husbandry Requirements

Do your animals have special needs to be addressed by DLAM?

☐ x **YES**

TEMPERATURE RANGE		(F)	Humidity:		(%)
LIGHT CYCLE (hours light/hours dark)	12/12				
CAGING	Type: individual hanging	Size:		Filter tops required? no	
BEDDING/LITTER	Type:	Autoclaved?		Changes/week: we change	
WATER	Sterile:	De-ionized:	Acidified:	Tap:	Other:
DIET	Special Feeding Requirements: we will feed animals				

Revised: March 2001

OTHER SPECIAL NEEDS	

☐ **NO** (If you indicate 'No', your animals will be cared for according to standard operating procedures of DLAM)

☐ **Not Applicable**

SECTION 7: Hazardous Materials

Will zoonotic or recombinant, radioactive, or hazardous chemical agents be **PRESENT IN THE ANIMAL ROOM?**

Zoonotic/Recombinant Agents?	Radioisotopes?	Hazardous Chemicals?
☐ YES X NO	☐ YES X NO	☐ YES X NO
Agent(s):_____	Isotope(s):_____	Compound(s):_____
☐ EXEMPT	Are you certified by the Radiation Safety Committee?	
IBRDS Chairman **Signature:** _____	☐ YES ☐ NO	

Note: If zoonotic (infectious to humans) or recombinant organisms are to be used, this protocol request must be submitted to the Biohazardous Materials Safety Committee for approval **PRIOR TO CONSIDERATION** by the IACUC. Similarly, if hazardous chemicals are to be used in the animal room, submit the proposal to the Chemical Safety Committee for prior approval. **P.I. MUST PROVIDE** health and safety measures for animal technicians and facility maintenance personnel. In Standard Operating Procedure (SOP) form, describe any precautions, procedures, or personal protection required in handling animals or waste containing listed agents or compounds, or in working in or around the animal room (including air handling system), and **attach a copy of your SOP(s) to this protocol proposal.**

Revised: March 2001

SECTION 8: Summary of Procedures

Your response in this section should provide the reader with a complete description of how every animal to be used in this project is to be treated during every phase of the study. Your target audience is a faculty member from a discipline unrelated to yours. Do not use jargon. Please include the following information:

4) The rationale for using animals. Why should this study be done? What hypothesis will be tested?
5) How and/or why you selected the animal species indicated.
6) How you arrived at the number of animals to be used.
7) A complete description of the proposed use of the animals. Describe the experimental design of the study. Include a list of any physical, chemical or biological agents **(name, dose, volume, route, frequency)** that may be administered. Tables and outlines are helpful to indicate group assignments and study progression.
8) A description of procedures designed to assure that discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research. Indicate how analgesic, anesthetic, and tranquilizing agents will be used where appropriate, to minimize discomfort and pain to the animals. It is advisable that you obtain input from LSU's Attending Veterinarian (Dr. David Baker) or from another veterinarian familiar with the species to be used.
9) A description of any euthanasia method to be used.

Revised: March 2001

Ovariectomized rats are used as an animal model for postmenopausal women for evaluating methods of reducing osteoporosis. We are testing diet variables as alternatives to estrogen replacement therapy and other drugs that reduce osteoporosis. It is important to obtain more information on the dose response to varying levels of soy isoflavones as well as specific affects of diet treatment on bone breaking strength which cannot be measured in humans. This study is a continuation of research begun last summer where we evaluated the effects of rice bran oil and 3 levels of soy protein on bone and heart disease parameters in ovariectomized retired breeder rats. Last year we choose to use a high protein, high fat diet so we could raise the levels of soy and rice bran oil in the diets. We found that the high protein, high fat control diet ameliorated the effects of ovariectomy on bone, preventing any loss of bone density in long bones (tibia, humerus and femur) but not preventing loss of bone density in vertebrae which are primarily trabecular bone. This year we will evaluate the effects of 3 levels of soy protein and of 2 levels of rice bran oryzanol in preventing ovariectomy-induced bone loss at the normal levels of protein and fat used in rat diets. We will also re-test the high protein, high fat diet to determine if the diet effects seen last year are repeatable. We have found that 10 rats per diet group is sufficient to detect significant differences in bone parameters. 85 Sprague-Dawley retired breeder rats approximately 9 months old will be used. Rats will be allowed a month's recovery time from shipping and from their last pregnancy/lactation cycle before surgery. Rats will be blocked by weight and randomly assigned to surgery/diet treatment groups.

ShC sham-operation, casein diet, n=10
OC ovariectomized (ovx), casein diet, n=10
O5S ovx, 5% soy diet, n=10
O10S ovx, 10% soy diet, n=10
OS ovx, 15% soy diet, n=10
O1O ovx, casein diet + 1% oryzanol, n=10
O2O ovx, casein diet + 2% oryzanol, n=10
OH ovx, high casein + high fat diet, n=10

The five extra rats ordered will be used to determine the optimum scan parameters for measuring bone density and body composition in live animals by a pDEXA (peripheral dual energy x-ray absorptiometer, SABRE, Norland). Rats will be either ovariectomized (75) or sham-operated (10) by LSU veterinarians.

Ovariectomy: Rats will be anesthetized via Isoflurane inhalation in an induction chamber, followed by transfer to the surgical table and fitting with an inhalation mask and continued Isoflurane inhalation. Following induction, a single injection of buprenorphine (0.05 mg/kg SQ) will be given to provide preoperative analgesia. The hair over the mid to dorsal aspects of the abdomen will be removed with a clipper

and an antiseptic (betadyne) applied to the skin surface. The following surgical procedure will be performed aseptically. A small dorsal midline incision will be made halfway between the middle of the back and the base of the tail. Entrance to the peritoneal cavity will be made by small bilateral incisions made 2/3 down the side of the body wall. The ovaries will be removed by sharp dissection and the horn returned to the abdominal cavity. No hemostasis is required. The muscle incisions do not require suturing unless they have been inordinately large, where a single suture (5-0, PDS) will be placed. Skin will be closed with "superglue". The rats will be returned to their cages and monitored until ambulatory. Daily observations by caretakers will determine if rats need additional analgesia. Rats showing signs of pain (lethargy, anorexia, rough coat) will be given buprenorphine (0.05 mg/kg SQ, tid) for as long as needed.

Sham-operation: The rats receiving the sham-operation will undergo all of the above procedures except that the ovaries will not be removed and the intact ovaries and uterine horns will be returned to the abdominal cavity.

Rats will be analyzed for body composition and bone density three times during the study: the week before surgery, 4 weeks after surgery and 8 weeks after surgery. For this analysis, individual rats will be anesthetized via Isoflurane inhalation in an induction chamber, followed by transfer to the pDEXA and fitting with an inhalation mask and continued Isoflurane inhalation. The 3 scans (similar to x-rays but lower dosage of radiation) will take about 15 minutes (exact time to be determined with test animals). After the last scan, the rats will be returned to their cages. In addition, during weeks 4 and 8 post-surgery rats will be placed in individual metabolism cages for 24 hours for collection of a 24-hour urine specimen.

Rats will be killed by exsanquination in week 9 after anesthesia is induced via Isoflurane inhalation in an induction chamber, transfer to a dissecting table and fitting with an inhalation mask for continued Isoflurane inhalation. Blood will be removed via cardiac puncture, followed by removal of the heart, liver, uterus, abdominal fat, and selected bones. Bone density of individual bones and specific bone regions will be measured postmortem, as will bone breaking strength.

Revised: March 2001

SECTION 9: Type of Project

	TYPE A - Pain or distress will not be induced; animals will only be used for injections, collections, or procedures causing nothing more than minor discomfort; or will be humanely euthanized prior to induction of pain or distress.
X	**TYPE B** - Pain or distress will be relieved by appropriate therapy.
	TYPE C - Drug intervention for pain or distress would interfere with the protocol. **(If this block is checked, specific justification MUST be provided.)**

SECTION 10: Check "Yes" or "No" to each of the following questions. On a separate page, provide an explanation for any "Yes" answers that are not included in the above summary.

Provide justification for **why** the action is needed, and include information in Section 8 above, such as **who** will perform procedures, **how** they will be performed, frequency, duration, drugs to be used, dosages, routes of administration, etc. Not all of this nformation may be needed for every "Yes" answer. The information you provide in this section is very important in highlighting specific points of your study that are important considerations for the IACUC in their review process.

YES	NO		Individual(s) Responsible
	X	Will animals be restrained? *(Restraint refers to immobilization or other restrictions to normal movement beyond momentary holding for injections, etc.)*	Not Applicable
	X	Will animals be fasted?	Not Applicable
X		Are any ANESTHETICS, ANALGESICS, or TRANQUILIZERS to be used? Who will administer?.....	Dr. Rhett Stout Dr. Maren Hegsted Dr. Mike Keenan _____
	X	Are neuromuscular blocking agents to be used? Who will administer agents?..... How will animals be monitored?_____ _____	_____

Revised: March 2001

X		Will surgical procedures be employed? Are they: Survival X Multiple_____ Terminal_____ Who will perform surgery?..... If survival: **1) Who** will be responsible for recovery of the animals?..... **2) Who** will maintain post-operative records?..... **3) Where** will records be maintained? With the animal records outside the animal room **4) Who** will provide post-op analgesics?..... **Note:** *Survival surgeries must be conducted aseptically, and major surgical procedures performed on non-rodent species must be conducted in a dedicated surgical facility.*	 Dr. Rhett Stout, Dr. Kem Singletary, Dr. Marie Grant, Adam Ralston, Matthew Wheelcock Dr. Marie Grant Dr. Maren Hegsted Dr. Marie Grant Dr. Marie Grant
X		Do you anticipate any adverse effects of the experimental procedures on the animals (e.g., pain, discomfort, reduced growth, fever, anemia, etc)? Possible pain in recovery from surgery. If rats show signs of discomfort they will be given additional analgesia as described in the summary of procedures.	Not Applicable
	X	Is death an endpoint in your experimental procedure? **Note:** *Death as an endpoint refers to acute toxicity testing, assessment of virulence of pathogens, neutralization tests for toxins, and other studies in which animals are not euthanized, but die as a direct result of the experimental manipulation.*	Not Applicable
	X	Are there emergency treatments by the DLAM veterinary staff that would not be allowed?	Not Applicable
	X	Are you using wild or exotic species for which permits are necessary? **(ATTACH COPY)** **Note:** *Permits are required for protocol approval.*	Not Applicable
X		Will animals be euthanized during or at the close of the study? Who will perform euthanasia?.....	Dr. Maren Hegsted Dr. Mike Keenan

Revised: March 2001

	X	Will animals be used for antibody production?	Not Applicable
	X	Will Complete Freund's Adjuvant be used? **If yes, please justify based on scientific reasons.**_____	Not Applicable
	X	Will other adjuvants be used? **If yes, please specify.** _____	Not Applicable
X		Will blood be collected? How often? Once, at sacrifice Volume? 8-10 ml Who will collect blood?..... **Note:** *Blood equal to 1.5% of the animal's body weight per 2 weeks represents the upper approvable limit, unless scientific justification is provided.*	Dr. Maren Hegsted Dr. Mike Keenan

SECTION 11: Animal Management

Individual (or groups of) animals are identified by (i.e. tag, tattoo): Cage labels

Check all applicable below:

CARE OF SICK ANIMALS	DISPOSAL OF DEAD ANIMALS	PEST CONTROL
X Call Investigator X Clinician to Treat ☐ Euthanasia	X Call Investigator ☐ Necropsy ☐ Disposal. List any special requirements for disposal?_____	☐ Call Investigator X Pesticides OK ☐ No Pesticides

Revised: March 2001

SECTION 12: Disposition of Animals

What will be done with any animals at the conclusion of the project?

X	Animals will be euthanized.
	DLAM/LAES has permission to **REASSIGN** animals to another IACUC-approved protocol.
	TRANSFER animals to the following IACUC-approved protocol(s). **Please list Protocol Number:**_____
	OTHER (Please state) _____

SECTION 13: Narrative Statement

Federal regulations mandate that you provide **written, narrative statements** for all projects:

1) that the activities do not unnecessarily duplicate previous experiments. In this statement, include sources used to make such a determination (e.g., Databases, workshops, expertise in the field, etc.) If an electronic database was used, include database, years and words searched, and date of search.

Note: Address the following items only if you indicated project Type B or C in SECTION 9.

2) that you have considered alternatives to procedures producing more than momentary or slight pain or distress. Indicate what those alternatives were and why they are not appropriate.

3) describing the methods you used to determine that alternatives to such procedures were not available (Databases, years and words searched, date of search etc.). Put your statements in the block below.

1) The effects of dietary treatments on bone density and breaking strength can only be measured in bones from live animals. The Web of Science search system was used with the key words: soy protein, isoflavones, oryzanol, phytoestrogens and bone and the years 1990-present. There have been a number of studies published testing soy protein in this animal model but none have tested a dose response to 3 levels of soy protein. No one has reported the effects of oryzanol on bone.
2) The only source of pain in this study is from the ovariectomy surgery which is required to produce an estrogen-deficient animal model for postmenopausal women. Since this surgery is required by FDA for testing of all osteoporosis treatment/prevention studies there is no alternative method at this time.
3) Web of Science, 1990-present, animal models and osteoporosis.

SECTION 14: Investigator Training

In accordance with IACUC policy, all personnel conducting animal-based research must attend a Rules and Regulations Course and verify their training, experience and skills in the care and use of the animals and techniques they are responsible for.

List all persons involved in animal care and use for this study below.

RULES AND REGULATIONS TRAINING COURSES					
Name	**Rules/Regulations Training Course**	**Date Attended**	**Species Wet Lab***	**Date Attended**	Training and Experience**
Maren Hegsted	X YES ☐ NO	3/8/00	☐ YES ☐ NO		X YES ☐ NO
Mike Keenan	X YES ☐ NO	3/9/99	☐ YES ☐ NO		X YES ☐ NO
Carol O'Neil	X YES ☐ NO	6/1/99	☐ YES ☐ NO		X YES ☐ NO
Rhett Stout	X YES ☐ NO		☐ YES ☐ NO		X YES ☐ NO
Kem Singletary	X YES ☐ NO		☐ YES ☐ NO		X YES ☐ NO

Marie Grant	X YES ☐ NO	9/7/99	☐ YES ☐ NO		X YES ☐ NO
Adam Ralston	? YES ☐ NO		☐ YES ☐ NO		X YES ☐ NO
Matthew Wheelcock	? YES ☐ NO		☐ YES ☐ NO		X YES ☐ NO

* Exemption based on previous experience with the study species may be obtained by written request to the IACUC.

** Training/Experience in assigned procedures for this protocol.

Who will train individuals for participation in protocol procedures? Rhett Stout

Personnel participating in the project that have not attended the Rules and Regulations Course or the applicable Species Wet Lab, will have **six (6) months** from the approval date of the project to complete them.

Rules and Regulations Courses will be held the first Tuesday of every month from 11:00 a.m. until Noon, in room 1212C, School of Veterinary Medicine. The Wet Labs will be held on the same day beginning at 1:00 p.m. in the DLAM facility. Please call Ms. Dawn Best-Desjardins at 578-9643 to sign up for these courses.

SECTION 15: Occupational Health and Safety

It is the responsibility of the principal investigator to conduct a hazard analysis and risk assessment to determine if personnel involved directly or indirectly in the study should participate in the Occupational Health Program administered through DLAM and the Student Health Center.

Will project personnel participate in the Occupational Health Program?

☐ **YES**
X **NO**

If yes, please name participants below, and have them contact Mr. Rick Ramsey at 578-9644 for information.

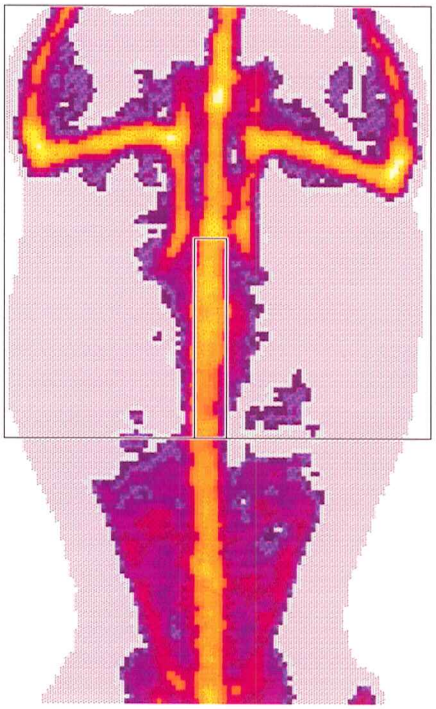
APPENDIX B

On the opposite page is a sample scan of a peripheral dual energy x-ray absorptiometry (pDEXA) scan of a vertebrae of an O15S rat.

LSU Human Ecology Nutrition Keenan, Hegsted, O'Neil

Name	15S3	*Ethnic*	SD RAT
ID	15S3090501	*Height*	17
Age	1 *Sex* N/A	*Weight*	372

L H Research on 09/05/01 11:56



09/05/01 0.206

Bone image not for diagnosis
Histogram Averaging Width: 0.0190 g/cm2

RES Scan

	BMD g/cm^2	BMC g	AREA cm^2	LENGTH cm	WIDTH cm
VERT	0.2063	0.6210	3.010	4.30	0.70
AB FAT	0.1286	4.548	35.37	9.30	9.20

See Operator's Guide for information on CVs.
1.0 x 1.0 mm, 40 mm/s, 11.40 cm Rev. 3.9.4/1.1.1 Calib. 09/05/01

COMMENTS

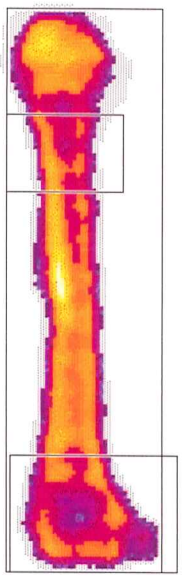
APPENDIX C

On the opposite page is a sample scan of a pDEXA scan of a humerus of an O15S rat.

LSU Human Ecology Nutrition Keenan, Hegsted, O'Neil

Name 15S3
ID 15S3 R HUMER
Age *Sex* N/A
Ethnic
Height
Weight

L H Research on 11/29/01 12:52



Bone image not for diagnosis

11/29/01 0.154

RES Scan

	BMD g/cm²	BMC g	AREA cm²	LENGTH cm	WIDTH cm
Humer	0.1540	0.1772	1.151	2.88	0.80
Elbow	0.1300	0.04222	0.3248	0.60	0.86
Shoulder	0.1426	0.01900	0.1332	0.40	0.60

See Operator's Guide for information on CVs.

0.2 x 0.2 mm, 10 mm/s, 2.00 cm Rev. 3.9.4/1.1.1 Calib. 11/29/01

COMMENTS

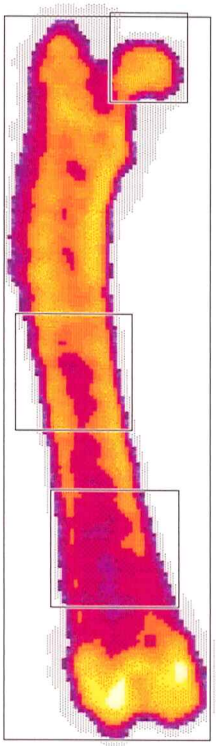
APPENDIX D

On the opposite page is a sample scan of a pDEXA scan of a femur of an O15S rat.

LSU Human Ecology Nutrition Keenan, Hegsted, O'Neil

Name	15S3		*Ethnic*
ID	15S3 R FEMUR		*Height*
Age		*Sex* N/A	*Weight*

L H Research on 11/29/01 13:04



Bone image not for diagnosis

11/29/01 0.186

RES Scan

	BMD g/cm²	BMC g	AREA cm²	LENGTH cm	WIDTH cm
Femur	0.1861	0.3764	2.023	3.70	1.06
Hip	0.1783	0.02007	0.1125	0.46	0.40
Knee	0.1428	0.04593	0.3215	0.60	0.66
Midshaft	0.1848	0.05121	0.2771	0.60	0.60

See Operator's Guide for information on CVs.

0.2 x 0.2 mm, 10 mm/s, 2.00 cm Rev. 3.9.4/1.1.1 Calib. 11/29/01

COMMENTS

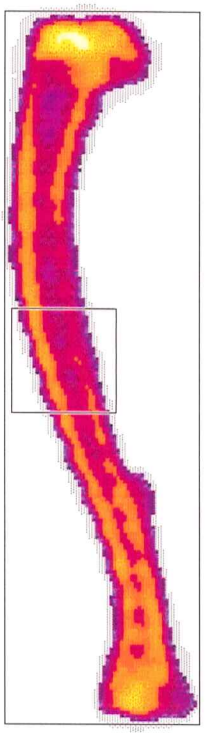
APPENDIX E

On the opposite page is a sample scan of a pDEXA scan of a tibia of an O15S rat.

LSU Human Ecology Nutrition Keenan, Hegsted, O'Neil

Name 15S3 *Ethnic*
ID 15S3 R TIBIA *Height*
Age *Sex* N/A *Weight*

L H Research on 11/29/01 13:22



Bone image not for diagnosis

11/29/01 0.170

RES Scan

	BMD g/cm²	BMC g	AREA cm²	LENGTH cm	WIDTH cm
Tibia	0.1696	0.2983	1.759	4.08	1.12
Midshaft	0.1539	0.03595	0.2337	0.60	0.60

See Operator's Guide for information on CVs.

0.2 x 0.2 mm, 10 mm/s, 2.00 cm Rev. 3.9.4/1.1.1 Calib. 11/29/01

COMMENTS