EFFECT OF FEEDING GRAPEFRUIT PULP ON BONE MICROARCHITECTURE AND STRENGTH IN ORCHIDECTOMIZED RATS

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NOMENCLATURE

Connectivity Density - indicates the average number of connections present between trabeculae in a specified volume.

Cortical Area - explains the amount of cortical bone per square millimeter.

Cortical Bone - smooth part of bone which is protective in nature and forms the outer layer of the bone as a shell.

Cortical Bone Thickness- explains the thickness of cortical bone.

Cortical Porosity - describes the porous or empty spaces in the bone.

Degree of Anisotropy - explains the degree of directional organization of bone's internal structure when a load is applied in a particular position. A higher DA indicates increasing disorganization when viewed from a given plane.

Medullary Area - describes the central volume of the bone per square millimeter.

Osteoblast - a mononucleate cell arising from osteoprogenitor cells which is associated with bone formation.

Osteoclast - a large multinuclear cell associated with the resorption of bone.

Structural Model Index - describes a bone's relative rod like property. A value of zero corresponds to pure plates and a value of three corresponds to perfect rods. Negative values indicate concave like structure and a value of four indicates a sphere. Plate-like properties are considered desirable and rod-like properties are mostly seen in ovariectomized models as cross struts are removed and trabeculae are eroded.

Trabeculae - thin strand like structures in cancellous bone.

Trabecular (Cancellous) bone - the lattice like bone, rigid structure that appears spongy.

Trabecular Number - describes the average number of trabeculae present per mm^3.

Trabecular Separation - describes the average separation or air space between trabeculae. Greater trabecular number (Tb. N) would result in less trabecular separation.

Trabecular Thickness - describes the average thickness of the trabeculae in a specified region.

LIST OF ABBREVIATIONS

2D…….……Two-Dimensional

3D…….……Three Dimensional

BMC…….……Bone Mineral Content

BMD…….……Bone Mineral Density

BV..……Bone Volume

BV/TV……Bone Volume Fraction

Co. Area…….Cortical Area

Co. P...…..……..Cortical Porosity

Co. Th……....Cortical Thickness

Conn. D.……Connectivity Density

DA…….Degree of Anisotropy

DXA……...Dual Energy X-Ray Absorptiometry

L_4……Fourth Lumbar Vertebra

Micro-CT/µCT…….Micro-Computed Tomography

FE (FE)…… Finite Element

SMI……..Structural Model Index

Tb .N…… Trabecular Number

Tb. Sp……...Trabecular Separation

Tb. Th…….……..Trabecular Thickness

CHAPTER I

INTRODUCTION

Background to the problem

Bone is a living, growing tissue that is mostly made of collagen, a protein that provides a soft framework. It also consists of calcium phosphate, a mineral that adds strength and hardens the framework (1) .

Throughout life, old bone is removed (resorption) and new bone is added to the skeleton (formation). During childhood and teenage years, new bone is added faster than old bone is removed. As a result, bones become larger, heavier, and denser. Bone formation outpaces resorption until peak bone mass (maximum bone density) is reached around age 30. After that time, bone resorption slowly begins to exceed bone formation. However continued resorption without adequate formation can lead to thinning and weakening of the bones to a point whereby they become fragile and break easily, a condition referred to as osteoporosis.

In the United States, 10 million individuals are estimated to already have the disease and almost 34 million more are estimated to have low bone mass, placing them at increased risk for osteoporosis. Of the 10 million Americans estimated to have osteoporosis, 80% are women and 20% are men (2).

The bones of the skeleton consist either of cortical (compact) or trabecular (cancellous) bone. The loss of cancellous bone that occurs with aging is not due to generalized thinning of the bone plates but is rather caused by complete perforation and fragmentation of the trabeculae. Osteoporosis is a systemic skeletal disease characterized by loss of bone mass and microarchitectural deterioration of the trabecular network (3). According to the National Osteoporosis Foundation, osteoporosis is also known as the silent disease of aging as bone loss occurs without symptoms until deterioration of the bone becomes so significant that bone fracture occurs. This disease can be prevented and treated.

Bone mass gain is mainly related to increases in bone size, that is in bone external dimensions with minimal changes in bone microarchitecture whereas bone loss results from thinning of both cortices and trabeculae and from perforation and eventually disappearance of the latter and significant alteration of the bone microarchitecture (4).

Male osteoporosis is understudied in bone research. As in females, hypogonadism seems to be the principal risk factor in men that leads to bone loss and increased fracture incidence (5). Men tend to achieve a greater peak bone size and mass (6) compared to women and hence have a greater protection once bone loss begins. Some of the risk factors associated with male osteoporosis include age (bone loss increases with age), race (Caucasian males have the greatest risk of disease) and genetics (NOF 2004). Other secondary risk factors include hypogonadism, alcohol abuse, and prolonged steroid therapy (7).

Osteoporosis is considered a disease of aging, and one of the hypotheses explaining the aging process is attributed to oxidative stress. Emerging evidence indicates

that the activation of redox sensitive transcription factors and dysregulated gene expression due to the age-related oxidative stress could be the underlying cause that links aging and age related pathological diseases such as osteoporosis (8). Oxidative stress could lead to progressive bone loss and osteoporosis could be eliminated if oxidative stress is prevented (9, 10). Oxidative stress is involved in bone resorption due to generation of superoxide by osteoclasts resulting in bone degradation.

Though there are several treatment options available approved by the Food and Drug Administration (FDA), the incidence of the disease has escalated with the main reason being that there is a lack of long term adherence to treatment (11). Hence there is a need for alternative therapies that have fewer side effects and that are part of the daily American diet (12).

Grapefruit is a sub-tropical citrus tree which is a rich source of bioactive compounds called flavonoids and limonoids which have potential health promoting properties (13).Flavonoids are biologically active compounds found in plants that have been associated with a decreased risk of some age related and chronic diseases in humans (14). Flavonoids are present in the pulpy, fibrous parts of major fruits (red grapes, oranges, pink grapefruit, strawberries and blueberries), fruit products, tea, and soy. Most common supplemental forms of flavonoids are the citrus flavonoids quercetin, rutin, and hesperidin. Flavonoids which are polyphenols are effective antioxidants that negate reactive oxygen species (ROS) and may or may not be colored. Yu et al studied the antioxidant activity of citrus flavonoids and concluded that several structural features were linked to the strong antioxidant activity of flavonoids. Citrus flavonoids demonstrated mild to strong antioxidant activity in comparison to limonoids (15). Some

of the flavonones found in grapefruit include hesperetin (metabolizes to hesperidin), naringenin (metabolized from naringin) and eriodictyol (16).

Grapefruit contains compounds such as naringin, naringenin, and 6,7-dihydroxybergamottin that act by blocking the activity of the cytochrome P-450 (CYP) 3A4 isoenzyme in the intestinal wall, thereby preventing the presystemic first-pass metabolism of a wide range of drugs (17). Calcium channel antagonists, neuropsychiatric medications, statins, and antihistamines are just a few of the drug classes whose actions are significantly affected by the consumption of grapefruit and grapefruit juice. Patients and other health care professionals need to be educated about potential drug interactions with grapefruit juice (18).

Grapefruit juices contain large quantities of bioactive compounds, which guarantee their high antioxidant potential, and the positive influence on plasma lipid metabolism and plasma antioxidant activity could make fresh grapefruit juice a valuable supplement for disease-preventing diets (19).

Research Objectives

The principal objective of this study was to determine the effects of feeding grapefruit pulp on the microarchitecture and strength of bone in an orchidectomized rat model of male osteoporosis.

The specific aims of the experiment include:

1. To examine the dose dependent effects of grapefruit in preventing bone loss by assessing bone mineral area (BMA), bone mineral content (BMC) and bone mineral density (BMD) in a rat model for male osteoporosis.

2. To determine the dose dependent role of grapefruit pulp in preventing loss of bone structure by assessing trabecular microstructural properties of the proximal tibia and the fourth lumbar vertebra.
3. To evaluate the dose dependent effect of feeding grapefruit pulp on the strength of bone in a rat model of osteoporosis using finite element analysis.

Hypothesis

The hypothesis of this study is that feeding grapefruit pulp that has antioxidant properties will prevent bone loss in an orchidectomized rat model of male osteoporosis.

Limitations

Unlike men who experience a gradual decline of gonadal hormones over time, orchidectomy induces an abrupt decrease in gonadal hormones in male rats. However, the desired hormonal deficiency to induce bone loss was achieved in this model.

Although, the orchidectomized rat model is considered acceptable for studies relevant to osteoporotic men, the findings of this study cannot be extrapolated directly to humans due to differences in the bone metabolism between the two species. Rats have minimal haversian systems in cortical bone thus making the pattern of bone remodeling in rats and humans different. However, the results of this study should provide information to warrant clinical trials to determine the use of grapefruit pulp as a treatment option for male osteoporosis.

Format of thesis

Chapter IV of this thesis is written in the form of a manuscript for submission to Journal of Nutrition using their authors' guidelines. The remaining chapters are based on the Oklahoma State University thesis guidelines.

CHAPTER II

REVIEW OF LITERATURE

Bone is a composite material composed of an organic and inorganic phase. The organic phase is synthesized by osteoblasts, and the inorganic phase is composed of calcium phosphate (20). The development of bone begins before birth and formation predominates until approximately the end of the second decade of life (21, 22). Osteoblasts are bone cells that deposit mineral salts onto a collagen matrix and increase bone mass (23) . Osteoclasts are cells responsible for bone resorption and are the only cell type able to breakdown the mineralized matrix. The osteoclasts foster bone modeling during growth and bone remodeling during adulthood hence playing a crucial role in bone physiology. Chondrocytes, the cells in endochondreal skeleton, deposit an extracellular matrix that is cartilage specific and help in the repair of degraded collagen matrix (24).

Osteoporosis is a systemic skeletal disease characterized by low bone mass as well as microarchitectural deterioration of bone tissue, which leads to an increase in bone fragility and susceptibility to fractures (25). Osteoporosis results from defective remodeling either when there is enhanced osteoclastic activity or decreased osteoblastic activity resulting in a net bone loss (26).

Epidemiology of osteoporosis

Bone loss with age is inevitable and universal, occurring in all skeletal sites, all races and cultures and in both sexes (27). The World Health Organization defines osteoporosis as a condition where the BMD is greater than 2.5 standard deviations below the young adult reference mean (WHO 1994). The National Osteoporosis Foundation estimates that 10 million Americans are already afflicted with osteoporosis and another 34 million individuals have low bone mass putting them at risk of osteoporosis related fractures. It is estimated that one in two women and one in eight men over the age 50 will have an osteoporosis related fracture (28). The annual expenditure for treating osteoporosis related fractures was $17.5 billion in 2002 (29) and is projected to exceed $60 billion by the year 2020 (30).Risk factors for the development of osteoporosis include genetics, sex, race, menopause, inadequate nutrition, use of medications such as glucocorticoids and lifestyle factors such as smoking and inappropriate strenuous exercise.

Estrogen deficiency is critical to the pathogenesis of osteoporosis as is evident by the fact that postmenopausal women who have naturally declining estrogen levels are at the greatest risk for developing the disease (31). Estrogen is critical for epiphyseal closure in puberty in both sexes and regulates bone turnover in men as well as women. It has more effect than androgen in inhibiting bone resorption in men although androgen may still play a role (32).

Bone remodeling

Bone is a dynamic tissue that constantly undergoes remodeling although growth and modeling of the skeleton have been completed. Remodeling of the bone involves localized removal of old bone (resorption) and replacement with newly formed bone. Bone remodeling occurs in small packets of cells called basic multicellular units which turn bone over on multiple bone surfaces (33). The process is regulated by biochemical and mechanical factors (34). Two principal cell types are found in the bone, the osteoclast and the osteoblast which are the major effectors in the turnover of bone matrix (35). Osteoblasts lay down bone matrix that ultimately becomes mineralized in a well regulated manner (36, 37) .These cells then either undergo apoptosis or become buried into the matrix to form osteocytes. The osteocytes express receptors for estrogen and vitamin D in their nuclei (38). Osteoclasts, which are multinucleated cells found in clusters or alone, are modulated by interleukin-1(IL-1), IL-3, IL-6, IL-11, TNF-α, and colony stimulating factors. The osteoclasts attach to the endosteal bone surface and create a tight seal and then secrete acid that solublizes the hydroxyapatite crystals and exposes the bone matrix. Digestive enzymes then dissolve the protein matrix and liberate previously deposited growth hormones and collagenases (39). Once resorption is completed the resorptive cavity becomes refilled with osteoblasts that initiate formation hence creating a repeating cycle.

Remodeling of Cortical Bone: Cortical bone which comprises 85% of the total bone in the body is regulated by the formation of periosteal bone, by remodeling within the haversian systems and by endosteal bone resorption. Endosteal bone resorption leads to increased porosity of the bone. Cortical bone loss begins probably after the age of 40,

and there is an accelerated loss of bone 5-10 years after menopause. The loss of cortical bone is the main predisposing factor for fractures that occur at the hip and around the wrist (40).

Remodeling of Cancellous Bone: In spite of cancellous bone comprising only 15% of the skeleton, mainly in the vertebral column, it is the main determinant of spinal osteoporotic fractures. The loss of cancellous bone that occurs with aging is not simply due to generalized thinning of the bone plates but due to complete perforation and fragmentation of trabeculae (40).

Bone remodeling is regulated by systemic hormones and local factors. The hormones regulate the synthesis, activation and effects of the local factors that have a direct action on cellular metabolism. Hormones also modify the replication function of the osteoclasts and osteoblasts (41).

Although the main function of bone remodeling is not fully understood, the two most common reasons attributed to it are that (1) it serves as a means of accessing minerals and growth factors stored in the bone but needed elsewhere and (2) it provides a replacement for mechanically compromised bone (42).

Changes in bone with age

The primary cause of osteoporotic fractures in the elderly is age-related bone loss (43, 44). Bone loss begins approximately at the age of 40 years and progresses linearly at a rate of 0.5 to 1% per year. Hence, by 70 years of age as much as 30 % of bone loss is accrued. This type of bone loss results in increased porosity in cortical and trabecular bone, decrease in mineralization and finally increases the risk of fracture (45, 46).

Most of the studies on age-related bone loss have focused on postmenopausal women since women start losing bone earlier than men. However, changes in bone mass with age are also observed in the male skeleton. Between the ages of 20-30 years (early adulthood), bone loss begins in women after the growth of long bones is stopped due to a negative balance in the remodeling process (47). Many studies have shown that the onset of menopause is associated with an increase in turnover and an increase in bone loss. The rate of bone remodeling doubles at perimenopause and triples after menopause. This rate of bone remodeling remains high in osteoporosis (48).

At the onset of menopause, estrogen production drops resulting in increased bone turnover rate (49). Consequently, more resorption cavities on the endosteal surface of bone are produced with an increase in bone turnover. Deficiency of estrogen increases the life span of osteoclasts resulting in the resorption of bone being higher than formation. As a result, net bone loss occurs since bone formation occurs at a slower rate than resorption. This increase in resorption leads to an increase in the resorption cavities and deeper resorption lacunae causing a net loss of trabecular connectivity. Loss of trabeculae changes the microarchitecture of bone leading to a decrease in overall strength of the bone (50). This negative balance in bone turnover, where more bone is resorbed than

formed, is linked to a higher rate of cancellous bone loss in women with osteoporosis (51). Eventually, rapid loss of bone after menopause results in the complete destruction of some structural trabecular elements. The remaining trabecular elements show a reduced thickness due to the continuous loss of trabecular bone leading to further trabecular architecture deterioration (52, 53).

Cortical thinning and an expansion of marrow cavity is another important contributing factor in human osteoporotic fractures at the femoral neck (54, 55). An increase in the cortical area at the femoral midshaft is observed until the seventh decade of life (56). A study reported by Stein et al (1999) showed that elderly patients did not have a greater number of pores than younger subjects in the cortical region but elderly subjects showed larger pores. However, this study did not assess the distribution of porosity throughout the cortical width or the porosity changes with age (57).

A significant decrease in osteoid mineralization was observed in male Wistar rats after marrow ablation in femur (58). A decrease in the response of bone cells with age was observed by bone morphometric and structural studies. Also these rats did not show a significant decrease in femur strength when compared to their peak values as the force required to fracture femurs at midshaft did not change with aging. Conversely, ultimate stress decreased 14% from 12 to 24 months. Ultimate stress is a parameter that normalizes for differences in bone geometry and size. Other biomechanical properties, modulus of elasticity, yield strain and ultimate deformation, were not significantly affected by age. Although the tissue strength increased with age, the strength of the femur was maintained due to architectural compensations. Based on these findings the authors concluded that bone status was compromised in the aged male rat (59).

Male osteoporosis

Preventive medicine for elderly men tends to focus on prostate and bowel cancer whereas the possibility of bone loss and fractures is usually neglected (60). The prevalence of low bone mass increases with age in both men and women, an age associated bone loss. However it is higher for women than men because accelerated bone loss occurs in the immediate post menopausal period (61). Although women have been the main focus of osteoporosis research, osteoporosis is a common occurrence in men as well. The life time risk of experiencing an osteoporotic fracture in men over the age of 50 is 13% (62) which is similar to the risk of developing prostate cancer (63). Hip fractures become more common with age and between the ages of 85 and 89 years comprise 33% of all osteoporotic fractures in men and 36% in women (64). The mortality associated with hip fracture in the elderly is higher in men than women due to more comorbid conditions at any specific age and lower life expectancy in men (65, 66). A Finnish study involving 75 -80 year old men and women showed that the risk of fracture was equivalent at a given BMD level in men and women (67). Also Selby and colleagues reported that the gender specific T-score of – 2.5 is appropriate in identifying both men and women likely to have a fracture (68). Although peak bone mass is higher in men than women because men have bigger bones, the peak bone mineral density is equivalent (69). The amount of trabecular bone lost at the spine and iliac crest is similar in both men and women during aging, however, cortical bone loss is less in men due to the reduced endocortical resorption and increased periosteal formation (70). Elderly men have an

accelerated bone loss because endocortical resorption and increased cortical porosity increase the surface available for resorption.

Menopause is the main risk factor for osteoporosis in women. The reason is attributed to estrogens that preserve bone by restraining the production of cytokines that promote bone resorption. Loss of estrogen at menopause removes the restraint and promotes the osteoclastic bone loss. In contrast to postmenopausal osteoporosis in women, the mechanism of age related bone loss in men has been less studied (Wang 2001). Part of the loss in bone has been linked to gonadal hormone deficiency (71). Serum total and free testosterone levels have been reported to be significantly lower in elderly men with hip fractures. In contrast to women, bone remodeling rate in men, as judged by indices of bone turnover, remains low in midlife and is variably increased in advanced age.

Androgen receptors have been found in osteoblasts, bone marrow stromal cells, osteocytes, hypertrophic chondrocytes and osteoclasts (72).These findings provide evidence that testosterone has a direct effect on male skeletal health.

Studies involving cross-sectional, longitudinal and direct interventional studies have established the key role that estrogens play in bone metabolism in men, young and old. Testosterone is important in the male skeleton for sexual dimorphism at the time of puberty. In adult men, it may also contribute to inhibition of bone resorption and the maintenance of bone formation, both directly and by serving as a substrate for aromatization to estrogen (73). Male osteoporosis has become a big problem due to the potential for longer life expectancies leading to an increased number of elderly individuals.

The orchidectomized rat as a model for male osteoporosis

Animal models show similar age related changes to humans which include changes in tissue function at the level of the whole organ and tissue as well as cellular and subcellular levels (74).Although an animal model of age-related bone loss may not precisely replicate the human situation, it may be useful for studying certain aspects of the bone loss (75). Rats are commonly used as animal models of human diseases because they are easily available, relevant and appropriate. Wang and colleagues recommend the use of the Sprague-Dawley rat as a model of male osteoporosis to study potential preclinical therapies of age related bone loss in men as well as to study the effects of aging on circulating sex hormones and the effect of therapy with female steroid hormones on bone in men.

In a study by Audran et al. the orchidectomized (ORX) rat was used to simulate male osteoporosis due to hypogonadism. Significant bone loss was found due to increased bone resorption with/without reduced bone formation using histomorphometric methods (76). The orchidectomized rats showed significant reductions in trabeculae number and an increase in trabecular separation, both of which are signs of compromised trabecular connectivity (77).

Another group investigated the effects of androgen deficiency and androgen replacement on bone density in 14 and 17 month old male rats. Bone mineral content (BMC) and density (BMD) measured with DXA were not decreased one month after orchidectomy but were lowered four months after orchidectomy by 10% and 8% respectively (78).

Treatment options for male osteoporosis

As the magnitude and character of osteoporosis in men became apparent, so did the awareness that few data were available concerning its treatment. For some time, male patients were managed with extrapolations of treatments for women (79). Some of the options considered before any therapeutic regimen include lifestyle management such as a calcium rich diet, limited alcohol consumption, smoking cessation, adequate exercise and treatment of secondary causes of osteoporosis (80).

Other treatment options include bisphosphonates which are used in the prevention and treatment of osteoporosis due to glucocorticoid excess or hypogonadism. The most commonly used bisphosphonates include alendronate and trials are underway with risedronate though it has shown positive effects on BMD (81). The use of parathyroid hormone (PTH) seems to have excellent effects on BMD. The increase in BMD induced by PTH is as marked in those with low testosterone levels as in eugonadal men (82). Hypogonadal men, when treated with sex steroid therapy such as testosterone have restoration of both serum testosterone and estradiol to levels of eugonadal men. A variety of trials have shown that sex steroid replacement in men with well established hypogonadism is associated with an increase in BMD (83).

Bone mineral density and fracture risk

Bone strength is determined by bone geometry, cortical thickness and porosity, trabecular bone morphology and intrinsic properties of bony tissue. Bone strength is indirectly estimated by BMD using dual energy X-ray absorptionmetry (DXA) (84). DXA is the most commonly used technique to measure bone mineral density. It uses two

X-ray beams of different energy levels to scan the region of interest and measure the attenuation as the beam passes through bone (85)

Based on various studies that have examined fracture risk in men and women according to BMD, it can be concluded that the relationship between BMD and fracture risk changes with age (86). That means the risk of a fracture at a T-score of -2.5 SD at the age of 50 yrs is lower than the risk at the age of 80 years with the same T-score. These problems can be overcome by sampling populations at random and expressing risk as a function of BMD or standardized T-scores and with age adjustment (87).

Approximately 5% of men over the age of 50 years have a BMD of the proximal femur more than 2 standard deviations below the young normal mean (88). A study by Orwoll et al found that BMD in the older men was positively related to weight and negatively related to age, gastrectomy, previous history of fracture and rheumatoid arthritis (89). Other studies have found that high body mass index and weight gain have been associated with high BMD and lower rates of BMD loss (90).

The role of androgens on bone in elderly men was studied by Johnell et al who found that free testosterone levels were independent positive predictors of BMD in total body, total hip, femur trochanter and arm but not in the lumbar spine. Free testosterone but not free total estradiol was a positive predictor of total body bone area and bone mineral concentration. The study concluded that not only estrogens but also androgens are of importance for bone health in elderly men (91).

Oxidative stress

Oxidative stress is caused by either an increase in the production of reactive oxygen species (ROS) and free radicals, or a decrease in the antioxidant defense system. The free radical theory of aging suggests that ROS cause time-sensitive structural and functional disorders that can lead to the development of age-related chronic diseases such as osteoporosis. Oxidative stress is defined as an imbalance in which the concentration of pro-oxidants is greater than that of antioxidants. In a state of oxidative stress, antioxidants are low and markers of oxidative damage are abundant. Ways to decrease oxidative stress include limiting exposure to outside oxidative agents such a smoke, UV light and air pollution, reducing production of free radicals that are by-products of mitochondrial respiration and consuming antioxidants.

A study by Hosokawa showed that higher oxidative stress was related with senescence acceleration and age dependent alterations in cell structure and function (92). Other studies by Isomura et al have shown that oxidative stress could be involved in the pathogenesis of metabolic bone diseases such as osteoporosis (93).

Previous studies have shown that certain risk factors for osteoporosis such as smoking, hypertension and diabetes mellitus are associated with increased oxidative stress (94). In addition, free radicals are involved in osteoclastogenesis and in bone resorption (95) in both in vitro and in vivo studies. The major problem in assessing free radical induced oxidative stress in various diseases has been the limitation in available assay methods for in vivo measurement of free radical generation or end products of free radical catalyzed oxidation of lipids (96).

Muthusami et al studied the effect of ovariectomy on the antioxidant systems of bone using a rat model (97). They found that there was an increase in hydrogen peroxide and lipid peroxidation in the femur of ovariectomized rats supporting the notion that estrogen deficiency activates ROS for the induction of bone loss (98). Apparently estrogen deficiency causes bone loss through increased expression of cytokines such as TNF-α, IL-1, and IL-6 in osteoclasts, supportive bone marrow stromal cells, monocytes and lymphocytes.

Antioxidants

Polyphenolic flavonoids are among a wide variety of phytochemicals present in the human diet. Flavonoids are low molecular weight, polyphenolic molecules which occur naturally in fruits, vegetables, legumes and seeds of vascular plants. Basic research in animal models as well as human studies suggests flavonoid intake may reduce the risk of several age related chronic diseases (99). Due to their polyphenolic structure, flavonoids can be potent scavengers of free radicals (100).

Experimental studies on animals or cultured human cell lines support a role for polyphenols in the prevention of cardiovascular diseases, cancers, neurodegenerative diseases, diabetes, or osteoporosis. However, it is very difficult to predict from these results the effects of polyphenol intake on disease prevention in humans. One of the reasons is that these studies have often been conducted at doses or concentrations far beyond those documented in humans (101).

Osteoporosis is associated with many etiological causes such as poor nutrition, increases in cytokines, loss of hormones, and aging. Recently, reactive oxygen species

(ROS) have been considered to be responsible for the aging process and osteoporosis. (102).

Vitamin C supplementation has been shown to increase bone mineral density in post menopausal women in two interventional studies. However, not much is known about associations between intakes of other antioxidants and bone health especially in men who are at a substantial risk of osteoporotic hip fractures (103). A study by Zhang et al found that higher intakes of antioxidants were associated with a lower risk of hip fractures in those who had ever been smokers (104). Other studies have shown that smoking accelerates bone loss and increases fracture risk and the adverse effects of smoking are attributed to increased oxidative stress (105).

Fruits and vegetables contain high amounts of known antioxidants such as polyphenols , vitamin C, Vitamin E , B-carotene and lycopene (106). Proteggente et al. and Henn et al have reported that fruits rich in flavones include orange and grapefruit and that these citrus fruits are second to fruits rich in anthocyanins. These studies are supported partially by Lichtenhaler as they found citrus juices superior against hydroxyl and peroxy radicals to have the same capacity as vitamin added juices against peroxynitrite radicals (107, 108).

Grapefruit as a source of antioxidants

Recent research, carried out by scientists at the Nutrition and Medical Research Centre at Scripps Clinic in San Diego, found that the simple act of adding grapefruit and grapefruit juice to the diet aided weight loss. The three-month study showed that adults who ate half a grapefruit with each normal meal, three times a day, lost 3.6 lbs on average

compared with people eating a similar diet without grapefruit who lost only 0.5 lbs. The researchers believe that enzymes in grapefruit affect the way the body deals with sugar and make it less likely to be laid down as fat.

Certain studies have looked at the antioxidant property of grapefruit and its advantages for lowering plasma lipid and its interaction with lipid lowering drugs. A study by Gorinstein et al found that naringin that was isolated from grapefruit had lipid lowering and plasma antioxidant capacity and grapefruit beneficially influenced plasma lipid levels and plasma antioxidant capacity (109).

Grapefruit contains compounds that may reduce atherosclerotic plaque formation and inhibit cancer cell proliferation (110). Naringin is the most abundant flavonoid in grapefruit and can account for 40-70% of the dry weight of the small fruit (111). It is one of the flavanones that is a consistent component of grapefruit and found in all varieties of grapefruit(112). The properties of naringin were studied in rats fed cholesterol, and it was found to have a plasma lipid lowering effect, a mechanism that involved an increase in the bile flow, biliary cholesterol and bile acid concentrations. In addition, a significant increase in the plasma antioxidant capacity was found in the grapefruit diet group (113). Naringin has also been reported to suppress cytotoxicity and apoptosis induced by H_2O_2 a proxidant in mouse leukemia cells (114).

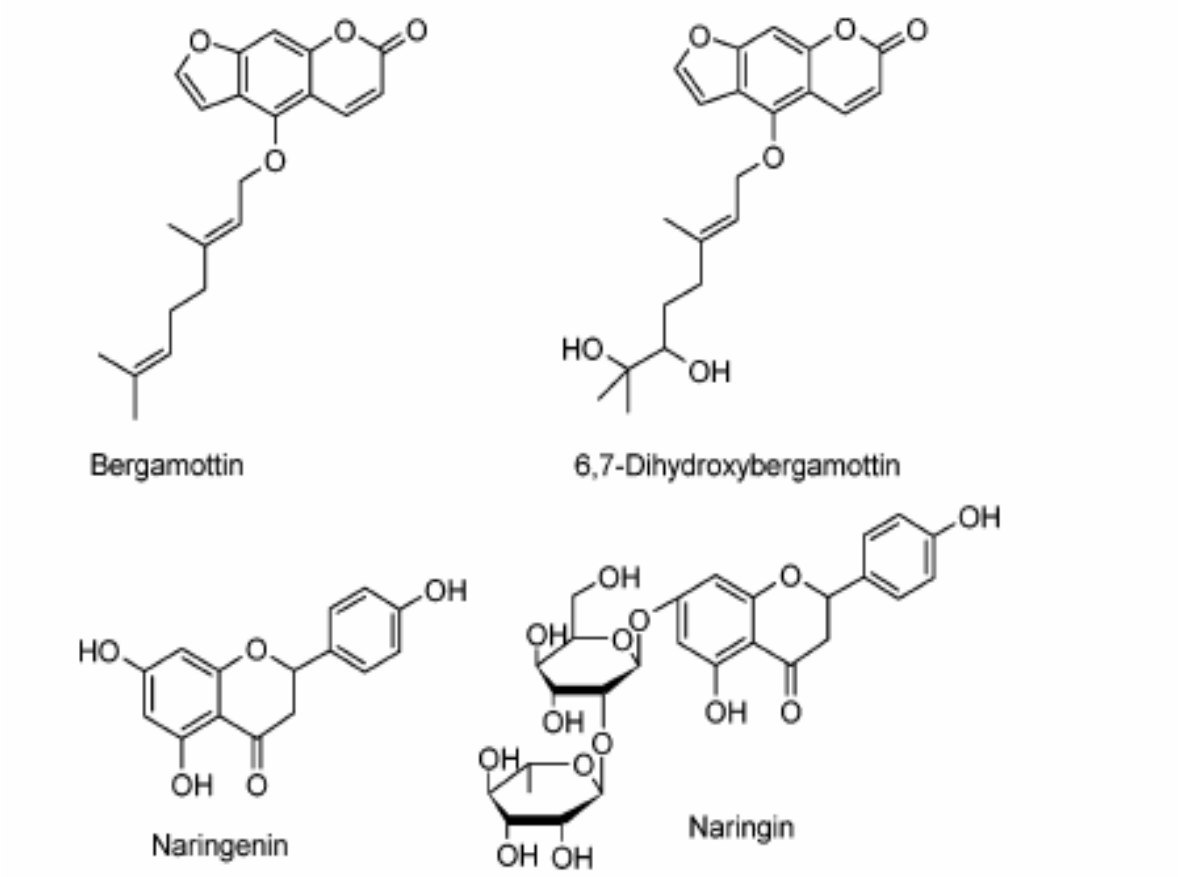

Figure 1. Structures of flavonoids and furanocoumarins present in grapefruit juice.

Zhang J, Brodbelt JS. Analyst. 2004 Dec;129(12):1227-33

Antioxidants and bone studies

Not much is known about associations between intake of antioxidants and bone health particularly in the area of the male skeleton. Two intervention trials involving vitamin supplementation have shown that vitamin C supplementation increased bone mineral density in post menopausal women (115, 116). Several studies have reported bone protective effects of soy protein and soy isoflavones, a good source of antioxidants, on the female skeleton (117-121). A recent study by Zhang et al investigated antioxidant intake and the risk of osteoporotic hip fracture and found that increased intake of vitamin

E, ß- carotene and selenium were associated with reduced risk of osteoporotic hip fracture in a dose responsive manner among those who had never smoked in a Utah population (122).

Deyhim et al evaluated the effect of citrus juice on enhancing serum antioxidant status and prevention of osteoporosis in orchidectomized rats. Their studies revealed that orchidectomy decreased total antioxidant capacity, femoral density and biochemical properties whereas orchidectomy plus orange juice and orchidectomy plus grapefruit juice reversed the orchidectomy induced antioxidant suppression and delayed time-induced femoral fracture (123)

Bone quality and structural properties

Bone quality is a set of characteristics influencing bone strength (124). These characteristics include structural properties (geometry and microarchitecture) and material properties (collagen and mineral) which are affected by bone modeling and remodeling (125).

The geometry of bone comprises the size and shape of the bone whilst the micro architecture refers to the trabecular architecture and cortical thickness of the bone (126).Bone strength also depends on the structure of collagen and the mineral content (127).

Bone structure or geometrical arrangement is genetically determined and controls the bone's ability to adjust and function accordingly with the existing loads of modeling and remodeling (128). Geometrical arrangement of a bone is one of the factors that determines the stress within a bone which is a predictor of fracture risk (129, 130).

Microarchitecture and assessment using the micro CT

Bone microarchitecture describes the cortical bone and the three dimensional network of trabeculae in the cancellous bone. Three dimensional micro CT data can be used to assess bone biomechanical properties using finite element analyses (131).

The parameters involved in microarchitecture measurements that help determine bone strength include trabecular thickness (Tb Th), trabecular number (Tb N), trabecular connectivity, degree of anisotropy (DA) as well as thickness and porosity of the cortical bone (132). Analysis using high resolution CT microstructure was more useful in identifying subjects at high risk of fracture than bone density measurements using DXA (131).

Biomechanical properties and assessment using finite element analysis

The finite element analysis model can be generated using the micro CT images that provide three dimensional geometric details and information about the material properties. The three dimensional images are developed from high resolution images obtained from a region of interest of the bone. The images from the micro CT are digitized and stacked in order to rebuild the original structure of the model in a 3D model. Compression testing is simulated on the region of interest and apparent stiffness, strains and stress for a given force are calculated from the results of the FE analyses (133).

CHAPTER III

MATERIALS AND METHODS

Animals and treatment groups

Three-month–old male Sprague-Dawley rats were maintained on a 12 hr light/dark cycle in an environmentally controlled animal laboratory and acclimated with a common control laboratory diet for 2 days. The animals were then divided into four weight-matched groups using a complete randomized design. One group of rats was sham operated (SHAM) and the other three groups were orchidectomized (ORX). All rats received a common control diet for 3 days of recuperation from the surgery. Thereafter, treatment intervention (Table 1) began for 57 days. Guidelines for the ethical care and treatment of animals from the Animal Care and Use Committee of Texas A&M University-Kingsville were strictly followed.

Table 1. Experimental groups

Surgery and Treatment group	n	Day 1-3	Day 4-60
SHAM	11	Control diet	Control diet
ORX	15	Control diet	Control diet
GF^{5}	11	Control diet	Control diet + 5% grapefruit pulp
GF^{10}	14	Control diet	Control diet + 10% grapefruit pulp

n=number of rats per treatment
ORX =orchidectomized rat
GF^{10} = ORX fed 10% grapefruit pulp
GF^{5} = ORX fed 5% grapefruit pulp

Composition of experimental diets

The rats were fed a semi-purified, powdered casein-based diet modification of the AIN-93M diet (Teklad, Madison, WI; Table 2).

Table 2. Composition of control diet

Ingredients	Control diet (g/100g diet)
Carbohydrate	
Total	70.95
Corn starch	45.0
Maltodextrin	15.5
Sucrose	10.45
Protein	
Total	14.65
Casein	14.45
Cystine	0.2
Fat	
Total	4.6
Soybean oil	4.6
Fiber	
Total	5.0
Cellulose	5.0
Vitamin premix[1]	1.0
Trace mineral[2]	1.34
Choline	0.20
Calcium carbonate	0.61
Di-calcium phosphate	0.873
Total Calories (Kcal)	3675.0
Total Protein (%)	12.6175

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

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

All rats were pair-fed to the mean food intake of the SHAM rats.Two treatment groups received either 5% or 10% grapefruit pulp from day 4-60. Before each feeding, the food remaining was weighed and the amount ingested was calculated. De-ionized water was provided *ad libitum*, and the animals were weighed at the beginning and at the end of the study which lasted for 60 days.

Surgical orchidectomy procedure

Testes were surgically removed to render the rats sex hormone deficient and to induce bone loss. Orchidectomy was performed on anesthetized animals using aseptic techniques. Sham-operated control animals were subjected to the same procedure, without removing their testes. Briefly, animals were anesthetized using a mixture of ketamine/xylazine (100/5 mg/kg body weight, respectively) and laid on their back and the skin of the scrotum was thoroughly cleaned using 70% ethanol and 0.1% betadine. A small median incision of about 1 cm was made through the skin at the tip of the scrotum. The subcutaneous connective tissue that was encountered was cleared. A 5 mm incision was made into the tip of each scrotal sac. The cauda epididymis, testes, vas deferens and spermatic blood vessels were pulled out. A single ligature was placed around the blood vessels and the vas deferens was severed distal to the ligature allowing removal of the testis and the epididymides. The remaining vas deferens and the fat were pushed back into the sac, and each muscle incision was closed with a single suture before closing the skin incision with tissue adhesive. Estimated blood loss was $\leq$0.5 ml. A heat lamp was used to keep the rats warm as they recuperated from anesthesia. Following surgery, the animals were carefully monitored until fully recovered. Thereafter, animals were cared for and monitored on a daily basis.

Necropsy

After 57 days of consuming the dietary treatments, the rats were anesthetized with a mixture of ketamine/xylazine (5 mg and 100 mg per kg body weight, respectively) and were bled from their abdominal aortas. The tibias and the fourth lumbar vertebrae were carefully removed, cleaned and stored at -20 degrees Celsius. The samples were then shipped to Oklahoma State University for analysis using the DXA and μCT.

BMD and DXA scans

All specimens after being received at Oklahoma State University were frozen at -20 ºC until scanned. The excised tibias and the 4 th lumbar vertebrae were scanned (DXA, model QDR-4500A Elite, Hologic, Waltham, MA) to assess bone mineral area (BMA), bone mineral content (BMC) and bone mineral density (BMD). The instrument was calibrated at each use with a phantom provided by the manufacturer. All DXA measurements and analyses were performed by the same investigator who was blind to the dietary/surgical treatments.

Bone microarchitecture using microcomputed tomography (μCT)

The effects of treatments on the tibias and the 4th lumbar vertebrae were evaluated using the μCT 40 scanner (Scanco Medical, Switzerland). The tibial bone was placed in a 16 mm plastic sample holder in the μCT, supported by sponge on the sides to prevent movement of the bone. The scout view scan was used to select the region of bone to scan for 3D imaging. This involved getting 300 images from each tibia and the CT images were reconstructed in 1024×1024 pixels using a medium resolution and an integration time of 150ms. The volume of interest was then selected as a region beginning 45 slices

away from the growth plate and was obtained by contouring every 10^{th} slice and morphing between each contoured slice.

The lumbar vertebrae were scanned from the caudal to the dorsal end (450 slices; 16.5µm). The VOI was selected to begin 10 slices away from the appearance of the growth plate at each end of the vertebral body. The parameters of interest obtained by analyzing the VOI were bone volume fraction or ratio of bone volume over total volume (BV/TV), trabecular number (Tb N.), trabecular separation (Tb Sp), trabecular thickness (Tb Th), connectivity density (Conn D) and structure model index (SMI) .

Finite element analyses

The information obtained from micro computed tomography was used to generate finite element models (134). The finite element analysis was performed using specialized computer software in which the microcomputed tomography (µCT) histomorphometric data was used to simulate compression of the VOI to predict the behavior of the bone in response to the compression. Analyses were performed on VOI of the tibia and the fourth lumbar vertebrae. The data on the microarchitecture of the bone was subjected to a high friction compression test in the z direction. This provided data on average strain, total force, physiological force, stiffness, size independent stiffness, and average Von Mises stresses of the trabecular bone. These data predict the strength of the bone specimen.

Statistical analyses

The data were analyzed using SAS (version 9.1, SAS Institute, Cary, NC). Significance level was set at $P \leq 0.05$. When the generalized linear model procedure

produced a significant F statistic ($p < 0.05$), Fisher's least significant difference square means procedure was used to identify treatment differences.

CHAPTER IV

Effects of feeding grapefruit pulp on bone microarchitecture and strength in orchidectomized rats

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Abstract

The aim of this study was to evaluate the effects of enhancing antioxidant status on bone microarchitecture and strength in a male rat model of osteoporosis. Three month-old male Sprague-Dawley rats were randomly assigned to four groups. Three groups were orchidectomized (ORX) and one group had sham surgery (SHAM). Three days post-surgery, ORX rats were assigned to the following treatments: ORX, ORX + 5% grapefruit pulp and ORX + 10% grapefruit pulp. The rats were fed a semi-purified, powdered casein based diet modified from AIN-93M for 2 months. At the end of the study, blood and bones were collected. The bone samples were assessed for bone density and microarchitecture using DXA and microcomputed tomography. The data were analyzed using SAS (version 9.1) and the generalized linear model (GLM) and Least Significant Differences (LSD) procedures were used to separate treatment differences ($p<0.05$). Microarchitectural changes seen in tibia showed a marked improvement in the 10% grapefruit group in certain measures whilst none were seen in the 5% group. ORX negatively impacted certain measures of bone microarchitecture that included bone volume fraction, connectivity density, trabecular number and trabecular separation of the tibial bone. In 4th lumbar trabecular assessment, a dose dependent effect was seen as ORX + 10% grapefruit pulp rats had significantly improved bone volume fraction, connectivity density, trabecular number and reduced trabecular separation. FE analysis of the tibial bone showed that a greater force was required to compress SHAM than any of the ORX groups though no significant difference was appreciated in the lumbar vertebrae.

Introduction

Although women have been the main focus of osteoporosis research, osteoporosis is a common occurrence in men as well. The mortality associated with hip fracture in the elderly is twice as high in men as women due to more comorbid conditions at any specific age and lower life expectancy in men (1). Though male osteoporosis is an understudied topic in bone research (2), reduced testosterone seems to be the principal risk factor in men that leads to increased bone loss and increased fracture incidence (3). Several cross-sectional (4) and longitudinal (5) studies have documented a decline in total and bioavailable circulating testosterone levels with aging in men. More than 60% of healthy, elderly men over 65 yr of age have free testosterone levels below the normal values of men aged 30–35 yrs (6).

Osteoporosis is considered a disease of aging, and one of the hypotheses explaining the aging process attributes aging to oxidative stress (7). Certain risk factors for osteoporosis such as smoking, hypertension and diabetes mellitus are associated with increased oxidative stress (8).

Free radicals have been shown to be involved in osteoclastogenesis and bone resorption in vitro and in rodents (9). Oxidative damage apparently increases with age and thus may overwhelm the natural repair systems in the elderly (10). Other studies have found that oxidative stress could be lowered 27-38% (11) by effective nutritional antioxidants which would possibly delay human aging and age related diseases.

The grapefruit, a tropical fruit that is rich in flavonoids has been shown to reduce the risk of several age related chronic diseases (12) by scavenging free radicals. Although grapefruit ingestion has been studied for its beneficial role in lowering plasma lipid levels

and interactions with lipid lowering drugs, knowledge of the role of grapefruit in prevention of osteoporosis is limited.

Deyhim et al evaluated the effects of citrus juice on enhancing serum antioxidant status and the prevention of osteoporosis in orchidectomized rats. Their studies revealed that orchidectomy decreased total antioxidant capacity, femoral density and biochemical properties whereas orchidectomy plus orange juice or orchidectomy plus grapefruit juice reversed orchidectomy-induced antioxidant suppression and delayed time-induced femoral fracture (12). This follow up study assessed bone mineral density, microarchitecture and biomechanical properties to determine if feeding grapefruit dose-dependently prevented bone loss.

Materials and methods

Animals and treatment groups

Three-month–old male Sprague-Dawley rats were maintained on a 12 hr light/dark cycle in an environmentally controlled animal laboratory and acclimated with a common control diet for 2 days. The animals were then divided into four weight-matched groups using a complete randomized design. One group of rats was sham operated (SHAM) and the other three groups were orchidectomized (ORX). All rats received a common control diet for 3 days of recuperation from the surgery. Thereafter, treatment intervention (Table 1) began for 57 days. Guidelines for the ethical care and treatment of animals from the Animal Care and Use Committee of Texas A&M University-Kingsville were strictly followed.

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Cellulose	5.0
Vitamin premix[1]	1.0
Trace mineral[2]	1.34
Choline	0.20
Calcium carbonate	0.61
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[1]Vitamin Mixture Composition (AIN-93; Harlan Teklad, Madison, WI).

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All rats were pair-fed to the mean food intake of the SHAM treated rats. The two treatment groups received either 5% or 10% grapefruit pulp from day 4-60. De-ionized water was provided *ad libitum*, and the animals were weighed at the beginning and at the end of the study.

Surgical orchidectomy procedure

Testes were surgically removed using aseptic techniques to render the rats sex hormone deficient and to induce bone loss. Sham-operated control animals were subjected to the same procedure, without removing their testes. Briefly, animals were anesthetized using a mixture of ketamine/xylazine (5/100 mg/kg body weight, respectively). Small incisions were made through the skin of the scrotum and the scrotal sac. The cauda epididymis, testes, vas deferens and spermatic blood vessels were isolated. A single ligature was placed around the blood vessels and the vas deferens was severed distal to the ligature allowing removal of the testis and the epididymides. The remaining vas deferens and fat were returned to the sac, and the muscle incision was closed with a single suture before closing the skin incision with tissue adhesive.

Necropsy

After 57 days of dietary treatments, the rats were anesthetized with a mixture of ketamine/xylazine (5mg and 100 mg per kg body weight, respectively) and were bled from their abdominal aortas. The tibias and the fourth lumbar vertebrae were removed, cleaned and stored at -20 ° C.

BMD and DXA scans

The excised tibias and the 4th lumbar vertebrae were scanned (DXA, model QDR-4500A Elite, Hologic, Waltham, MA) to assess bone mineral area (BMA), bone mineral content (BMC) and bone mineral density (BMD). The instrument was calibrated with a phantom provided by the manufacturer. All bones were scanned in water by the same investigator who was blind to the treatments.

Bone microarchitecture using microcomputed tomography (µCT)

All specimens were frozen at -20 ºC until scanned after being received at Oklahoma State University. The effects of treatments on the tibias and the 4th lumbar vertebrae were evaluated using the µCT 40 scanner (Scanco Medical, Switzerland). The tibia was placed in a 16 mm plastic sample holder in the µCT, supported by sponge on the sides to prevent movement of the bone. The scout view scan was used to select the region of bone to scan for 3D imaging. This involved getting 300 images at 16.5µm intervals from each tibia and the CT images were reconstructed in 1024×1024 pixels using a medium resolution. The volume of interest was then selected as a region beginning 45 slices away from the growth plate and was obtained by contouring every 10th slice and morphing between each contoured slice. The lumbar vertebrae were scanned from the caudal to the dorsal end (450 slices; 16.5µm). The VOI was selected to begin 10 slices away from the appearance of the growth plate at each end of the vertebral body. The parameters of interest obtained by analyzing the VOI were bone volume fraction or ratio of bone volume over total volume (BV/TV), trabecular number (Tb N.), trabecular separation (Tb Sp), trabecular thickness (Tb Th), connectivity density and structure model index (SMI) .

Finite element analyses

The information obtained from microcomputed tomography was used to generate finite element models (15). Analyses were performed on the VOI from the tibia and the fourth lumbar vertebrae. The data on the micro-architecture of the bone was subjected to a high friction compression test in the z direction. This provided data on average strain, total force, physiological force, stiffness, size independent stiffness, and average Von Mises stress of the trabecular bone. These data predict the strength of the bone specimen.

Statistical analyses

The data were analyzed using SAS (version 9.1, SAS Institute, Cary, NC). Significance level was set at $P \leq 0.05$. When the generalized linear model procedure indicated any significant difference among the means, the Fisher's least significant difference procedure was used to identify treatment differences.

Results

Bone mineral content and density

Bone mineral area and bone mineral content differed significantly amongst groups in the tibial bones (Table 3). The bone mineral area was highest in the ORX and SHAM groups and was significantly reduced in the group fed 10% grapefruit pulp. The bone mineral content followed a similar pattern as the BMA with both treatment groups being significantly lower than ORX. The bone mineral density tended to show similar trends ($p=0.053$) though it was not significantly affected by ORX or by grapefruit feeding. The lengths of the tibia of the different groups were not significantly different from each other ($p=0.072$). None of the treatments influenced bone mineral area, content or density in the fourth lumbar vertebrae (Table 4) significantly.

μCT analysis

Trabecular architecture of the tibia (Table 5) showed differences amongst the treatment groups. Total volume (TV) of the trabecular bone sample from the proximal tibia tended to be lower in the group fed 5% grapefruit and was significantly lower in the 10% grapefruit group compared to both ORX and SHAM. The bone volume (BV) was highest in the SHAM group and the ORX +10 % grapefruit pulp was not significantly different from SHAM although it tended to be lower and the ORX + 5% group was lower than SHAM. The ORX + 5% group showed a significant decrease in bone volume fraction compared to the SHAM and ORX +10% grapefruit pulp.

The connectivity density was significantly higher in the SHAM than in ORX, ORX+ 5% and ORX +10% treatment groups. The structural model index and trabecular

thickness were not significantly different from each other (p=0.32 and p= 0.25 respectively). Trabeculae numbers did not differ significantly amongst the ORX, ORX+ 5% and ORX + 10% treatment groups, although the ORX+ 10% treatment group was not significantly lower than the SHAM group.

Trabecular separation was highest amongst the ORX + 5% grapefruit group followed by the ORX group. However the ORX +10% grapefruit group was not significantly higher than the SHAM group.

Analysis of the microarchitectural properties of the lumbar vertebrae (Table 6) revealed that although total volume and bone volume were not significantly different amongst the groups the bone volume fraction showed significant differences. The bone volume fraction was the lowest in the ORX group and increased dose dependently with the two doses of grapefruit pulp. The ORX +10% group was not significantly lower than SHAM. Connectivity density was significantly different in the ORX, 5 and 10% groups from the SHAM group. Connectivity density in the vertebrae was significantly reduced by ORX and was not restored by either grapefruit treatments. The trabecular number followed a similar pattern as the connectivity density as it tended to increase dose-dependently and was highest in the SHAM, followed by ORX +10% and then ORX+ 5% grapefruit treatment group. Orchidectomy increased trabecular separation which was highest amongst the ORX group and lowest in the SHAM group, supporting a dose dependent pattern.

The tibial midshaft cortical bone microarchitecture (Table 7) also showed some significant results. Although bone volume fraction was not significant, both tissue volume and bone volume showed similar significant results. The grapefruit fed groups

were lower than the ORX group. Cortical thickness was increased by ORX but the 10% grapefruit pulp was not different from the ORX alone. Cortical porosity was not significantly different amongst the treatment groups. Total bone area and the cortical area were significantly lower in grapefruit treatment groups than in the ORX group. The marrow area was highest in the SHAM group and significantly lower in the groups fed grapefruit pulp.

FE analysis

Finite element models were generated using the μCT analyses and the material properties of the bone in response to compression were predicted. Both fourth lumbar vertebrae and proximal tibia were analyzed for strength parameters of physiological force, average strain, stiffness, size independent stiffness, Von Mises stresses and average cross section area.

Treatment showed significant effect on the strength of proximal tibia (Table 8). Greater force was required to compress or crush the bone of the SHAM group compared to ORX groups, indicating poor bone quality in orchidectomized rats. The groups fed grapefruit showed a significant reduction in the force required to crush the bone. Similarly, the size adjusted stiffness ($p=0.03$) required to deform the bone in the SHAM group was greater when compared to the ORX +5% and ORX+ 10% groups though did not differ from the ORX group. There were significant changes in the average cross section area as the ORX + 10% group differed from SHAM and the ORX group. The values were lower in the ORX + 10% group compared to the others. Size-independent stiffness tended to be higher in the SHAM than the treatment groups. Findings on the

biomechanical properties of lumbar vertebrae did not show any significant changes (Table 9). The physiological force required for compression of the vertebrae, stiffness and cross sectional area did not differ in any of the treatment groups. Size-independent stiffness tended to be lower in ORX and to show a dose-dependent increase with grapefruit feeding.

Discussion

The use of grapefruit for the prevention of male osteoporosis is an appealing natural alternative to taking pills everyday. In this study, freeze dried grapefruit pulp prevented some aspects of microarchitectural deterioration and loss of strength in bone. The main method of action is not known, though it might be through the presence of antioxidants that enhance bone formation and reduce bone resorption. Flavonoids are bioactive compounds found in grapefruit that improve bone health by improving antioxidant status, hence protecting bone resorption and enhancing bone formation. This is a follow up study on the work done by Deyhim et al (14) showing that lack of testosterone led to a decrease in antioxidant status, thus potentiating oxidative stress in the orchidectomized rats. In addition, they also concluded that drinking citrus juice prevented the decrease in serum antioxidant capacity in ORX rats. They also found a marked effect in decreasing bone resorption in the orange group versus the grapefruit group. Their finding correlates with some of the measures of bone structure and biomechanical properties found in this study.

Although in this study, some trends were seen to improve bone structure dose dependently, this was not a consistent finding through out the study. Orchidectomy

negatively impacted some of the measures of bone though treatment with grapefruit pulp did not always reverse bone loss. Some of the limitations of the study are the age of the rats used and also the duration of study. It would be recommended to use a much older population of rats so that bone changes are significantly noticed. In addition feeding time should last at least four months to appreciate noticeable changes in bone structure.

Conclusion

We conclude from the above study that there are certain beneficial effects of grapefruit in preventing bone loss as evident by assessing trabecular microstructural and biomechanical properties of the proximal tibia and the fourth lumbar vertebra. The effects of a lower dose of grapefruit could not be easily discerned from our study although some of the data suggests that grapefruit could be a potential candidate as a natural alternative to prevention of male osteoporosis as treatment options for males are few and often have side effects.

Further research is required to determine the effects of feeding grapefruit pulp to orchidectomized rats in an older population and for a longer time frame.

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TABLE 3

Bone mineral area (BMA), bone mineral content (BMC) and bone mineral density (BMD) determined by DXA of tibia of rats fed grapefruit pulp [1, 2]

Bone Structure Variable	SHAM N=11	ORX N=15	ORX+5% Grapefruit Pulp N=11	ORX+10% Grapefruit Pulp N=14	P Value
Length (mm)	44.85 ± 0.38	44.98 ± 0.31	44.47 ± 0.27	43.92 ± 0.28	0.072
BMA (cm^2)	1.96 ± 0.03[a]	2.02 ± 0.02[a]	1.91 ± 0.02[ab]	1.79 ± 0.08[b]	0.0114
BMC (g)	0.462 ± 0.010[ab]	0.478 ± 0.008[a]	0.437 ± 0.006[b]	0.438 ± 0.009[b]	0.0017
BMD (g/cm^2)	0.235 ± 0.002	0.236 ± 0.002	0.229 ± 0.002	0.235 ± 0.002	0.053

[1]Measurements based on DXA scans.
[2]Values are means ± SEM .
[a-c]Means ± SEM within a row not sharing a common letter differ significantly, (P <0.05).

TABLE 4

Bone mineral area (BMA), bone mineral content (BMC) and bone mineral density (BMD) determined by DXA of lumbar vertebrae of rats fed grapefruit pulp

Bone Structure Variable	SHAM	ORX	ORX+5% Grape fruit Pulp	ORX+10% Grape fruit Pulp	P value
	N=11	N=15	N=11	N=14	
BMA (cm^2)	0.80±0.01	0.80±0.02	0.76±0.01	0.79±0.02	0.1825
BMC (g)	0.195±0.004	0.189±0.004	0.178±0.004	0.187±0.006	0.1593
BMD (g/cm^2)	0.233±0.002	0.236±0.002	0.235±0.003	0.238±0.004	0.2761

Measurements based on DXA scans.
Values are means ± SEM .

TABLE 5

Trabecular bone morphology of proximal tibia from sham operated and orchidectomized rats fed diets containing 5% or 10% freeze dried grape fruit [1, 2]

Bone Structure Variable	SHAM, N=11	ORX, N=15	ORX+5% Grapefruit Pulp, N=15	ORX+10% Grape fruit Pulp, N=14	P value
Total Volume, mm^3	29.40±0.91[a]	29.86±0.67[a]	28.51±0.69[ab]	27.15±0.59[b]	0.0356
Bone Volume, mm^3	4.80± 0.36[a]	4.24±0.20[a]	3.49±0.31[b]	4.08±0.18[ab]	0.0159
Bone Volume Fraction, %	16.35± 1.2[a]	14.25±0.6[ab]	12.28±1.1[b]	15.08±0.7[a]	0.0290
Connectivity Density,1/ mm^3	52.6± 5.2[a]	38.8±2.8[b]	32.6±4.9[b]	40.7±2.4[b]	0.0083
SMI	2.23±0.09	2.35±0.04	2.41±0.09	2.29± 0.05	0.3284
Trabecular Number,1/mm	3.40±0.19[a]	2.96±0.13[b]	2.74±0.19[b]	3.04± 0.09[ab]	0.0410
Trabecular Thickness, *mm*	0.073±0.001	0.075±0.001	0.072±0.002	0.076±0.001	0.2475
Trabecular Separation, *mm*	0.29±0.02[b]	0.34±0.02[ab]	0.38±0.03[a]	0.32±0.01[b]	0.0426

[1]Measurements based on µCT analysis.
[2]Values are means ± SEM.
[a-c]Means ± SEM within a row not sharing a common letter differ significantly, (P <0.05)

TABLE 6

Effects of feeding grapefruit pulp on trabecular bone in sham operated and orchidectomized rats fed diets containing 5% or 10% freeze dried grape fruit on the lumbar vertebrae [1, 2]

Bone Structure Variable	SHAM N=11	ORX N=15	ORX+5% Grape fruit Pulp N=11	ORX+10% Grape fruit Pulp N=14	P value
Total Volume, mm^3	19.79±0.8	21.97±0.88	22.76±1.18	21.96±0.67	0.1634
Bone Volume, mm^3	4.461±0.2	3.98±0.17	4.50±0.26	4.69±0.32	0.2097
Bone Volume Fraction. %	22.7±1.1[a]	18.3±0.7[b]	19.9±0.8b[bc]	21.2±1.0[ac]	0.0096
Connectivity Density 1/ mm^3	44.2±2.8[a]	34.6±2.2[b]	35.8±1.8[b]	35.9±1.9[b]	0.0200
SMI	0.86±0.11	1.22±0.06[a]	1.08±0.07[a]	1.05±0.09[ab]	0.0399
Trabecular Number.1/mm	3.225±0.0	2.726±0.097[b]	2.841±0.097[b]	2.955±0.088[b]	0.0041
Trabecular Thickness, *mm*	0.083±0.0	0.081±0.001	0.083±0.001	0.085±0.001	0.0841
Trabecular Separation, *mm*	0.308±0.0	0.374±0.014[a]	0.353±0.014[ac]	0.336±0.012[ac]	0.0057

[1]Measurements based on µCT analysis.

[2]Values are means ± SEM .

[a-c]Means ± SEM within a row not sharing a common letter differ significantly, (P <0.05)

TABLE 7

Effects of feeding grapefruit pulp on tibia midshaft cortical bone architecture in sham operated and orchidectomized rats fed diets containing 5% or 10% freeze dried grape fruit [1, 2]

Bone Structure Variable	SHAM N=11	ORX N=15	ORX+5% Grapefruit Pulp N=11	ORX+10% Grapefruit Pulp N=14	P Value
TV [mm^3]	2.59 ± 0.04^{a}	2.60 ± 0.04^{a}	2.42 ± 0.05^{b}	2.44 ± 0.05^{b}	0.0052
BV [mm^3]	2.50 ± 0.03^{a}	2.53 ± 0.03^{a}	2.34 ± 0.04^{b}	2.37 ± 0.05^{b}	0.0043
BVTV [%]	96.55 ± 0.48	97.16 ± 0.08	96.92 ± 0.12	97.11 ± 0.06	0.2237
CortTh [mm]	0.618 ± 0.011^{b}	0.643 ± 0.008^{a}	0.615 ± 0.007^{b}	0.639 ± 0.008^{ab}	0.0501
Poros [%]	3.45 ± 0.48	2.84 ± 0.08	3.08 ± 0.12	2.89 ± 0.06	0.2237
Bone Area [mm^2]	5.24 ± 0.08^{a}	5.26 ± 0.07^{a}	4.88 ± 0.09^{b}	4.93 ± 0.11^{b}	0.0052
Cort Area [mm^2]	3.81 ± 0.07^{ab}	3.93 ± 0.05^{a}	3.62 ± 0.05^{b}	3.74 ± 0.07^{b}	0.0092
Marrow Area [mm^2]	1.43 ± 0.06^{a}	1.33 ± 0.04^{ab}	1.26 ± 0.06^{bc}	1.18 ± 0.06^{c}	0.0186

[1]Measurements based on µCT analysis.

[2]Values are means ± SEM .

[a-c]Means ± SEM within a row not sharing a common letter differ significantly, ($P < 0.05$)

TABLE 8

Effects of feeding grapefruit pulp on the tibial biomechanical properties in sham operated and orchidectomized rats fed diets containing 5% or 10% freeze dried grape fruit [1, 2]

Bone Structure Variable	SHAM N=11	ORX N=15	ORX+5% Grape fruit Pulp N=11	ORX+10% Grape fruit Pulp N=14	P value
Average strain	0.258±0.013	0.245±0.013	0.230±0.015	0.227±0.008	0.3233
Physiological force [N]	24.6±3.2[a]	20.1±1.8[ab]	15.6±1.9[b]	17.6±0.9[b]	0.0276
Stiffness [N/m × 10^3]	4964609±640752[a]	4054303±369385[ab]	3140809±391375[b]	3557430±201580[b]	0.0276
Cross sectional area	17.79±0.55[a]	18.07±0.40[a]	17.26±0.42[ab]	16.44±0.36[b]	0.0356
Independent stiffness [N/m]	458±57[a]	369 ±31[ab]	299±36[b]	359±21[ab]	0.0492
Von Mises stress [MPa]	20.66±1.59	23.04±1.28	41.86±2.27	24.16±1.17	0.1423

[1]Measurements based on FE analysis.
[2]Values are means ± SEM .
[a-c]Means ± SEM within a row not sharing a common letter differ significantly, (P <0.05)

TABLE 9

Effects of feeding grapefruit pulp on the vertebral biomechanical properties in sham operated and orchidectomized rats fed diets containing 5% or 10% freeze dried grape fruit [1, 2]

Bone Structure Variable	SHAM N=11	ORX N=15	ORX+5% N=11	ORX+10% N=14	P value
Average strain	0.156±0.019	0.110±0.012	0.140±0.016	0.139±0.017	0.2344
Physiological force [N]	4.4±0.7	3.2±0.3	4.0±0.5	4.7±0.6	0.1606
Stiffness	325035±50972	236854±35057	271378±35891	316106±41344	0.3714
Cross sectional area	4.14±0.18	4.75±0.27	4.62±0.23	4.46±0.10	0.2168
Independent stiffness [N/m]	377±54	228±24	296±42	347±47	0.0653
Von Mises stress [MPa]	16.71±1.19	19.30±1.19	17.08±1.13	17.44±1.88	0.5655

[1]Measurements based on FE analysis.
[2]Values are means ± SEM .

CHAPTER V

SUMMARY

CONCLUSION AND RECOMMENDATIONS

The study found that feeding grapefruit pulp prevented bone loss in some measures of bone microarchitecture by improving the antioxidant status in the orchidectomized rats.

Conclusion

We conclude from the above study that there is a positive effect of grapefruit in preventing bone loss as evident by assessing certain measures of trabecular microstructural and biomechanical properties of the proximal tibia. In addition the microCT was found to be more sensitive in assessing the changes due to grapefruit diet as compared to the DXA machine.

These data suggest that grapefruit could be a potential candidate as a natural alternative to prevention of male osteoporosis. Treatment options for males are few and often have side effects.

Although further research is required to determine the results of this animal studies in human subjects with a probable higher dose of grapefruit, these results are promising in grapefruits ability to reduce bone loss in an osteoporotic model.

Recommendations

Taking into consideration the findings of this study, there are a lot of research questions to be addressed about the exact mechanism of action of these antioxidants. Further research needs to look at urine and serum biochemical markers of bone turnover including deoxypyridinoline crosslinks and osteocalcin. In addition dose dependent studies need to be done with higher concentrations of grapefruit to seek if this has an impact on greater bone loss prevention.

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VITA

Meenu Pandey

Candidate for the Degree of

Master of Science

Thesis: EFFECT OF FEEDING GRAPEFRUIT PULP ON BONE MICROARCHITECTURE AND STRENGTH IN ORCHIDECTOMIZED RATS

Major Field: Nutritional Sciences

Biographical:

Personal Data: Born in Pratapgarh, India, on 15th August 1978, daughter of Girja and Savitri Pandey.

Education: Graduated from University of Zambia in 2002 with bachelor of medicine and bachelor of surgery. Completed requirements for the Master of Science degree at Oklahoma State University, July 2006. Accepted to pursue residency at OU Medical Centre with major in Family Practice July, 2006.

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Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EFFECT OF FEEDING GRAPEFRUIT PULP ON BONE MICROARCHITECTURE AND STRENGTH IN ORCHIDECTOMIZED RATS

Pages in Study: 69 Candidate for the Degree of Master of Science

Major Field: Nutrition Sciences

Scope and Method of Study: The aim of this study was to evaluate the effects of enhancing antioxidant status on bone microarchitecture and strength in a male rat model of osteoporosis. Three month old male Sprague-Dawley rats were randomly assigned to four groups. Three groups were orchidectomized (ORX) and one group had sham-surgery (SHAM). Three days post-surgery, ORX rats were assigned to the following treatments: ORX, ORX + 5% grapefruit pulp and ORX + 10% grapefruit pulp. The rats were fed a semi-purified, powdered casein-based diet, AIN-93M for 2 months. At the end of the study, blood and bones were collected. Bone density, structure and strength were assessed using the DXA, μCT and finite element analysis. The data was analyzed using SAS (version 9.1) and the generalized linear model (GLM) procedure was used to separate treatment differences ($p<0.05$).

Findings and Conclusions:

Microarchitectural changes seen in tibia showed a marked improvement in the 10% grapefruit group in certain measures whilst none were seen in the 5% group. ORX negatively impacted certain measures of bone microarchitecture that included bone volume fraction, connectivity density , trabecular number and trabecular separation of the tibial bone. In 4th lumbar trabecular assessment, a dose dependent effect was seen as ORX + 10% grapefruit pulp rats had significantly improved bone volume fraction, connectivity density ,trabecular number and reduced trabecular separation. FE analysis of the tibial bone showed that a geater force was required to compress SHAM than any of the ORX groups though no significanct difference was appreciated in the lumbar vertebrae. Grapefruit has shown to improve bone microarchitecture and further research with a longer feeding time frame and older rats is needed to assess dose dependent effects.

ADVISER'S APPROVAL: Dr. B.J. STOECKER