The effects of oryzanol on bone mineral density in ovariectomized, retired breeder rats

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The effects of oryzanol on bone mineral density in ovariecotomized, retired breeder rats

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science in The School of Human Ecology

By

Heather Compton Colona
B.S., Louisiana State University, 2000 August, 2002
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ABSTRACT

The accelerated rate of bone loss that occurs after menopause may be reduced by consuming rice bran oil (RBO), which contains natural oryzanol. Three different forms of oryzanol (natural oryzanol, NO; crystalline oryzanol, CO; and solubilized oryzanol, SO) were 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 a thirteen week study. Rats were either ovariectomized (O, n=37) or sham-operated (ShC, n=10) and assigned to control (C) diets (OC, n=10 or ShC, n=10) or one of three forms of oryzanol (NO, n=9; CO, n=9; or SO, n=9). Bone mineral density of the vertebrae, humerus, femur, and tibia were measured by pDEXA. Results demonstrated that the diet consisting of natural oryzanol (RBO) was slightly protective in preventing bone loss at several bone sites including the elbow, total femur, hip, knee, and femoral mid-shaft. Also, the NO-containing diet was effective in preventing loss of bone mineral density of the tibia. Although the NO-containing diet seemed to have a positive effect on long bones, it did not demonstrate similar effects in the vertebrae. This suggests that NO primarily affects cortical bone. The benefits seen with the natural oryzanol were not seen with the crystalline or solubilized oryzanol. One explanation might be that the natural oryzanol was absorbed more effectively than the crystalline and solubilized oryzanol. Another reason could be that the benefits of the NO diet are due to other components of the unsaponifiable fraction of rice bran oil. Rice bran oil, fed as a sole source of dietary fat reduced bone loss in long bones of ovariectomized rats suggesting there may be beneficial effects in humans.
CHAPTER 1
INTRODUCTION

Osteoporosis affects 25 million Americans and causes 1.5 million fractures of the hip, spine, wrist, and other bones each year (Kelsey and Hoffman, 1987). In 1995, an estimated $13.8 billion in health care costs was spent on osteoporotic fractures. Usually, the incidence of fractures increases with increasing age, and many more women than men suffer from fractures related to osteoporosis (Ray, et al., 1997).

The occurrence of osteoporosis is usually related to menopause and reduced production of estrogen (Bingham, et al., 1998). Type I, or postmenopausal osteoporosis, commonly occurs in women about 15-20 years after menopause. The disease is usually accompanied by an increased loss of trabecular bone which leads to a high incidence of low trauma fractures (Riggs and Melton, 1986).

Currently, hormone-replacement therapy (HRT) is the method of choice for preventing osteoporosis after menopause. Hormone-replacement therapy is effective in maintaining bone and decreasing the chances of sustaining osteoporotic fractures associated with menopause (Greendale, et al., 1999). However, compliance with hormonal therapy is poor because of possible negative side effects such as breast and endometrial cancer (Riggs and Melton, 1986; Wark, 1993; Toniolo, et al., 1995).

As a result of the low HRT participation by postmenopausal women, efforts are being made to find substances that can offer the benefits of hormone therapy, including those related to skeletal health, but without the negative side effects. For example, some recent investigations have focused on a group of cholesterol-lowering drugs known as statins. These drugs were shown to decrease the risk of heart disease while
simultaneously promoting bone formation (Meier, et al., 2000; Wang, et al., 1994; Chan, et al., 2000; Edwards, et al., 2000). However, the exact mechanism by which statins work to improve bone metabolism is not well understood at this time.

Another group of compounds gaining interest are isoflavones found naturally in soy foods. These compounds are structurally similar to estrogen and have been shown to bind to estrogen receptors at comparatively low levels and exhibit estrogenic properties (Miksicek, et al., 1994). Research has shown that soy supplementation is associated with a reduction in plasma total and low-density lipoprotein (LDL) cholesterol concentrations (Potter, et al., 1998; Merz-Demlow, et al., 2000). Further, it was shown that isoflavones effect bone cells resulting in little or no net bone loss after menopause (Potter, et al., 1998). These effects were comparable to treatment with estrogen, but without the negative consequences. Therefore, it was suggested that the addition of soy products containing isoflavones to the diet may provide a possible alternative mode of therapy to the improvement of health in postmenopausal women. (Potter et al., 1998).

Oryzanol is a group of compounds consisting of a mixture of ferulic acid esters with plant sterol or triterpene alcohol (Jariwalla, 2001). These compounds are unique to rice bran oil and, like soy, have been shown to have important physiological effects on the cardiovascular system. These include oryzanol’s ability to reduce plasma cholesterol (Lichenstein, et al., 1994), reduce cholesterol absorption (Rong, et al., 1997), inhibit platelet aggregation (Seetharamaiah, et al., 1990), and increase fecal bile acid excretion (Seetharamaiah and Chandrasekhara, 1988).

Although a number of investigations have focused on the cholesterol-lowering properties of oryzanol, there are no current studies demonstrating the effects of oryzanol on bone metabolism. Interestingly, the recent discovery that cholesterol-
lowering substances such as statins, soy, and HRT regimens also increase bone mineral density (BMD) begs the question of whether oryzanol has a similar effect on bone. Therefore, the purpose of this research was to evaluate the effects of oryzanol on BMD in ovariectomized rats.

In this study, forty-seven rats were divided into five treatment groups for twelve weeks. Two groups, control and sham-operated, were fed control diet without oryzanol. The remaining three groups were fed control diets containing 0.3% oryzanol in one of three different forms: natural, crystalline, or dissolved in oil. After two weeks on treatment diets, four of the five groups were ovariectomized (ovx) while the fifth group was subjected to a sham operation.

Measurements of BMD and body composition were conducted three times throughout the study: three weeks before surgery, four weeks after surgery, and eight weeks after surgery. In addition, a twenty-four hour urine and feces specimen was collected from each rat during weeks three and seven post-surgery. During week thirteen of the study, all rats were blocked by group and then sacrificed to evaluate BMD of the vertebrae, humerus, femur, and tibia. In addition, markers of bone turnover, such as serum osteocalcin and urinary deoxypyridinoline, were measured.

- The hypotheses put forth in this research were:

1. Ovariectomies would initiate bone loss due to estrogen deficiency.
2. Oryzanol supplementation would prevent the bone loss associated with estrogen deficiency after ovariectomy.
3. The sham-operated group would not experience changes in bone mass.

- Limitations of this research include:

1. The same concentration of oryzanol, 0.3%, was used in the natural oryzanol (NO), crystalline oryzanol (CO), and solubilized oryzanol (SO) treatment groups.
The small sample size of the treatment groups and the short duration of the study could be responsible for the lack of significant results in BMD of the humerus, femur, and vertebrae.

(3) The peripheral dual-energy x-ray absorptiometer (pDEXA) was replaced by another pDEXA between measurements of BMD, which might have caused imprecise measurements.

Assumptions in this study:

(1) Ovx rats were a good model for postmenopausal women.

(2) The ovariectomized treatment groups would sustain a significant increase in bone loss due to estrogen deficiency.

(3) Any treatment that reduced bone loss in ovx rats was assumed to have similar effects in postmenopausal women.
CHAPTER 2
LITERATURE REVIEW

BONE

Composition

The skeleton, representing about 15% to 17% of body weight, provides structure and support for organs and muscles (Ott, 1991). There are two types of bone. The outer layer, cortical bone, surrounds the inner, trabecular bone. Long bones contain primarily cortical bone with a cavity to hold bone marrow. Cortical bone is dense and makes up about 75% of the body’s total bone content. Cortical bone contains layers of mineralized collagen which form canals containing the blood vessels that nourish the bone (Groff and Gropper, 2000; Ott, 1998).

Trabecular bone, however, has a sponge-like appearance, comprises about 25% of bone in the body, and is found mainly in bones of the wrist, vertebrae, pelvic areas, and head of long bones (Ott, 1991). Although trabecular bone is lightweight, it provides substantial strength and weight-bearing properties of bone (Ott, 1998). Due to its high metabolic turnover rate, trabecular bone responds readily to unfavorable conditions that affect the skeleton such as calcium depletion (Riggs and Melton, 1986).

Remodeling

Throughout a lifetime, bone is continuously subjected to a process of resorption and formation known as remodeling. Bone remodeling, or turnover, is required for the maintenance and overall health of bone (Marcus, 1991). The two types of bone cells primarily involved in bone remodeling are osteoblasts and osteoclasts. Osteoblasts are
responsible for the formation of bone tissue, whereas osteoclasts aid in resorption or breakdown of bone. Normally, osteoblastic activity (bone build-up) and osteoclastic activity (bone breakdown) balance each other, so there are no obvious changes in bone (Mahan and Escott-Stump, 2000). Bone mass increases until about the age of thirty. After that, a period of stability occurs, where osteoblast and osteoclast activity remain in balance until the onset of age-related bone loss (Riggs and Melton, 1986). The decrease in bone mass is due to accelerated osteoclastic activity resulting in a gradual loss of bone-mineral mass (Mahan and Escott-Stump, 2000).

MENOPAUSE

Reproductive Cycle

In a newborn female, the ovaries contain about two million oocytes, or immature ovum. Primary oocytes are contained within 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 (Fox, 1996).

The anterior pituitary gland produces and secretes two hormones involved in the structure and function of the ovaries: follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Under the stimulation of FSH, an ovarian follicle matures and 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 the LH hormone, which forces the follicle to rupture and release its oocyte into the uterine tube. Therefore, ovulation occurs due to the sequential effects of FSH and LH on the ovarian follicles (Fox, 1996).
After ovulation occurs, LH stimulates the empty follicle to undergo structural and biochemical changes to become a corpus luteum. Unlike developing follicles which secrete only estrogen, the corpus luteum secretes both estrogen and progesterone. These high levels of estrogen and progesterone result in a negative feedback inhibition of FSH and LH secretion. This serves to stop the development of new follicles and prevent multiple ovulations during the same cycle (Fox, 1996).

Levels of estrogen and progesterone fall to very low levels when the corpus luteum regresses and stops functioning. This withdrawal of ovarian hormones causes menstruation and allows a new cycle of follicle development to occur (Fox, 1996).

**Perimenopause**

The menopause is defined as the permanent termination of menstruation due to cessation of ovarian follicular function. Menopause is diagnosed after 12 months of amenorrhea; and the average age at menopause is 51 years (Greendale, et al., 1999).

Although the beginning of the postmenopausal period is designated as a distinct point in time, the shift to this phase is not quick. According to most theories, perimenopause, a period of altering ovarian function, occurs before the final menses. Even though perimenopause has not been clearly defined, this period may begin between 2 and 10 years before the postmenopausal period (Seifer and Naftolin, 1998). Menstrual cycles during the perimenopause are irregular and unpredictable, with extremes of short and long cycles (Metcalf, 1979).

In addition to irregularity of menstruation that occurs in perimenopause, some women experience common postmenopausal symptoms such as hot flashes. Moreover, an increased risk for chronic disease, such as osteoporosis, may occur during the
perimenopause (Greendale, 1999). For example, a study by Chapurlat et al found that untreated perimenopausal women had significant bone loss from trabecular and cortical sites after being measured at one year intervals for three years (Chapurlat, et al., 1993).

**Physiology of Menopause**

Once menopause occurs, the ovaries contain very few follicles and production of estrogen diminishes rapidly to extremely low levels. It is the withdrawal of estrogen secretion from the ovaries that causes 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. These symptoms include “hot flashes” with extreme flushing of the skin, irritability, fatigue, anxiety, loss of memory, depression, insomnia, urogenital atrophy, and occasional psychotic states. There is also increased risk of heart disease and increased progression of osteoporosis (Ratner and Ofri, 2001; Al-Azzawi, 2001).

**Effects of Menopause**

Estrogen receptors are widely distributed throughout many parts of the central nervous system (CNS) (Taylor and Al-Azzawi, 2000). Estrogen regulates the synthesis and secretion of neuropeptides, neurotransmitters, and other hormones transmitted in the brain. It enhances sensory perception, locomotor activity, limb coordination, and balance (Backstrom, 1995). A deficiency of estrogen reduces synthesis of serotonin, which may cause insomnia (Luine and Mc Ewen, 1977; Crowley, 1982).

**Vasomotor symptoms**

One of the most obvious symptoms of estrogen deficiency is the instability of body temperature resulting in hot flashes and sweating (Greendale, et al., 1998).
Hot flashes result from vasodilation of the skin capillaries, and typically affect the chest and facial areas. Skin temperature increases one to two degrees Celsius. Episodes can differ in frequency and severity, usually lasting between 0.5 minutes and ten minutes (Greendale, 1999). Hot flashes usually disappear spontaneously; about 25-50% of women will continuously suffer from symptoms for up to five years following menopause (Al-Azzawi, F).

**Urogenital symptoms**

The loss of estrogen affects the reproductive tract. This loss results in vaginal dryness, leading to atrophy of the endometrium, cervix, and vagina. The bladder and urethra are also affected by the reduction of estrogen resulting in urinary incontinence, increased frequency of cystitis, and dysuria (Ratner and Ofri, 2001; Al-Azzawi, 2001).

**Cardiovascular system**

Estrogen deficiency results in several changes in the cardiovascular system. Coronary artery disease (CAD) is the leading cause of death in American women. The risk of CAD increases significantly after menopause, suggesting that estrogen may play a protective role in preventing heart disease (Ratner and Ofri, 2001). Other factors contributing to the increased risk of cardiovascular disease due to estrogen deficiency after menopause include hypertension, increased total cholesterol and triglyceride levels, and increases in coagulation factors (Matthews, et al., 1989).

**Bone Metabolism**

Osteoclastic activity significantly increases within six to eight weeks after loss of estrogen. As a result, several biochemical changes in bone metabolism may occur. These changes include reduction in serum osteocalcin levels, increase in urinary
hydroxyproline: creatinine ratio, increase in urinary calcium, and reduction in intestinal absorption of calcium (Al-Azzawi, 2001).

RATS AS A BONE MODEL OF HEALTH

The main focus of the current study was to examine the effects of ovx and diet on bone density. 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 (Kalu, 1991).

Ovariectomized non-primate animals are often used as models of menopause because they develop pathophysiologica changes similar to those seen in postmenopausal women (Bellino, 2000). For example, rats can be ovx to make them sex-hormone deficient, and to stimulate the accelerated bone loss that occurs in women after menopause (Kalu, 1991). Bone loss in ovx rats and postmenopausal bone loss share many 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 bone loss by obesity; and similar responses to hormonal therapies (Miller, et al., 1995; Miller and Wrons, 1993).

This broad range of similarities provides powerful evidence that the ovx rat bone loss model is appropriate for studying problems associated with postmenopausal bone loss in women (Kalu, 1991). In spite of the increasing use of the ovx rat for studying ovarian hormone deficiency, the ovx rat model is not a perfect one. Reasons for this include: the rat is perceived to be growing continuously and has been suggested as
being unsuitable as a model of a human disease that starts after the attainment of skeletal maturity, rats do not appear to have the same pattern of bone remodeling as humans, and the overwhelming number of women affected by osteoporosis provides a ready pool of subjects for studying the disease (Kalu, 1991).

OSTEOPOROSIS

Osteoporosis, a term meaning “porous bone”, is defined as a clinically detectable decrease in bone density (Gamble, 1995; Ott, 1998). It is a condition in which bone composition is unchanged, but the amount of bone per unit volume is reduced. The bone becomes porous due to the imbalance of bone formation and bone resorption in which resorption exceeds formation (Bingham, et al., 1998). The decrease in bone mass results in the inability of the skeleton to sustain ordinary strains causing a high incidence of low trauma fractures (Riggs and Melton, 1986).

Diagnosis

The World Health Organization has identified criteria for diagnosing osteoporosis based on a comparison between measured bone mass and the optimal peak adult bone mass. Using the term T score, a value of 0 is given to the mean peak bone mass, with normal bone mass ranging within 1 standard deviation of this mean. Therefore, an individual with a T score from +1 to –1 is thought to have normal bone mass. Osteopenia, low bone density, is defined as a score between –1 and –2.5 below the mean. A person with a bone mineral density 2.5 standard deviations or more below the mean is classified as having osteoporosis. This definition is important because it permits diagnosis of osteoporosis before a fracture occurs (WHO, 1994).
Prevalence

Osteoporosis affects 25 million Americans, and causes 1.5 million fractures of the hip, spine, wrist, and other bones each year (Kelsey and Hoffman, 1987). Of the 1.5 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 (Wark, 1993).

Health care costs due to osteoporotic fractures in 1995 were estimated at $13.8 billion, of which $10.34 billion (75.1%) was for the treatment of white women (Ray, et al., 1997). Generally, risk of fractures increases significantly with increasing age. Further, over twice as many women as men suffer from fractures related to osteoporosis (Ray, et al., 1997).

After age 65, 1 in 2 women and 1 in 5 men develop osteoporosis-related fractures (Gamble, 1995). Osteoporotic fractures are an important cause of disability (Greendale, et al., 1999). A hip fracture increases the likelihood by 5 to 20% that an older person will die within one year (Wark, 1993; Gamble, 1995). Among survivors, 15 to 20% 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 (Wark JD).

Fractures

Osteoporotic fractures commonly occur due to the deterioration of bone tissue, particularly trabecular bone tissue. Depletion of trabecular bone tissue is responsible for the high incidence of fractures of the vertebrae, hip, and wrist which are high in trabecular bone (Mahan and Escott-Stump; Al-Azzawi, 2001). Hip fractures are
especially important because they are associated with incapacitation, long-term nursing care, and frequent morbidity and mortality (Bingham, et al., 1998). Hip fractures affect nearly 20% of post-menopausal women up to age 80 and almost 50% of those over age 80 (Anderson, 2002). Due to its high content of trabecular bone, the lumbar spine is highly susceptible to osteoporotic fractures. These fractures typically lead to back pain, loss of height, and spinal deformities such as dorsal kyphosis (Al-Azzawi, 2001).

**Types of Osteoporosis**

The occurrence of osteoporosis is usually related to menopause and estrogen deficiency (Bingham, et al., 1998). However, the condition is also associated with other consequences of aging, certain diseases, and surgical procedures or medications. Thus osteoporosis has been divided into primary and secondary categories according to those factors that either directly cause bone loss in individuals or act as additional risk factors for bone loss. Such factors include hypogonadism, hyperthyroidism, malabsorption syndrome, and rheumatoid arthritis. Primary osteoporosis is classified into idiopathic osteoporosis, which is a rare form found in children and young adults, and involutional osteoporosis, which is the more common condition that occurs with aging (Ott, 1998).

Two types of involutional osteoporosis have been suggested according to clinical features and hormonal status. Type I, or postmenopausal osteoporosis occurs primarily in postmenopausal women 15 to 20 years after the onset of menopause. Typical symptoms include fractures of the vertebral and wrist. Some cortical bone loss occurs, but not as much as trabecular bone loss (Riggs, et al., 1981). Type I osteoporosis is related to menopause and reduced production of estrogen. Type II, or senile osteoporosis occurs in both men and women over 75 years of age and is characterized by demineralization of the
vertebrae, hip, pelvis, humerus, and tibia. Both trabecular and cortical bone are affected due to an age-related decrease in osteoblast activity. Also, decreased synthesis of vitamin D and intestinal transport of calcium occur with aging and promote Type II osteoporosis (Riggs and Melton, 1986; Harward, 1993).

**Risk Factors**

Although several risk factors have been identified, the development of osteoporosis in an individual patient cannot be reliably predicted. Some of the risk factors associated with osteoporosis include: increasing age, female gender, white or Asian ethnicity, family history, estrogen deficiency, calcium deficiency, vitamin D deficiency, low body weight for height, sedentary lifestyle, alcoholism, and smoking. Most of these risk factors are primarily related to low BMD, which is a strong predictor of fracture risk. Usually, each standard deviation drop in BMD below the adult mean increases the risk of fracture two to three times (Lenchik and Sartoris, 1998).

**Management**

The ability to measure BMD is important because fracture risk is associated with bone density. Quantitative assessment of bone mass is particularly important for osteoporosis because it is a preventable rather than a curable disease. Osteopenia, or low bone mineral density, is a precursor to osteoporosis. Preventing osteoporosis involves detecting the asymptomatic disease in its early stages (Eastell, 1998).

The process of measuring bone density, bone densitometry, is useful in predicting sites of high fracture risk and monitoring response to treatment. Bone density refers to mass of bone per unit volume. Bone densitometry techniques measure bone mineral content as either surface density (g/cm$^2$) or volumetric density (g/cm$^3$). Reduced bone
mass is an important predictor of fracture risk even though it is not the only indicator of bone strength (Seeger, 1997).

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 of the same sample over time. Regardless of the bone densitometry technique chosen, it is necessary that measurements be made using the same method, the same instruments and the same testing site (Seeger, 1997). Several techniques available for the measurement of bone mass include ultrasound scans, computed tomography, single energy x-ray absorptiometry, and dual energy x-ray absorptiometry (DEXA). Among them the most useful in clinical practice is DEXA (Eastell, 1998).

Ultrasound Scans

The method using ultrasound scans may be suitable for screening to predict osteoporotic fracture risk. However, it is less precise and may have limitations in the follow up assessment of response to treatment. Common sites measured include the heel, fingers, and tibia. This method is inexpensive, free of radiation, fairly portable, and does not need a skilled operator (Eastell, 1998).

Computed Tomography

Computed tomography provides a volumetric assessment of bone density, measures sites such as the spine and forearm, and provides follow up assessments of sites responsive to treatment. However, limitations include high radiation dose, high costs, and the inability to assess trabecular bone alone (Al-Azzawi, 2001; Eastell, 1998).
Single Energy X-ray Absorptiometry

The single energy x-ray absorptiometry offers high precision with a low dose of radiation at sites such as the wrist, forearm, and heel (Eastell, 1998).

Dual Energy X-ray Absorptiometry

Since its introduction into clinical practice in 1987, DEXA has become the optimal method for measuring bone mineral density (Ott, 1998; DeMott, 1999; Gamble, 1995). This method calculates bone mass using photons produced by an x-ray tube located inside the instrument (DeMott, 1999; Gamble, 1995). Dual energy x-ray absorptiometry produces two energy peaks or radiation that allow for different levels of absorption by bone and soft tissue. The DEXA uses a radiation dosage that is slightly higher than background radiation, which enables the technologist to remain in the exam room without shielding. As a two-dimensional technique, DEXA does not provide volumetric density, but does represent area density (g/cm$^2$) (DeMott, 1999; Ott, 1998).

Generally, DEXA is used to measure BMD at the lumbar spine, proximal femur, and distal radius (Ott, 1998). Advantages to this noninvasive technique include high accuracy and precision within 0.4%, reduced radiation exposure, minimal scanning time, and better spatial resolution (Seeger, 1997; Eastell, 1998). Clinically, the high precision of DEXA in measuring bone mineral density is important because it allows the clinician to track the course of bone loss and response to treatment of osteoporosis over time (Gamble, 1995). DEXA printouts provide BMD values, as well as scores representing the number of standard deviations above or below the mean in order to assess risk of fracture risks (Ott, 1998).
Prevention

Bone mineral density declines most rapidly within two years of menopause, when estrogen deficiency causes increased trabecular bone resorption (Ratner and Ofri, 2001). Estrogen-replacement therapy (ERT) prevents the increased resorption of trabecular bone, which provides protection from further bone loss as long as it is used. Standard oral or transdermal ERT doses are used to relieve symptoms of menopause and/or prevent chronic diseases such as osteoporosis (Greendale, 1998).

Estrogen replacement therapy is associated with reduction of menopausal symptoms, and reducing the risk of osteoporosis by preventing bone loss and subsequent fractures (Grady, 1992). The effective dose of estrogen is 0.625 mg daily. Positive outcomes are usually associated with initiating ERT within five years of menopause and continuing it indefinitely (Cauley, 1995).

According to results from the Postmenopausal Estrogen/Progestin Interventions (The writing group for the PEPI Trial), postmenopausal women assigned to placebo demonstrated decreased BMD at the spine and hip, whereas women assigned to estrogen therapy increased BMD during a three-year period (PEPI trial). A study by Felson et al also suggested that long-term use of ERT is necessary to prevent osteoporosis. In that study, women receiving estrogen therapy for at least 7 years after menopause had significantly higher (11.2% higher) bone density than women who had not received estrogen therapy. However, in women 75 years of age and older in whom the duration of therapy was comparable, bone density was only 3.2% higher than in women who had never taken estrogen. Therefore, it was concluded that at least seven years of estrogen therapy after menopause is necessary for a long-term protective effect on BMD.
However, therapy of this duration may be insufficient to protect women seventy-five years and older from fracture (Felson, et al., 1993).

Although postmenopausal estrogen therapy has many advantages associated with menopause and osteoporosis, its effects on other aspects of women’s health need to be considered. For example, the continuous administration of this hormone has been associated with the development of endometrial cancer (Riggs and Melton, 1986). Therefore, long-term estrogen therapy alone should only be given to women who have had a hysterectomy (Al-Azzawi, 2001). Hormone-replacement therapy (HRT), estrogen and progestins, is needed to offset the effect of continuous estrogen stimulation of the endometrium and should be used for women with intact uteri (Wark, 1993).

Another disadvantage of long-term ERT and HRT is the potential increase in the risk of breast cancer (Toniolo, et al., 1995). According to several studies, postmenopausal women with high serum concentrations of estrogen have a high risk of breast cancer (Toniolo, et al., 1995; Cauley, et al., 1995). An increased relative risk of breast cancer in women receiving ERT was shown in a follow-up from the Nurses’ Health Study (Colditz, et al, 1996). However, there is not enough evidence at this time to conclude whether ERT increases the risk of the breast cancer (Wingo, et al., 1987; Kaufman, et al., 1991).

Other impediments of HRT include increased uterine bleeding, mood changes, breast tenderness, bloating, weight gain, and the concept of interfering with a “natural” process (Ravnikar, 1987). Unscheduled heavy bleeding, with poor predictability has been reported as the most common side effect of HRT. The increased incidence of uterine bleeding frequently occurs among all preparations of HRT in use. The frequency
of these cycles may be regulated by increasing the dose of progestin, but this may increase the occurrence of progestin-induced side effects.

The potential public health effect of HRT is reduced because only about 35% to 40% of menopausal women ever begin HRT and only about 15% continue taking it long-term. There are many reasons for this low participation including physician failure to approve it and patient concerns such as fear of cancer and the unacceptable adverse effects (Ravniker, 1987).

Given the downfalls in current therapy and the improbability of restoring osteoporotic bone to normal, preventive measurements should assume first priority (Eisman, 1991). To reduce the incidence of osteoporosis, preventive strategies are needed throughout life. For example, risk factors such as smoking, physical inactivity, excess alcohol consumption, and inadequate dietary calcium and vitamin D intake should be avoided (Seeman and Allen, 1989). For most individuals, attention to lifestyle factors, along with careful consideration of HRT in the postmenopausal years can prevent or reduce osteoporosis (Wark, 1993).

**Raloxifene**

A number of investigations are focusing on synthetic estrogen analogs, such as raloxifene, which appear to decrease bone loss while not adversely affecting the uterus. Raloxifene, a nonsteroidal benzothiophene, is characterized as a selective estrogen receptor modulator (SERM) (Bryant and Dere, 1998; Ettinger, et al., 1999). SERMs are compounds that exert estrogenic effects in certain body tissues but not others. Raloxifene binds to estrogen receptors and inhibits bone resorption similar to estrogen. However, at the same time, the drug acts as an estrogen antagonist on the uterus and
breasts (Delmas, et al., 1997; Ettinger, et al., 1999; Cummings, et al., 1999; Johnston, et al., 2000). Complications related to raloxifene use include an increase in the incidence of hot flashes and an increased risk of thromboembolism. Therefore, treatment with raloxifene is appropriate for postmenopausal women who do not complain of hot flashes and who are at an increased risk for osteoporosis (Ettinger, et al., 1999).

STATINS

Statins, a class of cholesterol-lowering drugs, have been shown to decrease the risk of cardiovascular disease (LaRosa, et al., 1999). They act by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting step in cholesterol synthesis. By inhibiting this enzyme, production of mevalonic acid is decreased (Van Staa, et al., 2001). Mevalonic acid has been found to be a precursor of cholesterol as well as geranylgeranyl pyrophosphate, which is essential in the control of osteoclast-related bone resorption (Coxon, et al., 2000).

Recent studies have shown that the use of statins to reduce cholesterol levels also promotes bone formation and is related to a lower incidence of fractures (Meier et al., 2000; Wang, et al., 2000; Chan, et al., 2000; Edwards, et al., 2000). These cholesterol-lowering drugs were also shown to promote bone formation in mice (Mundy, et al., 1999).

In a study by Edwards et al, there was an association between BMD and statin use in a population-based cohort of postmenopausal women. Bone mineral density at the spine and hip (femoral neck) was significantly higher in the statin users compared with the control group from the same population. The results remained significant after adjusting for age, height, weight, and HRT use. In addition, a group of women with
hypercholesterolemia, on no cholesterol-lowering treatment, were analyzed and did not differ at the hip or spine for BMD when compared with controls (Edwards, et al., 2000).

Chan and coworkers conducted a population-based case-control study using the health-maintenance records from 928 women aged 60 or over who had experienced a fracture of the hip, humerus, distal tibia, wrist, or vertebrae and compared them with 2747 controls with no fracture. All women taking drugs to treat osteoporosis were excluded from the study. The results showed that women who had taken statins for at least one year had a deceased risk of fracture after adjustment for age, number of hospital admissions, chronic disease score, and use of non-statin lipid-lowering drugs. No relationship was found between fracture risk and use of non-statin lipid-lowering drugs (Chan, et al., 2000).

Meier et al recorded a population-based, nested case-control study using 91,611 individuals at least 50 years of age (28,340 men and women 50 years of age or over taking lipid-lowering drugs, 13,271 with hyperlipidemia not taking lipid-lowering drugs, 50,000 randomly selected subjects without hyperlipidemia, 3,940 individuals with a previous bone fracture, and 23,379 control patients from the same population). After controlling for weight, height, smoking, number of physician visits, and corticosteroid and estrogen use, current use of statins was associated with a significantly reduced fracture risk compared with nonuse of lipid-lowering drugs. Once again, current use of other lipid-lowering drugs was not related to a decreased fracture risk (Meier, et al., 2000).

Wang et al published a case-control study of 6,110 individuals aged 65 and over who were registered with Medicare and Medicaid or the Pharmacy Assistance for the
Aged and Disabled Program. Of these patients, 1,222 of them had experienced surgical repair of a hip fracture. Use of statins in the previous six months or previous three years was associated with a decreased hip fracture incidence. These results remained significant after adjusting for race, insurance status, estrogen use, ischemic heart disease, cancer, and diabetes mellitus. No reduction in fracture risk was seen with use of non-statin lipid-lowering drugs. Interestingly, this study showed a significant dose-response effect when the patients were divided into quartiles on the basis of the length of statin use. Individuals who used the most statins also had the lowest fracture risk (Wang, et al., 1994).

Possible mechanisms by which statins work to reduce fracture risk and increase BMD are emerging. One hypothesis is that the inhibition of the mevalonate pathway by statins leads to a decrease in the prenylation of key regulatory GTP-binding proteins, farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which block bone resorption by inhibiting osteoclast activity (Russell and Rogers, 1999).

Another possible explanation is that statins are potential stimulators of bone formation. Mundy et al analyzed more than 30,000 compounds for the ability to increase the synthesis of bone morphogenic protein 2 (BMP-2). BMP-2 is a powerful growth factor that causes osteoblasts to proliferate, mature, and form new bone. Mundy and his colleagues used rats to show that injecting lovastatin and simvastatin into the subcutaneous tissue overlying bone for 5 days caused formation of cortical bone. In addition, oral administration of statins in intact or ovariectomized rats increased trabecular bone volume by 90%. However, the doses of statins used in the rat studies were higher than those used for cholesterol-lowering in humans (Mundy, et al., 1999).
These studies indicate that products of mevalonate metabolism in the cholesterol synthesis pathway may have important effects on both bone formation and resorption. These observations, although not clearly defined, propose new advantages for the treatment of osteoporosis. However, more research needs to be conducted before prescribing statins for bone maintenance. Until then, patients with osteoporosis should be treated with agents that have been rigorously tested and proven to reduce the risk of fractures (Cummings and Bauer, 2000).

SOY

The importance of soy foods to the health of populations throughout the world has recently been recognized, primarily because soy protein contains isoflavones (Knight and Eden, 1996). Isoflavones are unique organic substances that have been shown to bind to estrogen receptors at comparatively low levels and exhibit estrogenic properties (Miksicek, 1994).

Only in Asian nations have soy products historically been used to any great extent for human consumption. In the U.S., the use of these products has had a slight upswing, but estimates of human intake remain very low (Anderson, 1997). Therefore, information on the benefits of soy and foods derived from soy to human health is based mostly on experimental results using animals rather than on human investigations. The unmistakable effects of isoflavones on bone tissue in rodent models provide promising evidence for similar benefits in humans. Experiments with animals have been encouraging enough to suggest that human adults will receive similar improvements in BMD (Makela, et al., 1994). There are few published studies looking at isoflavone consumption and their potential health benefits. Observations of populations such as the
high soy-consuming Japanese provide important suggestions about the benefits of soy consumption (Anderson and Garner, 1997).

**Chemistry**

The similarities in the structures of the isoflavones and $17\beta$-estradiol, the most potent naturally occurring estrogen, are sufficient to allow occupancy of the estrogen receptor by the isoflavones. But, occupancy time or affinity for the receptor is significantly lower for isoflavones compared to $17\beta$-estradiol (Miksicek, 1994). The structures of the isoflavones, genistein and daidzein, differ only by the absence of a hydroxy group on the A ring (Anderson and Garner, 1997).

**Sources of Isoflavones**

Of the many different plants that make up the legume family, only soy and soy products provide significant amounts of the isoflavones in the diet (Knight and Eden, 1996). In addition, the consumption of soy protein products is highly related to consumption of isoflavones because these molecules are closely linked with the protein. Therefore, isoflavones continue to affiliate with the proteins in different products made from soybeans. Asian populations, especially the Japanese, consume the greatest quantity of soy and soy products. Japanese consume between thirty and fifty grams of soy products per day, but Americans only take in about three grams per day (Anderson and Garner, 1997). Most of this is from the use of soy meal in small quantities in breads and other baked goods. The intake of soy is nearly zero for most people, but a small percentage of the population, especially vegetarians, consume more than three grams of soy a day. Clearly, the difference in intake of soy products between the U.S. and Asian populations is large (Anderson and Garner, 1997).
Table 1 - Food sources of isoflavones (Wang and Murphy, 1994)

<table>
<thead>
<tr>
<th>Major Soy Sources of Isoflavones</th>
<th>Daidzein (mg/100g)</th>
<th>Genistein (mg/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybeans</td>
<td>84</td>
<td>111</td>
</tr>
<tr>
<td>Soybeans (roasted)</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Soy Flour23</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>Tempeh</td>
<td>27</td>
<td>32</td>
</tr>
<tr>
<td>Tofu</td>
<td>15</td>
<td>16</td>
</tr>
</tbody>
</table>

Soy and Bone

The potential of isoflavones to prevent bone loss after menopause has given them a lot of attention. Researchers are focusing on many investigations around the world regarding the effects of isoflavones on human physiology. Unfortunately, there are a limited number of studies done in the United States regarding effects of isoflavones on bone in humans (Anderson, 1997).

Potter et al studied the effects of feeding soy protein containing 0 mg, 56 mg, or 90 mg of isoflavones per day on BMD in 66 postmenopausal women for six months. They found that the treatment groups receiving moderate (56mg) or high amounts (90mg) of isoflavones had significantly higher BMD than the group receiving the soy protein supplement alone (isoflavones removed). Significant gains were observed in BMD of lumbar vertebrae, but not of the proximal femur. The subjects receiving the lower dose of isoflavones had gains in the measurement of lumber vertebrae, but they were not significant. This study shows a differential response of the two skeletal sites of the women in the study, as well as a dose-dependent effect of the isoflavones (Potter, et al., 1998).
Alekel et al gave the results of a second 6-month feeding trial with soy that specifically evaluated the effects of soy with isoflavones (80 mg/day) and with isoflavones reduced to 4.4 mg/day on bone sparing in perimenopausal women. Although there were some differences between this trial and that of Potter et al, Alekel et al also found that soy with high levels of isoflavones attenuated bone loss from the lumbar spine. According to Alekel et al, 24 weeks is a short time for a dietary study of bone loss because the bone remodeling cycle ranges up to 80 weeks in length (Miksicek, 1994). It would be premature to assume that soy with 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 of great benefit to peri- and postmenopausal women if soy with isoflavones alone or with other therapy was shown to prevent bone loss in the spine. This would provide these women with an alternative approach to hormone therapy for maintaining bone (Alekel, et al., 2000).

Experimental animal models, especially rats, have been employed to establish dose-response effects and general changes in tissues after periods of treatment with isoflavones. The published reports using ovariectomized (ovx) rat models of osteoporosis show the addition of isoflavones in the animal feed contributes to improved bone mass in comparison to animals fed diets that do not contain the isoflavones (Anderson and Garner, 1997; Arjamdi, et al., 1998).

A positive effect of genistein on bone was shown to be dose-dependent in rat studies (Fanti, et al., 1998; Ishimi, et al., 2000). Genistein-rich soy preparations had a modest bone-conserving effect at low doses, but less skeletal retention at high doses.
Ishimi et al also observed uterine hypertrophy with the higher dose (Fanti, et al., 1998; Ishimi, et al., 2000).

Arjmandi et al examined the effect of soy protein in reversing bone loss in ovx rats one month after surgery (after bone loss had occurred). Slight differences were noticed (not significant) in femoral density only within the soy groups. Overall, the findings of this study supported the idea that the best and most effective approach to combating osteoporosis is through prevention. It was stated that once bone loss has occurred, its reversal is difficult. Arjmandi et al also suggested that long-term consumption of soy or its isoflavones produce small but continued increments in bone mass (Arjmandi, et al., 1998).

**Effects of Isoflavones on Bone Cells**

The number of estrogen receptors in different mammalian cells varies widely. For example, reproductive tissues have about 100-1000 fold more receptors than bone cells and most other cells in the body (Migliaccio, et al., 1992). Isoflavones combine with estrogen receptors but at much lower affinities than 17β-estradiol (Miksicek, 1994).

Compared with estradiol as the model estrogen molecule, isoflavones act as weak agonists or antagonists of estrogen,. Studies with osteoblast-like cells showed that an optimal dose range of pure genistein exists for optimal responses, but doses that are too high can produce cell death by apoptosis (Anderson, et al., 1998). Doses that are too low have little or no effect on bone-forming cells. It is generally accepted that the dose range of isoflavones to provide the best results is between 50 and 100 mg per day, but the intake within this dose range must occur almost every day and indefinitely (Anderson, 1998).
**Mode of Action**

Isoflavones are known as weak estrogen agonists or antagonists depending on cell type. Isoflavones affect bone cells resulting in little or no net bone loss. This effect is comparable to treatment with estrogen, which maintains homeostasis between osteoblastic activities and osteoclastic activities. The actual dose of the isoflavone may be 100 to 1000-fold higher than that of estradiol. In the end, 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 (Anderson, 1997).

**Soy and Cholesterol**

The consumption of soy protein also results in a reduction in plasma total and low-density lipoprotein cholesterol concentrations. However, the components of soy responsible for these effects are unclear (Wangen, et al., 2001). Clinical trials have shown that soy supplementation is associated with a reduction in lipids and lipoproteins in hypercholesterolemic (Anderson, 1995; Potter, et al., 1981) as well as nonhypercholesterolemic subjects (Potter, et al., 1993). The research generally agrees that the decreases in plasma or serum cholesterol levels observed in subjects fed soy-protein diets occur mainly in the LDL fraction (Potter, et al., 1993; Scheiber, et al., 2000; Wangen, et al., 2001). Levels of high-density lipoprotein (HDL) cholesterol are usually not affected to any significant extent by the soy protein diets (Carroll, 1991).

The isoflavones contained in soy protein, genistein and diadzein, are antioxidants speculated to be responsible for the hypocholesterolemic effects of soy supplementation (Wiseman, et al., 2000; Merz-Demlow, et al., 2000). For example, it was found that the soy isoflavone, genistein, acts as a SERM and preferentially binds to the form of estrogen
receptor found mainly in the cardiovascular system and bone. This raises the question of whether a soy-rich diet can offer protection to the cardiovascular system and bone to women in periods of estrogen deficiency (Finkel, 1998; Scheiber, et al., 2000).

Scheiber et al examined the effects of consuming 60 to 70 mg/day total isoflavones for 12 weeks in 43 postmenopausal women. Their results showed a decreased ratio of total to HDL cholesterol, a significant decrease in LDL oxidation, decreased osteoclast activity, and increased serum osteoblast activity. It was concluded by stating that consuming 60 to 70 mg/day of isoflavones had a significant positive effect on healthy postmenopausal women. Furthermore, these results suggested a potential, more natural form of ERT with fewer unwanted side effects (Scheiber, et al., 2000).

**RICE BRAN OIL**

In the United States, rice bran oil has been commercially produced for food since 1994 (McCaskill and Zhang, 1999). Total annual world production of vegetable oil in 1997 was estimated to be approximately 84 million metric tons. However, the actual current annual world RBO production is estimated to be less than 800,000 metric tons, or about 1% of all vegetable oils (McCaskill and Zhang, 1999).

The use of RBO is very common in the Japanese population. Although the amount consumed is only about 2.5% of total vegetable oils, it has strong support as being a healthy oil not only in Japan, but also in India and other Asian countries (Sugano, 1999).

The bran fraction of the rice kernel contains about 75% of the total oil content (McCaskill and Zhang, 1999). The odorless oil is pale yellow and has a pleasant, lightly sweet flavor (Cicero and Gaddi, 1997). The typical composition of
crude RBO is 68%-71% triglycerides, 2%-3% diglycerides, 5%-6% monoglycerides, 2%-3% free fatty acids, 2%-3% waxes, 5%-7% glycolipids, 3%-4% phospholipids, and 4% unsaponifiables (non-fatty acid components) (McCaskill and Zhang, 1999).

The unsaponifiable portion is made up of various sterols (43%), triterpene alcohols (28%), 4-methyl-sterols (10%), and less polar components such as squalene and tocotrienols (19%) (Sayre and Saunders, 1990). Table 2 compares the fatty acid composition of RBO with that of peanut, soybean, and cottonseed oils (McCaskill and Zhang, 1999). Oleic and linoleic acids make up more than 80% of the fatty acids of the glycerides. The much lower linolenic acid content of RBO makes it more stable to oxidation than soy oil (McCaskill and Zhang, 1999).

Table 2: Fatty acid composition of selected oils (McCaskill and Zhang, 1999).

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Rice bran</th>
<th>Peanut</th>
<th>Soybean</th>
<th>Cottonseed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic (16:0)</td>
<td>15.0</td>
<td>8.1</td>
<td>10.7</td>
<td>27.3</td>
</tr>
<tr>
<td>Stearic (18:0)</td>
<td>1.9</td>
<td>1.5</td>
<td>3.9</td>
<td>2.0</td>
</tr>
<tr>
<td>Oleic (18:1)</td>
<td>42.5</td>
<td>49.9</td>
<td>22.8</td>
<td>18.3</td>
</tr>
<tr>
<td>Linoleic (18:2)</td>
<td>39.1</td>
<td>35.4</td>
<td>50.8</td>
<td>50.5</td>
</tr>
<tr>
<td>Linolenic (18:3)</td>
<td>1.1</td>
<td>-</td>
<td>6.8</td>
<td>-</td>
</tr>
<tr>
<td>Arachidic (20:0)</td>
<td>0.5</td>
<td>1.1</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Behenic (22:0)</td>
<td>0.2</td>
<td>2.1</td>
<td>0.1</td>
<td>-</td>
</tr>
</tbody>
</table>

Rice bran oil is unique due to its relatively high content of unsaponifiable matter (De Decker and Korver, 1996). Unlike most vegetable oils, refined RBO may contain 1.5% to 2.6% of unsaponifiable lipids, and crude RBO may contain up to 5% of this matter (Rong, et al., 1997). Among the many sterols present in the unsaponifiable fraction of RBO, oryzanols and tocotrienols are two distinctly different groups that have been widely studied for their health benefits (Rogers, et al., 1993; Rong, et al., 1997).
Oryzanol

Crude RBO contains about 2% or more oryzanol, which represents about 20% to 30% of the unsaponifiable matter. Oryzanols consist of a mixture of esters of ferulic acid with plant sterol or triterpene alcohol (Jariwalla, 2001). Cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campestenyl ferulate are the three primary components and make up about 80% of the oryzanol in RBO. The content of oryzanol differs with the source of RBO and depends on the degree and possibly the method of processing (Rogers, et al., 1993).

The prototype member of the oryzanol group is gamma oryzanol, which has been extensively characterized and studied (Naruse and Takeshita, 1999). Studies have shown several physiological effects related to gamma oryzanol and RBO components. These include its ability to reduce plasma cholesterol (Lichenstein, et al., 1994), reduce cholesterol absorption and decrease early atherosclerosis (Rong, et al., 1997), inhibit platelet aggregation (Seetharamaiah, et al., 1990), and increase fecal bile acid excretion (Seetharamaiah, et al., 1990). Oryzanol has also been used to treat nerve imbalances and disorders of menopause (Nakayama, et al., 1987). The nutritional function of gamma oryzanol components may be related to their antioxidant property because of the ferulic acid structure (Cuvelier, et al., 1992).

Tocotrienols

Another important substance in the unsaponifiable fraction of RBO is tocotrienol, a member of the vitamin E family (Jariwalla, 2001). Tocotrienols occur in at least four known forms (alpha, beta, gamma, sigma) and are similar to the tocopherols in chemical structure. Like the tocopherols, tocotrienols exhibit antioxidant activity (Serbinova
and Packer, 1994). Other physiological benefits of tocotrienols include decreasing serum cholesterol (Qureshi, et al., 1991), decreasing hepatic cholesterol synthesis by suppression of hydroxy methyl glutaryl coenzyme A reductase, and anti-tumor activity (Komiyama, et al., 1989).

**Oryzanol and Cholesterol**

Rice bran oil, possibly because of its unsaponifiable fraction or its fatty acid content, lowers cholesterol levels in rats, hamsters, humans, and non-human primates (Seetharamaiah and Chandrasekhara, 1989; Rong, et al., 1997; Nicolosi, et al., 1992). Although human studies of RBO are limited, one study conducted recently by Raghuram et al investigated the hypolipidemic effect of RBO in 12 human subjects with total cholesterol levels (TC) (>225mg/dl) or triglyceride levels (>190 mg/dl). Subjects were instructed to use RBO in place of their usual cooking oil. A significant decrease in TC and triglyceride levels was observed when compared with control subjects 15 and 30 days after the use of RBO. The decreases in TC and triglycerides were highly significant as compared to initial values (Raghuram, et al., 1989).

Similar results were found in other studies where gamma oryzanol was compared with probucol, a cholesterol-lowering drug. When gamma oryzanol (300mg/day) was administered for three months to hyperlipidemic subjects, a significant decrease in plasma TC and LDL cholesterol was observed in both hypercholesterolemic and hypertriglyceridemic patients, while an increase in high density lipoprotein (HDL) cholesterol was noted only in the hypercholesterolemic group (Yoshino, et al., 1989).
Nicolosi et al investigated the hypolipidemic effect of RBO in nonhuman primates fed semi-purified diets containing blends of oils including RBO at 20%-25% calories as dietary fat. That study demonstrated that the degree of serum TC and LDL cholesterol reduction was highly correlated with initial serum cholesterol levels in monkeys fed a standard diet. In addition, RBO supplementation in the diet significantly influenced serum TC and LDL cholesterol without significantly affecting HDL plasma levels when RBO was used as the sole dietary oil. These results suggested the cholesterol-lowering capability of RBO cannot be explained by its fatty acid composition, suggesting a possible participation of unsaponifiable components (Nicolosi, et al., 1992).

Researchers demonstrated in rats that unsaponifiable matter from RBO enhanced the hypocholesterolemic effect of vegetable oil. The cholesterol-lowering effect was primarily due to reduction of cholesterol in very low density lipoproteins (VLDL) and LDL fractions. High-density lipoprotein cholesterol was not decreased (Sharma and Rukmini, 1987; Seetharamaiah and Chandrasekhara, 1988).

The mechanism of action of RBO on lipid metabolism has not yet been defined. However, literature reports speculate that the various substances of the unsaponifiable portion of RBO, in addition to the fatty acid composition, are responsible for the global effect on plasma lipids (Nicolosi, et al., 1992). A possible anti-atherosclerotic role is directed at gamma oryzanol. It is possible that the anti-hypercholesterolemic effect of gamma-oryzanol is partially due to its sterol moiety, cycloartenyl ferulate, which is partly split off from the ferulic acid part in the small intestine by cholesterol esterase (Fujiwara, et al., 1983). Cycloartenyl ferulate’s chemical structure is similar to cholesterol; and it may compete for the cholesterol binding sites, resulting in increased
excretion of bile salts and pigments. Therefore, it was suggested that the hypocholesterolemic action of dietary RBO does not solely depend on the fatty acid content of the oil, but also, and possibly to a greater extent, on the minor components of the unsaponifiable fraction (Rukmini and Raghuram, 1991).

**SUMMARY**

In summary, it can be concluded that:

1. Bone loss due to estrogen deficiency is a primary factor in the development of osteoporosis in postmenopausal women.

2. Hormone-replacement therapy is effective in preventing bone loss associated with estrogen deficiency after menopause; however, compliance is poor because of possible negative side effects such as endometrial and breast cancer.

3. Statins, cholesterol-lowering drugs, have recently been shown to increase BMD and reduce fracture risk in some studies.

4. Isoflavones, compounds contained in soy protein, are structurally similar to estrogen and in some cases exhibit estrogenic properties; for example, they have been shown to reduce cholesterol levels as well as increase bone mineral density.

5. Oryzanols, a group of compounds found in RBO, have a hypocholesterolemic effect in human and animal studies.

The benefits of ERT and HRT for reducing risk of osteoporosis are well established in postmenopausal women. However, the risks and unwanted side effects involved with this treatment method have led to the search for more natural forms of estrogen replacement and for agents that will have selective actions in different tissues. Statins, as well as the soy-containing isoflavones, are being investigated to fill this niche.
Although numerous studies have investigated the effects of oryzanol on the cardiovascular system, no studies have been published regarding the effects of oryzanol on bone metabolism. Therefore, little is known about the possible bone-sparing potential of these compounds. So the purpose of this study was to investigate the effects of three forms of oryzanol (dissolved in oil, crystalline, and natural) on bone mineral density in ovariectomized rats.
ANIMALS

Nine-month old, retired female breeder Sprague-Dawley rats (Harlan Co.; Indianapolis, IN) were used. Upon arrival, eighty-seven rats were weighed and housed individually in 24 centimeters x 28 centimeters x 18 centimeters hanging stainless steel wire cages. The rats were kept in room 654 of the Life Sciences, animal care facility of Louisiana State University and Agricultural and Mechanical College (LSU). The room temperature was 22 degrees Celsius with a humidity level of 60% and a 12-hour light/dark cycle (0700 light/1900 dark). The animals were provided with food and water ad libitum.

DIET HISTORY

Initially, the rats were used to test the effects of novel diets on recovery from travel to LSU and from their most recent pregnancy 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 used at the Harlan breeding facility. Seventeen rats were placed in cages containing the American Institute of Nutrition (AIN)-93M diet (Table 3); and the remaining seventeen rats were placed in cages containing both the chow and the AIN-93M diets. Two “extra” rats were given only the chow diet.

After two weeks, one-third of the rats on chow were changed to AIN-93M diet, one-third were given both chow and AIN-93M, and one-third remained on chow. The rats continued on these diets for two more weeks. At the end of the fifth week, the rats were stratified by weight and randomly assigned to one of the nine surgery/diet
treatment groups for the remaining studies. For this study, five of the treatment groups (47 rats) of the overall study were used.

**Table 3:** American Institute of Nutrition (AIN)-93 Diet for the maintenance of adult rodents.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>AIN-93M (g/kg diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macronutrients:</strong></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>180</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>425.7</td>
</tr>
<tr>
<td>Dextrinized cornstarch</td>
<td>155</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>40</td>
</tr>
<tr>
<td><strong>Micronutrients:</strong></td>
<td></td>
</tr>
<tr>
<td>Mineral mix (AIN-93M)</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mix (AIN-93M)</td>
<td>10</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.5</td>
</tr>
<tr>
<td>L-cystine</td>
<td>1.8</td>
</tr>
</tbody>
</table>

**TREATMENT GROUPS**

To ensure similarity of animal size among the groups, rats were divided into five treatment groups, each group containing rats with similar weights. Each group was fed a specified diet during the twelve weeks’ feeding period.

1. **Control group (OC):** ten rats were ovariectomized and fed control (AIN-93) diet.
2. **Sham Group (ShC):** ten rats were sham-operated and fed control (AIN-93) diet.
3. **Natural oryzanol (NO) group:** nine rats were ovariectomized and fed control diets containing rice bran oil.
4. **Crystalline oryzanol (CO) group:** nine rats were ovariectomized and fed control diets containing crystallized oryzanol.
(5) Solubilized oryzanol (SO) group: nine rats were ovariectomized and fed control diets containing oryzanol solubilized in vitamin E-stripped corn oil.

**DIET CONSUMPTION AND WEIGHT GAIN**

Diet intake and weight gain were quantified and recorded three times per week (Monday, Wednesday, and Friday). Rat weights were recorded to the nearest gram using a 500-gram analog scale (Toledo Scale Co.; Toledo OH). Food intake was determined by using the weight of the empty food cup and food spillage to the nearest gram. The following formula was used to calculate food intake: full food cup - [empty food cup + food spillage].

**DIETS**

Diets for each treatment group were prepared using the mixing instructions given in Table 4. The diets were mixed using a Hobart mixer (model no. A-200-FD; Hobart Mfg. Co.; Troy, OH) with a blade attachment. Ingredients were mixed on low speed in a 20-quart stainless steel mixing bowl. The diets were prepared, as needed, in six kilogram batches. They were frozen in large ziplock bags from which all excess air was removed.

The OC and ShC diets consisted of a modified AIN 93-M diet with 15% casein and without oryzanol. The NO diet contained 15% casein plus 0.3% rice bran oil. The CO diet consisted of 15% casein plus 0.3% crystallized oryzanol. The SO diet contained 15% casein plus 0.3% oryzanol solubilized in 240g of stripped corn oil. Information regarding ingredients used for each diet is presented in Table 5.
Table 4. Mixing instructions

(Note: Add butylated hydroxytoluene (BHT) to stripped corn oil and rice bran oil at a concentration of 200 mg/kg oil. Mix them well and store the oil at room temperature for duration of experiment.)

1. Add macronutrients (sucrose, casein, cornstarch, dextrinized cornstarch, cellulose fiber) to large mixing bowl.

2. Mix at low speed (#1 on mixer) for 10 minutes. Scrape bowl. Mix another 5 minutes.

3. Premix all of the micronutrients (mineral mix, vitamin mix, choline bitartrate, Cystine). First, sieve them sequentially into a large bowl, using a sieve with a small mesh. Be sure to grind, with a mortar and pestle, any crystals found in the sieve, and add the crushed ingredients to the bowl. Mix all of these ingredients by hand until they look uniform in distribution.
   (Note: For the diet with crystalline oryzanol, add the crystallized oryzanol in with the micronutrients and sieve.)

   Add micronutrient mixture to the large mixing bowl with the macronutrients. Mix 10 minutes. Scrape bowl. Mix another 5 minutes.

4. Add the fat (with BHT) to the dry ingredients in the large mixing bowl.
   (Note: For the diet with solubilized oryzanol, the oryzanol is solubilized in stripped corn oil and added to the dry mixture at this point.)

   First, mix for 5 minutes. Scrape the bowl and the paddle very well. Mix another 10 minutes.

5. Store diets frozen in tightly sealed Ziplock bags (Glad, 1qt) from which all the excess air has been removed. Label with diet type, date of preparation, and batch number.

   When storing diet, take a small sample from the last bag in the mixing bowl and put it (labeled with batch information) in another plastic bag. This sample will be used for later diet analysis. These small bags are kept together in a larger bag in the freezer.
Table 5. Diet composition of five treatment groups (g/6 kg batch)

<table>
<thead>
<tr>
<th>Ingredient (gm)</th>
<th>Control</th>
<th>Sham</th>
<th>NO</th>
<th>CO</th>
<th>SO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>900</td>
<td>900</td>
<td>900</td>
<td>900</td>
<td>900</td>
</tr>
<tr>
<td>Sucrose</td>
<td>600</td>
<td>600</td>
<td>600</td>
<td>600</td>
<td>600</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>2734</td>
<td>2734</td>
<td>2734</td>
<td>2734</td>
<td>2734</td>
</tr>
<tr>
<td>Dextrinized cornstarch</td>
<td>930</td>
<td>930</td>
<td>930</td>
<td>930</td>
<td>930</td>
</tr>
<tr>
<td>Corn oil, stripped</td>
<td>240</td>
<td>240</td>
<td></td>
<td>240</td>
<td>240</td>
</tr>
<tr>
<td>Rice bran oil</td>
<td></td>
<td></td>
<td>240</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellulose</td>
<td>300</td>
<td>300</td>
<td>300</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>Mineral mix AIN-93M</td>
<td>210</td>
<td>210</td>
<td>210</td>
<td>210</td>
<td>210</td>
</tr>
<tr>
<td>Vitamin mix AIN-93M</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>L-cystine</td>
<td>10.8</td>
<td>10.8</td>
<td>10.8</td>
<td>10.8</td>
<td>10.8</td>
</tr>
<tr>
<td>Oryzanol</td>
<td></td>
<td></td>
<td></td>
<td>16.8</td>
<td>16.8</td>
</tr>
</tbody>
</table>
OVARIECTOMY AND SHAM SURGERIES

Two weeks after placing the rats into treatment groups, students and faculty from the LSU School of Veterinary Medicine performed ovariectomy and sham surgeries. All rats were anesthetized via isoflurane inhalation (Abbott Labs, N. Chicago, IL) and oxygen via an induction chamber. After induction, they were fitted with an inhalation mask to ensure continued isoflurane inhalation.

Several preoperative steps were taken to ensure successful operations. First, a lubricant ophthalmic ointment (AKWA Tears, Buffalo Grove, IL) was applied to the rats’ eyes to prevent drying out while the animal was under anesthesia. Next, their identification numbers were written on their tails. Then, the hair over the mid to dorsal areas of the back was then shaved with a clipper; and an antiseptic (betadyne) was applied to the skin surface. Finally, a single injection of buprenorphine hydrochloride (Buprenex by Reckitt & Colman Products; Hull England) (0.05 mg/kg) was administered subcutaneously to provide analgesia.

The ovariectomies were initiated by a small dorsal midline incision made halfway between the middle of the back and the base of the tail. Entrance to the peritoneal cavity was made by 1-cm bilateral incisions made halfway between the middle of the back and the base of the tail approximately 1½ to 2 cm off the dorsal midline. Ovaries were removed; and the uterine horn was returned to the abdominal cavity. A single suture (5-0 PDS II, Ethicon, Inc.; Somerville, NJ) was used to seal muscle incisions. The skin was closed with “Superglue” (Instant Krazy Glue, Elmers’ Products, Inc.; Columbus, OH). Following surgery, rats were allowed to recover from anesthesia in a holding tank.
The tank was equipped with heating pads and a heat lamp to minimize heat loss. The rats were returned to their cages after recovery.

Rats in the sham group underwent the same surgical procedure as the other treatment groups except that the ovaries were not removed. The intact ovaries and uterine horn were returned to the abdominal cavity. Note: One rat in the NO group died several minutes after the surgical procedure, which left 8 rats in this treatment group.

**PDEXA – WHOLE BODY**

**Whole Body Scans**

pDEXA was used to conduct measurements of BMD and body composition of the rats three times during the study: three weeks before surgery, four weeks after surgery, and eight weeks after surgery. Isoflurane was used to anesthetize the rats before individually placing them on the scanning platform of the pDEXA. A nose-cone was used to continue isoflurane administration while each rat was scanned. Lubricant ophthalmic ointment was applied to the eyes to prevent them from drying out while under anesthesia, as described above.

Using a ruler, the rats were measured from the back of the ear to the base of the tail. This measurement, along with body weight, was recorded in a laboratory manual and entered in the pDEXA computer to be used for later analysis. While anesthetized, the rats were carefully placed on the pDEXA platform and scanned from the upper torso to the base of the tail using the following parameters:

- Resolution = 1.0 x 1.0 mm
- Speed = 40 mm/second
- Scan width = 11.40 cm
- Scan length = 15.0 cm
This area was chosen to obtain an estimate of total body fat, lean tissue, and bone density of the vertebrae. After each scan was completed, the nose-cone was removed and rats were returned to their cages to recover from the anesthesia.

**Whole Body Analysis**

Whole body scans were analyzed by pDEXA to determine BMD of the vertebrae and fat mass of the abdomen. Length of each rat, obtained during each scanning session, was used to calculate the portion of the vertebrae to be analyzed. To ensure consistency of measurements, 25% of each rat’s length was used to obtain BMD of the vertebrae. To analyze BMD of the vertebrae, a region was added which began from the point at which the pelvis joined the spine and extended up the vertebrae to the calculated length. The width of the region included only the vertebrae. For example, if the length of the rat from the back of the ear to the base of the tail was 16.0 cm, then the length of the region would be 4.0 cm.

To determine abdominal fat mass, a second region was added beginning from the base of the tail and extending to the end of the first region, including the entire first region. The width of this region extended outward to the rats’ widest point. See Appendix A for illustration.

**METABOLISM CAGES**

During weeks three and seven post-surgery, the rats were rotated in plastic metabolism cages (Lab Products, Seafood, DE) for twenty-four hours to collect twenty-four hour urine and feces samples from each rat (see Appendix B for schedule). Fecal samples were collected, weighed to the nearest gram, placed in labeled storage bottles and frozen for later analysis of oryzanol absorption. Urine samples were filtered to
remove any powdered diet using pre-moistened filter paper (#2 European-style cone coffee filters; Boise, ID).

After initial urine filtration, deionized water was used to rinse the remaining urine from the filter and allowed to drain again. Graduated cylinders were used to determine volumes before the urine samples were transferred to labeled Falcon tubes and frozen for later analysis.

**SACRIFICE**

During week thirteen of the study, rats were anesthetized with isoflurane prior to sacrifice. Rats were euthanised by cardiac puncture, and blood was removed with a 10-cc syringe fitted with a 22-gauge needle. Cervical dislocation was performed to ensure that the rats were dead. Whole blood was centrifuged; and the serum was separated and frozen for later analysis of osteocalcin.

The heart, liver, spleen, and kidneys were removed. Each organ was weighed and weights were recorded to the nearest 0.1 gram. The organs were individually wrapped in labeled foil packets and frozen in liquid nitrogen.

All rats were inspected visually for presence or absence of ovaries. Uteri and were removed and weighed to confirm sham operation or ovariectomy before being discarded. Abdominal fat was removed, weighed, and recorded to the nearest 0.1 gram. The fat from each rat was divided into three separate foil packets and frozen in liquid nitrogen. The front legs, rear legs, and the vertebrae were removed and frozen in Ziplock bags (Glad, 1qt) with saline solution.
PDEXA – INDIVIDUAL BONES

Individual Bone Scans

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

- **Resolution**: 0.2 X 0.2
- **Speed**: 10mm/second
- **Width**: 2.0 cm
- **Length**: 4.0 cm

Individual Bone Analysis

Analyses for the humerus included three regions: total humerus, elbow, and shoulder. Analyses for the femur included: total femur, hip, knee, and mid-shaft. Regions of the tibia included the whole tibia and the mid-shaft. To maintain consistency, regions of each bone had specific areas. See Table 6.

**Table 6. Regions and areas for humerus, femur, and tibia**

<table>
<thead>
<tr>
<th>Bone</th>
<th>Area (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Humerus</td>
<td></td>
</tr>
<tr>
<td>Total humerus</td>
<td>length and width of entire humerus</td>
</tr>
<tr>
<td>Elbow</td>
<td>length = 0.60 cm; width = 0.86 cm</td>
</tr>
<tr>
<td>Shoulder</td>
<td>length = 0.40 cm; width = 0.60 cm</td>
</tr>
<tr>
<td>(2) Femur</td>
<td></td>
</tr>
<tr>
<td>Total femur</td>
<td>length and width of entire femur</td>
</tr>
<tr>
<td>Hip</td>
<td>length = 0.46 cm; width = 0.40 cm</td>
</tr>
<tr>
<td>Knee</td>
<td>length = 0.60 cm; width = 0.66 cm</td>
</tr>
<tr>
<td>Mid-shaft</td>
<td>length = 0.60 cm; width = 0.60 cm</td>
</tr>
<tr>
<td>(3) Tibia</td>
<td></td>
</tr>
<tr>
<td>Total tibia</td>
<td>length and width of entire tibia</td>
</tr>
<tr>
<td>Mid-shaft</td>
<td>length = 0.60 cm; width = 0.60 cm</td>
</tr>
</tbody>
</table>
ANALYSIS OF THE DATA

Means and standard deviations of all variables were computed for each of the
groups.  Analysis of variance (ANOVA) was performed to determine whether there were
significant (p<0.05) differences among the groups.  When ANOVA indicated statistical
significance, an LSD test was used to determine which means were significant.  Data
were analyzed using the SPSS.
CHAPTER 4
RESULTS

FOOD INTAKE, WEIGHT GAIN AND FEED EFFICIENCY

As shown in Figure 1, the ShC group (1365g ± 92), NO group (1372g ± 149), and SO group (1363g ± 115) consumed significantly more food (p<0.05) when compared with the CO group (1227 ± 119) during the thirteen week feeding period.

There were no significant differences in initial body weight among the treatment groups at the beginning of the study (p>0.05) (Table 7). However, the NO group (404g ± 29) had a significantly higher final body weight (p<0.05) when compared with the ShC group (366g ± 29) (Figure 2).

Table 7 – Mean initial body weight (g) among treatment groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean weight (g) ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ShC</td>
<td>345 ± 27</td>
</tr>
<tr>
<td>OC</td>
<td>350 ± 36</td>
</tr>
<tr>
<td>NO</td>
<td>343 ± 33</td>
</tr>
<tr>
<td>CO</td>
<td>344 ± 28</td>
</tr>
<tr>
<td>SO</td>
<td>347 +/- 24</td>
</tr>
</tbody>
</table>

Rats in the NO group (61g ± 53) had a significantly higher (p<0.05) weight gain when compared with the ShC group (21g ± 27) at the end of the study.

There were no significant differences observed in weight gain among other treatment groups at the end of the study (p>0.05) (Figure 3).
Figure 1 – Total food intake (g) of treatment groups throughout 13-week feeding period.

Table 8 – Significant differences in total food intake (g) among treatment groups at the end of the study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Significance Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>ShC &gt; CO</td>
<td>0.010</td>
</tr>
<tr>
<td>NO &gt; CO</td>
<td>0.010</td>
</tr>
<tr>
<td>SO &gt; CO</td>
<td>0.013</td>
</tr>
</tbody>
</table>

Figure 2 – Final body weight (g) among treatment groups at the end of the study.

Table 9 – Significant differences in final body weight (g) among treatment groups at the end of the study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Significance Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO &gt; ShC</td>
<td>0.021</td>
</tr>
</tbody>
</table>
Figure 3 – Mean weight gain (g) among treatment groups throughout the study.

Table 10 – Significant differences in mean weight gain among treatment groups at the end of the study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Significance Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO &gt; ShC</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Feed efficiency (%) (Figure 4), i.e., the weight gained per gram of food intake, was significantly lower (p<0.11) in the ShC group (1.52 ± 1.89) when compared with the NO group (4.45 ± 3.98). There were no significant differences in feed efficiency among other treatment groups (p>0.05).

Figure 4 – Mean feed efficiency (%) among treatment groups at the end of the study.
**ORGAN WEIGHTS**

Table 11 shows mean organ weights (grams) of the uterus, liver, heart, spleen, and kidneys of each treatment group at the end of the study.

- The mean weight of the uterus was significantly higher in the ShC group when compared with the other treatment groups (p<0.05) at the end of the study (Fig 5).
- There were no significant differences in mean weight of the liver among the treatment groups at the end of the study (p>0.05).
- The mean weight of the heart in the CO group (1.34 ± 0.27) was significantly higher than the OC group (1.18 ± 0.13) at the end of the study (p<0.05).
- There were no significant differences in mean weight of the spleen among treatment groups at the end of the study (p>0.05).
- The mean weight of the kidneys in the ShC group (1.88 ± 0.21) was significantly higher than the CO group (1.70 ± 0.11) at the end of the study (p<0.05).

![Graph showing mean uterine weights of each treatment group at the end of the study.](image-url)

**Figure 5** – Mean uterine weights of each treatment group at the end of the study.
Table 11 – Mean organ weights (uterus, liver, heart, spleen, and kidneys) of each treatment group at the end of the study.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Organ</th>
<th>Mean Weight (g)</th>
<th>Standard Deviation</th>
<th>Level of Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ShC*</td>
<td>Uterus</td>
<td>0.69</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>OC</td>
<td>Uterus</td>
<td>0.27 *</td>
<td>0.14</td>
<td>0.000</td>
</tr>
<tr>
<td>NO</td>
<td>Uterus</td>
<td>1.21 *</td>
<td>0.04</td>
<td>0.000</td>
</tr>
<tr>
<td>CO</td>
<td>Uterus</td>
<td>0.22 *</td>
<td>0.06</td>
<td>0.000</td>
</tr>
<tr>
<td>SO</td>
<td>Uterus</td>
<td>0.26 *</td>
<td>0.10</td>
<td>0.000</td>
</tr>
<tr>
<td>ShC</td>
<td>Liver</td>
<td>10.33</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>OC</td>
<td>Liver</td>
<td>9.94</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>NO</td>
<td>Liver</td>
<td>10.23</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>CO</td>
<td>Liver</td>
<td>9.69</td>
<td>1.64</td>
<td></td>
</tr>
<tr>
<td>SO</td>
<td>Liver</td>
<td>9.86</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>ShC</td>
<td>Heart</td>
<td>1.23</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>OC</td>
<td>Heart</td>
<td>1.18 *</td>
<td>0.13</td>
<td>0.049</td>
</tr>
<tr>
<td>NO</td>
<td>Heart</td>
<td>1.24</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>CO*</td>
<td>Heart</td>
<td>1.34</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>SO</td>
<td>Heart</td>
<td>1.29</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>ShC</td>
<td>Spleen</td>
<td>0.73</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>OC</td>
<td>Spleen</td>
<td>0.78</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>NO</td>
<td>Spleen</td>
<td>0.71</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>CO</td>
<td>Spleen</td>
<td>0.83</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>SO</td>
<td>Spleen</td>
<td>0.77</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>ShC*</td>
<td>Kidneys</td>
<td>1.88</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>OC</td>
<td>Kidneys</td>
<td>1.84</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>NO</td>
<td>Kidneys</td>
<td>1.73</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>CO</td>
<td>Kidneys</td>
<td>1.70 *</td>
<td>0.11</td>
<td>0.022</td>
</tr>
<tr>
<td>SO</td>
<td>Kidneys</td>
<td>1.75</td>
<td>0.15</td>
<td></td>
</tr>
</tbody>
</table>
BONE MINERAL DENSITY OF HUMERUS, FEMUR, TIBIA AND VERTEBRAE

Humerus BMD (g/cm²)

Figure 6 illustrates BMD (g/cm²) at the end of the study for the total humerus, elbow, and shoulder for rats in each treatment group.

Table 12 shows significant differences in BMD (g/cm²) of the total humerus, elbow, and shoulder of among each treatment group at the end of the study. Mean BMD of the total humerus in the ShC rats (0.17 ± 0.01) was significantly higher than the OC group (0.16 ± 0.01), NO group (0.16 ± 0.01), CO group (0.16 ± 0.01), and SO group (0.16 ± 0.01) (p<0.05).

Mean BMD of the elbow in the ShC rats (0.14 ± 0.01) was significantly higher than the SO group (0.13 ± 0.01) (p<0.05). However, BMD of the ShC group was not significantly higher than the NO group (0.13 ± 0.01) (p>0.080). In addition, mean BMD of the NO group was not significantly different from the other treatment groups (p>0.05).

No significant differences in mean BMD of the shoulder were observed among treatment groups (p>0.05).
Figure 6 – Mean BMD (g/cm$^2$) of total humerus, elbow, and shoulder in the ShC, OC, NO, CO, and SO treatment groups.

Table12 – Significant differences in mean BMD (g/cm$^2$) of the total humerus, elbow, and shoulder among treatment groups at the end of the study.

<table>
<thead>
<tr>
<th>Bone site (humerus)</th>
<th>Group</th>
<th>Level of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total humerus</td>
<td>ShC &gt; OC</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>ShC &gt; NO</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>ShC &gt; CO</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>ShC &gt; SO</td>
<td>0.001</td>
</tr>
<tr>
<td>Elbow</td>
<td>ShC &gt; OC</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>ShC &gt; CO</td>
<td>0.021</td>
</tr>
<tr>
<td></td>
<td>ShC &gt; SO</td>
<td>0.012</td>
</tr>
<tr>
<td>Shoulder</td>
<td>Not significantly different</td>
<td></td>
</tr>
</tbody>
</table>

Femur BMD (g/cm$^2$)

Figure 7 illustrates mean BMD (g/cm$^2$) at the end of the study for the total femur, hip, knee, and femoral mid-shaft for rats in the ShC, OC, NO, CO, and SO treatment groups. Table 13 shows significant differences in mean BMD of the total femur, hip, knee, and mid-shaft among treatment groups at the end of the study.

Mean BMD of the total femur in the ShC group ($0.20 \pm 0.02$) was significantly higher than the OC group ($0.18 \pm 0.01$), CO group ($0.19 \pm 0.01$), and the SO group ($0.18 \pm 0.01$) ($p<0.05$). However, BMD of the ShC group was not significantly different from the NO group ($0.02 \pm 0.01$). Also, mean BMD of the NO group was not significantly different from the other treatment groups.

Mean BMD of the hip in the ShC group ($0.19 \pm 0.02$) was significantly higher than the OC group only ($0.17 \pm 0.01$) ($p<0.05$). However, neither the ShC group BMD nor the OC group BMD was significantly different from the NO group ($0.18 \pm 0.01$), CO group ($0.18 \pm 0.01$), or the SO group ($0.18 \pm 0.01$).
Mean BMD of the knee in the ShC group (0.16 ± 0.14) was significantly higher than the OC group only (0.15 ± 0.01) (p<0.05). However, neither the ShC group BMD nor the OC group BMD was significantly different from the NO group (0.15 ± 0.004), the CO group (0.15 ± 0.01), or the SO group (0.15 ± 0.01).

Mean BMD of the femoral midshaft in the ShC group (0.20 ± 0.02) was significantly higher than BMD of the CO group (0.18 ± 0.02) and the SO group (0.18 ± 0.01) (p<0.05). No significant differences in mean BMD were observed among other treatment groups.

![Figure 7](image.png)

**Figure 7 – Mean BMD (g/cm²) total femur, hip, knee, and femoral mid-shaft in the ShC, OC, NO, CO, and SO treatment groups.**

**Table 13 – Significant differences in mean BMD (g/cm²) of total femur, hip, knee, and femoral mid-shaft of each treatment group at the end of the study.**

<table>
<thead>
<tr>
<th>Bone site (femur)</th>
<th>Group</th>
<th>Level of Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total femur</td>
<td>ShC &gt; OC</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>ShC &gt; CO</td>
<td>0.034</td>
</tr>
<tr>
<td></td>
<td>ShC &gt; SO</td>
<td>0.006</td>
</tr>
<tr>
<td>Hip</td>
<td>ShC &gt; OC</td>
<td>0.004</td>
</tr>
<tr>
<td>Knee</td>
<td>ShC &gt; OC</td>
<td>0.034</td>
</tr>
<tr>
<td>Mid-shaft</td>
<td>ShC &gt; CO</td>
<td>0.033</td>
</tr>
<tr>
<td></td>
<td>ShC &gt; SO</td>
<td>0.046</td>
</tr>
</tbody>
</table>
Tibia BMD (g/cm²)

Figure 8 illustrates mean BMD (g/cm²) at the end of the study for the total tibia and tibia mid-shaft of rats in the ShC, OC, NO, CO, and SO groups. Table 14 shows significant differences in mean BMD (g/cm²) of the total tibia and tibia mid-shaft among treatment groups.

Mean BMD of the total tibia in the ShC rats (0.17 ± 0.01) was significantly higher than the OC group (0.17 ± 0.010) and the SO group (0.17 ± 0.01) (p<0.05). Furthermore, BMD of the NO group (0.17 ± 0.01) was significantly higher than the OC group (p>0.05), but it was not significantly different from any other treatment group.

No significant differences in mean BMD of the tibia mid-shaft were observed among treatment groups.
Table 14 – Significant differences in mean BMD (g/cm²) of total tibia and tibia mid-shaft among treatment groups at the end of the study.

<table>
<thead>
<tr>
<th>Bone Site (tibia)</th>
<th>Group</th>
<th>Level of Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total tibia</td>
<td>ShC &gt; OC</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>ShC &gt; SO</td>
<td>0.048</td>
</tr>
<tr>
<td></td>
<td>NO &gt; OC</td>
<td>0.045</td>
</tr>
<tr>
<td>Mid-shaft</td>
<td>Not Significantly Different</td>
<td></td>
</tr>
</tbody>
</table>

Vertebral BMD (g/cm²) Among Treatment Groups

Table 15 provides values of mean vertebral BMD among the ShC, OC, NO, CO, and SO treatment groups 3 weeks prior to surgery, 4 weeks post-surgery, and 8 weeks post-surgery. No significant differences were observed in BMD of the vertebrae among treatment groups 3 weeks before surgery and 4 weeks after surgery.

Table 15 – Mean vertebral BMD (g/cm²) of treatment groups 3 weeks prior to surgery, 4 weeks post-surgery, and 8 weeks post-surgery.

<table>
<thead>
<tr>
<th></th>
<th>ShC</th>
<th>OC</th>
<th>NO</th>
<th>CO</th>
<th>SO</th>
</tr>
</thead>
<tbody>
<tr>
<td>3wks before surgery</td>
<td>0.18 ± 0.17</td>
<td>0.18 ± 0.02</td>
<td>0.18 ± 0.12</td>
<td>0.18 ± 0.01</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>4 wks after surgery</td>
<td>0.18 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>8 wks after surgery</td>
<td>0.18 ± 0.01</td>
<td>0.18 ± 0.02</td>
<td>0.17 ± 0.02</td>
<td>0.17 ± 0.01</td>
<td>0.16 ± 0.01</td>
</tr>
</tbody>
</table>

Figure 9 illustrates mean BMD of the vertebrae (g/cm²) of each treatment group 8 weeks after surgery. Mean BMD of the vertebrae in the ShC group (0.18 ± 0.01) was significantly higher than the SO group (0.16 ± 0.01) (p<0.05). There were no significant differences found among any other treatment groups.

Table 16 shows significant differences in mean BMD (g/cm²) of the vertebrae among treatment groups at the end of the study.
Figure 9 – Mean BMD of the vertebrae (g/cm²) of each treatment group 8 weeks after surgery.

Table 16 – Significant differences in mean BMD (g/cm²) of the vertebrae among treatment groups at the end of the study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Level of Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ShC &gt; SO</td>
<td>.034</td>
</tr>
</tbody>
</table>

Vertebrae BMD (g/cm²) Within Treatment Groups

Table 16 shows mean BMD (g/cm²) of the vertebrae within treatment groups 3 weeks prior to surgery, 4 weeks after surgery, and 8 weeks after surgery. No significant differences in mean BMD (b/cm³) of the vertebrae were observed within treatment groups between the three sets of BMD measurements.
CHAPTER 5
DISCUSSION

This study was designed to examine the effects of three different forms of oryzanol in preventing bone loss following ovariectomy (ovx) in retired breeder Sprague Dawley rats. In this study, there were no significant differences in the average weight of the liver and spleen. Mean heart weight was significantly higher in the ovx crystalline oryzanol (group) than in the ovx control (OC) group. Mean weight of the kidneys was significantly lower in the CO group when compared to the sham-operated (ShC) group. Uterine weights of the ShC group were significantly higher than all other groups. The ovx natural oryzanol (NO) group exhibited a numerically higher food intake, weight gain, and feed efficiency when compared to the other treatment groups. Finally, BMD of the elbow, total femur, hip, knee, and tibia were higher in the NO group when compared to the OC, CO and SO groups.

ORGAN WEIGHTS

Uterine atrophy resulting from estrogen deficiency after ovx has been reported in several rat studies (Ishimi et al; Arjmandi et al; O’Fanti et al). In agreement with these studies, the present investigation also showed that ovx rats, regardless of diet treatment, had significantly lower uterine weights when compared to sham-operated rats. These results confirmed successful ovx among the OC, NO, CO, and SO treatment groups. Further, these observations suggest that oryzanol had no estrogen-like effect on uterine weight. Although there were no significant differences in weight of the liver and spleen among treatment groups, rats in the CO group were shown to have larger hearts and smaller kidneys. No suitable explanation could be determined for these observations.
FOOD INTAKE

The results of this 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 revealed inconsistent intakes of food following ovx. It has been reported recently that ovx rats experience an increase in food intake within four weeks after ovx (Ishida et al, McElroy JF and Wade GN). In contrast to this literature, rats in the ShC group did not exhibit greater food intake when compared to ovx rats in the OC, NO, and SO groups.

Results from this study showed that the NO, OC, and SO groups consumed significantly more food when compared to the CO group. Due to the relatively lower intake of food by the CO group, it is suggested that the rats disliked the crystalline oryzanol. One possible reason why the rats disliked this diet could be that they detected potential adverse effects from consuming crystalline oryzanol, which might explain the abnormal weights of the heart and kidneys in the CO group.

In contrast to the CO group, however, the NO group numerically had the highest food intake throughout the study when compared with the other diet treatment groups. During *ad libitum* feeding, rats in the NO group had less spillage outside the feeding containers. Unlike the other treatment diets that contained corn oil, the NO diet contained RBO. Therefore, these findings suggest that the rats preferred the natural oryzanol (RBO) over the other treatment diets.

WEIGHT GAIN AND FEED EFFICIENCY

In addition to increased preference and consumption of the NO diet, rats in this group gained more weight and exhibited a numerically higher feed efficiency when compared to rats in other treatment groups. This suggests that
rats fed natural oryzanol were able to metabolize energy more effectively than rats fed other treatment diets.

Our results did not support the hypothesis that ovx would increase weight in Sprague Dawley rats. Weight gain in rats following ovx, but not in sham-operated rats, has been reported in previous studies (Ishida et al and Arjmandi et al). This suggested that hormonal changes after removal of the ovaries stimulated weight gain.

Contrary to the literature (Ishida et al and Arjmandi et al), the ovx rats did not necessarily have an increased weight gain or higher final body weight when compared to the sham-operated rats. This could be due to the composition of the treatment diets. For example, all of the treatment groups were fed a low-fat diet. Each treatment diet consisted of 4% fat by weight, which was only 10% of energy from fat.

**BONE MINERAL DENSITY**

The results of this research demonstrated that the diet consisting of natural oryzanol was slightly protective in preventing bone loss at several bone sites including the elbow, total femur, hip, knee, and femoral-midshaft. In addition, the natural oryzanol-containing diet was significantly effective in increasing BMD of the tibia. The mechanism by which the natural oryzanol-containing diet could have influenced BMD in these particular bone sites is unknown.

The spine, rather than the long bones of the limbs, is the bone site primarily affected by soy and isoflavones (Potter et al). Results from the Postmenopausal Estrogen/Progestin Interventions Trial showed a greater responsiveness to various estrogen treatments in the spine than in the hip. Duan et al suggested that hormone replacement therapy may be more effective in reducing the risk of spinal rather than hip
fractures. Trabecular bone is known to have a higher turnover rate than does cortical bone. Thus, the lumbar spine, which is relatively high in trabecular bone, should be more sensitive to compounds that are thought to affect remodeling, such as estrogens and isoflavones.

Although the natural oryzanol-containing diet seemed to have a positive effect on the long bones of the limbs, it did not demonstrate similar effects in the vertebrae. Therefore, this suggests that natural oryzanol is acting differently than soy. This could have been due to several factors such as inadequate concentration of oryzanol, insufficient duration of the study, small sample size, or a little of effect on trabecular bone in non-weight bearing bones.

According to our results, natural oryzanol could have affected both trabecular and cortical bone tissues in long bones, but not the vertebrae. However, these effects were not seen with the crystalline oryzanol or solubilized oryzanol. One explanation might be that the natural oryzanol was absorbed more efficiently than the crystalline and solubilized oryzanol. Absorption levels of the natural, crystalline, and solubilized oryzanol are presently being analyzed by a student in the Human Ecology department of this university. Another explanation could be that the positive effects of natural oryzanol on bone metabolism are due to other fractions of the unsaponifiable fraction of rice bran oil. For example, the natural oryzanol, which is high in vitamin E and tocotrienols may have beneficial effects on bone (Jariwalla RJ). The crystalline oryzanol and solubilized oryzanol only have the oryzanol, not vitamin E and tocotrienols.

The role of dietary antioxidants, such as vitamin E, in osteoporosis has not been well explored. Some recent investigations have suggested that dietary antioxidants
influence factors related to bone turnover. For example, new evidence supports the idea that dietary antioxidants can attenuate osteoclastic activity to reduce the severity of bone and joint disease (Norazlina M, Ima-Nirwana S, Gapor MT, and Khalid BAK; Xu H, Watkins BA, and Seifert MF; Maiorano G, Manchisi A, Salvatori G, Filetti F, and Oriani G; Melhus G, Michaelsson K, Holmberg L, Wolk A, and Ljunghall S).

A study by Norazlina et al investigated the effects of supplementing two forms of vitamin E in ovx rats. Their results indicated that 60 mg/kg (palm oil), as well as 30 IU/kg alpha tocopherol maintained BMD in ovx rats. However, further research is needed in order to determine the mechanisms involved (Norazlina et al).

Xu et al evaluated the effects of using two levels of dietary vitamin E (30 IU/kg diet and 90 IU/kg diet) and two different dietary lipids (anhydrous butter oil – 40 g/kg diet plus soybean oil – 60 g/kg diet or soybean oil - 100 g/kg diet) on trabecular bone formation. Their results showed that the interaction effect between the vitamin E and the anhydrous butter oil plus soybean oil treatments led to the higher trabecular bone formation rate among the groups (Xu et al).

Maiorano and coworkers investigated the effect of vitamin E on metacarpal growth plate evolution in suckling lambs. Researchers compared four levels of weekly injections of DL-alpha-tocopherol acetate (Control group, 0 IU; Group 1, 625 IU; Group 2, 1,000 IU; and Group 3, 1,500 IU) on metacarpal growth plate width. Their results showed there was a dose-response effect in the thickness of the metacarpal growth plate. Specifically, Groups 2 and 3 had significantly wider growth plates when compared to Group 1 and the controls (Maiorano et al).
Melhus et al. examined whether the dietary intake of antioxidant vitamins may modify the increased hip fracture risk associated with smoking. They studied 66,651 women who were 40-76 years of age. The relative risk of hip fracture for current versus never smokers was analyzed in relation to dietary intake of antioxidant vitamins stratified into two categories (low/high). Their results suggested that oxidant stress in smokers is associated with adverse effects on the skeleton. Also, an insufficient dietary intake of vitamin E may substantially increase the risk of hip fracture in current smokers, whereas a more adequate intake seems to be protective (Melhus et al).

Although some recent investigations suggest a positive role of vitamin E on bone metabolism, a study by Leveille et al. found no association between vitamin E and femoral neck BMD in 1,892 postmenopausal women aged 55-80 years of age. However, the potential role of antioxidants and other nutrients in postmenopausal bone loss warrants further research, including research of other bone sites.

The hypothesis that the NO diet is affecting both trabecular and cortical bone stems from the results observed in BMD of the long bones such as the humerus, femur, and tibia. These bones contain primarily cortical bone, however, trabecular bone is also present around the ends, or heads, of these bones.

With respect to the tibia, rats in both the ShC and NO groups had significantly higher BMD when compared to rats in the OC group. These observations strongly suggest that NO may play an effective role in preventing trabecular, as well as, cortical bone loss in ovx rats.

The results also suggested that natural oryzanol slightly prevented bone loss in sites such as the elbow, hip, and knee. This suggests that natural oryzanol is
moderately protective in preventing loss of trabecular and cortical bone in long bones. However, the observation that natural oryzanol has a slightly protective effect on BMD of the femoral midshaft suggests that it may have a preventive effect on ovx-induced bone loss in primarily cortical bone tissue.

Bone mineral density of the vertebrae in the ShC rats was not significantly higher than the OC rats at the end of the study. This observation contradicts previous literature which suggests that ovarian-hormone deficiency due to ovx causes rapid bone loss and increased bone turnover in rats.

Due to the absence of an effect of ovx on vertebral BMD, natural oryzanol could not be effective in preventing bone loss in that particular site. For example, natural oryzanol could not prevent loss of BMD in bones that did not lose BMD. In addition, there were no significant differences in BMD of the vertebrae three weeks prior to ovx, four weeks after ovx, and eight weeks after ovx. These observations also contradict previous literature which states that estrogen deficiency due to ovx is associated with rapid bone loss and increased bone turnover in rats. However, the original PDEXA machine was replaced by a newer model shortly before the second series of BMD measurements. Thus, this could have resulted in imprecise measurements of BMD.

FUTURE DIRECTIONS

It is important to determine whether natural oryzanol, derived from rice bran oil, can be effective in maintaining bone mass in the long bones, as well as in the vertebrae. Hip fracture, 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 prevent bone loss in the femur would be important. Studies of variable duration, using high versus low
concentrations of natural oryzanol and/or isolated vitamin E and tocotrienols could be conducted. Studies evaluating biochemical markers of bone turnover, such as osteocalcin and urinary deoxypyridinoline could confirm the mechanism by which bone turnover is affected by oryzanol diets in ovx rats.
REFERENCES


Crowley WR. Effects of ovarian hormones on norepinephrine and dopamine turnover in individual hypothalamic and extra-hypothalamic nuclei. *Neuroendocrinology.* 1982; 34: 381-386.


Duan Y, Tabensky A, DeLuca V, Seeman E. The benefit of hormone replacement therapy on bone mass is greater at the vertebral body than posterior processes or proximal femur. *Bone.* 1997;21:447-51.


Metcalf MG, Donald RA. Fluctuating ovarian function in a perimenopausal woman. *NZ Med J.* 1979; 89: 45-47.


Migliaccio S, Davis VL, Gibson MK, Gray TK, Korach KS. Estrogens modulate the responsiveness of osteoblast-like cells (ROS 17/2.8) stably transfected with estrogen receptor. *Endocrinology.* 1992;130:2617-2624.


Miller SC, Bowman BM, Jee WSS. Available animal models of osteopenia-small and large. *Bone.* 1995;17:117S-123S.


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).

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 |

<table>
<thead>
<tr>
<th>Number of animals needed:</th>
<th>Maximum number needed at one time: 85</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year 1: 85</td>
<td>85</td>
</tr>
<tr>
<td>Year 2: 0</td>
<td></td>
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<tr>
<td>Year 3: 0</td>
<td></td>
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<tr>
<td>TOTAL: 85</td>
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</table>

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
G NO

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

Signature (required):

Location of Animals

<table>
<thead>
<tr>
<th>DLAM Vivarium</th>
<th>X</th>
<th>Life Sciences Vivarium</th>
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</thead>
<tbody>
<tr>
<td>SVM Barns</td>
<td></td>
<td>SVM Fish Building</td>
</tr>
<tr>
<td>LAES (List Site):</td>
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<td>Other (List Site):</td>
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</tbody>
</table>
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.

Professor 5/2/01
Principal Investigator Signature
Maren Hegsted
(Type Name of Principal Investigator)

Professor 5/2/01
Principal Investigator Signature
Carol O’Neil
(Type Name of Principal Investigator)

Associate Professor 5/2/01
Co-Investigator Signature
Mike Keenan
(Type Name of Co-Investigator)

Instructor 5/2/01 Surgeon
(if applicable) Signature
Rhett Stout
(Type Name of Surgeon)
SECTION 6: Special Husbandry Requirements

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

Gx YES

<table>
<thead>
<tr>
<th>TEMPERATURE RANGE</th>
<th>Humidity:</th>
</tr>
</thead>
<tbody>
<tr>
<td>(F)</td>
<td>(%)</td>
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</tbody>
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<table>
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<table>
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<th>Type:</th>
<th>Autoclaved?</th>
<th>Changes/week: we change</th>
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<th>Acidified:</th>
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<table>
<thead>
<tr>
<th>DIET</th>
<th>Special Feeding Requirements: we will feed animals</th>
</tr>
</thead>
</table>

GNO (If you indicate ‘No’, your animals will be cared for according to standard operating procedures of DLAM)

GNot Applicable

SECTION 7: Hazardous Materials

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

<table>
<thead>
<tr>
<th>Zoonotic/Recombinant Agents?</th>
<th>Radioisotopes?</th>
<th>Hazardous Chemicals?</th>
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</thead>
<tbody>
<tr>
<td>G YES X NO</td>
<td>G YES X NO</td>
<td>G YES X NO</td>
</tr>
</tbody>
</table>

Agent(s): ________________

Isotope(s): ________________

Are you certified by the Radiation Safety Committee?

G YES G NO

Compound(s): ________________
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.

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:

1) The rationale for using animals. Why should this study be done? What hypothesis will be tested?
   2) How and/or why you selected the animal species indicated.
   3) How you arrived at the number of animals to be used.
4) 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.
5) 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. A description of any euthanasia method to be used.
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 finally a (betadyne) applied to the skin surface.
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 exsanguination 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.
### SECTION 9: Type of Project

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TYPE A</td>
<td>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.</td>
</tr>
<tr>
<td><strong>X</strong> TYPE B</td>
<td>Pain or distress will be relieved by appropriate therapy.</td>
</tr>
<tr>
<td><strong>X</strong> TYPE C</td>
<td>Drug intervention for pain or distress would interfere with the protocol. <em>(If this block is checked, specific justification MUST be provided.)</em></td>
</tr>
</tbody>
</table>

### 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 information 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.

<table>
<thead>
<tr>
<th>YES</th>
<th>NO</th>
<th>Individual(s) Responsible</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>X</strong></td>
<td>Will animals be restrained? <em>(Restraint refers to immobilization or other restrictions to normal movement beyond momentary holding for injections, etc.)</em></td>
<td>Not Applicable</td>
</tr>
<tr>
<td><strong>X</strong></td>
<td>Will animals be fasted?</td>
<td>Not Applicable</td>
</tr>
</tbody>
</table>
| March 2001 | Are any ANESTHETICS, ANALGESICS, or TRANQUILIZERS to be used? | Dr. Rhett Stout  
Dr. Maren Hegsted  
Dr. Mike Keenan |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Who will administer?...............................................</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Are neuromuscular blocking agents to be used?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Who will administer agents?........................................</td>
<td></td>
</tr>
<tr>
<td></td>
<td>How will animals be monitored?______________________________</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>Will surgical procedures be employed? Are they: Survival X Multiple _____ Terminal_____</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>----------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Who will perform surgery? .......................................................................................................................................................</td>
<td></td>
</tr>
<tr>
<td></td>
<td>If survival:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1) Who will be responsible for recovery of the animals? ...........................................................................................................</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2) Who will maintain post-operative records?...............................................................................................................................</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3) Where will records be maintained? With the animal records outside the animal room ......................................................................</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4) Who will provide post-op analgesics?....................................................................................................................................................</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Note: Survival surgeries must be conducted aseptically, and major surgical procedures performed on non-rodent species must be conducted in a dedicated surgical facility.</td>
<td></td>
</tr>
</tbody>
</table>

|   | 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 |

<table>
<thead>
<tr>
<th>X</th>
<th>Do you anticipate any adverse effects of the experimental procedures on the animals (e.g., pain, discomfort, reduced growth, fever, anemia, etc)?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>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.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>X</th>
<th>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.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not Applicable</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>X</th>
<th>Are there emergency treatments by the DLAM veterinary staff that would not be allowed?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not Applicable</td>
</tr>
</tbody>
</table>

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| March 2001 | **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 | |
| **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? | Dr. Maren Hegsted  
Dr. Mike Keenan | **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. |
SECTION 11: Animal Management

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

Check all applicable below:

<table>
<thead>
<tr>
<th>CARE OF SICK ANIMALS</th>
<th>DISPOSAL OF DEAD ANIMALS</th>
<th>PEST CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>X Call Investigator</td>
<td>X Call Investigator</td>
<td>G Call Investigator</td>
</tr>
<tr>
<td>X Clinician to Treat</td>
<td>G Necropsy</td>
<td>X Pesticides OK</td>
</tr>
<tr>
<td>G Euthanasia</td>
<td>G Disposal.</td>
<td>G No Pesticides</td>
</tr>
</tbody>
</table>

List any special requirements for disposal?

SECTION 12: Disposition of Animals

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

<table>
<thead>
<tr>
<th>X Animals will be euthanized.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLAM/LAES has permission to REASSIGN animals to another IACUC-approved protocol.</td>
</tr>
<tr>
<td>TRANSFER animals to the following IACUC-approved protocol(s).</td>
</tr>
<tr>
<td>Please list Protocol Number: ____________________________</td>
</tr>
<tr>
<td>OTHER (Please state) ____________________________</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Name</th>
<th>Rules/Regulations Training Course</th>
<th>Date Attended</th>
<th>Species Wet Lab*</th>
<th>Date Attended</th>
<th>Training and Experience**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maren Hegsted</td>
<td>X YES G NO</td>
<td>3/8/00</td>
<td>G YES G NO</td>
<td>X YES G NO</td>
<td></td>
</tr>
<tr>
<td>Mike Keenan</td>
<td>X YES G NO</td>
<td>3/9/99</td>
<td>G YES G NO</td>
<td>X YES G NO</td>
<td></td>
</tr>
<tr>
<td>Carol O’Neil</td>
<td>X YES G NO</td>
<td>6/1/99</td>
<td>G YES G NO</td>
<td>X YES G NO</td>
<td></td>
</tr>
<tr>
<td>Rhett Stout</td>
<td>X YES G NO</td>
<td></td>
<td>G YES G NO</td>
<td>X YES G NO</td>
<td></td>
</tr>
<tr>
<td>Kem Singletary</td>
<td>X YES G NO</td>
<td></td>
<td>G YES G NO</td>
<td>X YES G NO</td>
<td></td>
</tr>
<tr>
<td>Marie Grant</td>
<td>X YES G NO</td>
<td>9/7/99</td>
<td>G YES G NO</td>
<td>X YES G NO</td>
<td></td>
</tr>
<tr>
<td>Adam Ralston</td>
<td>? YES G NO</td>
<td></td>
<td>G YES G NO</td>
<td>X YES G NO</td>
<td></td>
</tr>
<tr>
<td>Matthew Wheelcock</td>
<td>? YES G NO</td>
<td></td>
<td>G YES G NO</td>
<td>X YES G NO</td>
<td></td>
</tr>
</tbody>
</table>

* 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**
March 2001

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.

________________________________________

________________________________________

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VITA

Heather Compton Colona was born in Hammond, Louisiana, on July 9, 1978. She graduated from Independence High School in Independence, Louisiana. In May 2000, Heather completed a Bachelor of Science degree in dietetics from Louisiana State University (LSU), Agricultural and Mechanical College. Throughout her education, Heather worked in the foodservice industry. While working on her graduate thesis at LSU, she worked in various labs throughout the LSU campus. Currently, she is living in Hammond, Louisiana, and is pursuing the Master of Science degree in human ecology from LSU with an emphasis in human nutrition and food. Heather will receive the degree on August 8, 2002. In the future, she plans to complete the LSU dietetic internship program and later take the registration exam to become a registered dietitian.