FEEDING ORANGE PULP IMPROVED BONE

QUALITY IN A RAT MODEL OF MALE

OSTEOPOROSIS

By

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ABBREVIATIONS

AF2: Activator Function 2

ALP: Alkaline Phosphatase

BMA: Bone Mineral Area

BMC: Bone Mineral Content

BMD: Bone Mineral Density

DHEA: Dehydroepiandrosterone

DHEA-S: Dehydroepiandrosterone Sulfate

FEA: Finite Element Analysis

GH: Growth Hormone

IL-1: Interleukin-1

IL-6: Interleukin-6

M-CSF: Macrophage-Colony Stimulating Factor

OP: Orange Pulp

OPG: Osteoprotegerin

ORX: Orchidectomy

ORX_0_OP: Orchidectomized group receiving 0% Orange Pulp

ORX_2.5_OP: Orchidectomized group receiving 2.5% Orange Pulp

ORX_5_OP: Orchidectomized group receiving 5.0% Orange Pulp

ORX_10_OP: Orchidectomized group receiving 10.0% Orange Pulp

PGE_2: Prostaglandin E_2

PTH: Parathyroid Hormone

RANK: Receptor Activator of NFκB

RANKL: Receptor Activator of NFκB Ligand

SHAM: Sham-operated

SHBG: Sex-Hormone Binding Globulin

TGF-β: Transforming Growth Factor-beta

TNF-α: Tumor Necrosis Factor-alpha

TRAF: TNF (Tumor-Necrosis Factor) Receptor Activating Factor

TRAP: Tartrate-Resistant Acid Phosphatase

μCT: Microcomputed Tomography

CHAPTER I

INTRODUCTION

According to the National Institutes of Health's Osteoporosis and Related Bone Diseases Resource Center, approximately ten million people are affected by osteoporosis each year. Twenty percent of these are men (1). Comprehension of osteoporosis among men is limited. Within a sample of elderly men, most interviewees recognized the term osteoporosis, yet only about 44% of the men were able to correctly define it (2). This condition of porous bone is distinguished by a loss of bone mass, structure, and strength. Prevention of osteoporosis focuses on increasing peak bone mass at an early age and maintaining bone mass later in life. Treatments may include lifestyle modifications, including changing dietary habits, and pharmaceutical therapy (3).

Bone is a living tissue that continually experiences transformation. Continued degradation of bone without adequate tissue repair implies a risk for fracture, the most debilitating complication of osteoporosis. In elderly or medically unstable populations, full recovery from fracture is rare. In populations who develop osteoporosis, perceptions of high stress, low self-concept, high psychological distress and other psychosocial factors influence the ability of men and women to perform activities of daily living (4). In addition to painful symptoms, osteoporosis-related fractures contribute to a lower survival rate. For men, the reduction in life expectancy due to osteoporosis outweighs the

reduction in life expectancy for women (5). Goals for treating osteoporosis and improving bone health ultimately include reducing the occurrence and severity of fractures.

Osteoporosis is diagnosed clinically using dual-energy X-ray absorptiometry (DXA) to estimate bone mineral density (BMD). In women, a T-score that is less than 2.5 standard deviations (SD) below the mean of a young adult qualifies as a diagnosis of osteoporosis. Due to the relatively few studies of male osteoporosis, diagnostic standards are less clearly defined. Treatment may be indicated if a male patient is over the age of 70, has a BMD less than 2.5 SD below the mean, or has a history of long-term corticosteroid use with a BMD lower than 1.5 SD (6). However, due to the complexity of bone, an assessment based solely on DXA measurements of BMD may be inadequate for determining bone health. Assessments of the microarchitecture, structure, strength, and material properties of bone provide a more in-depth analysis of bone quality and fracture risk but may not be feasible in humans (7).

While certain risk factors can not be modified, i.e. race, age, and family history of osteoporosis, others are quite pliable. Lifestyle factors affected by behaviors may include adequate calcium and vitamin D intakes, an appropriate body mass index (BMI), medications, and nutrition-supported hormone replacement therapy and weight-bearing exercise (8). A complete and balanced intake of macronutrients, micronutrients, and other bioactive food components provides for optimal bone health. Without an adequate diet, treatment success is limited.

The aim of this thesis is to investigate the role of orange pulp in bone health. The etiology and pathophysiology of osteoporosis, specifically male osteoporosis, will be

briefly discussed. Potential mechanisms of action for nutrients and bioactive compounds in citrus will also be briefly reviewed. Clinical implications of these food components are relevant considering the possibility for prevention of morbidity and mortality associated with male osteoporosis.

Research Objective

The objective of this study was to evaluate effects of enhancing citrus intake on bone quality in a rat model of male osteoporosis (orchidectomized one-year-old retired-breeder Sprague-Dawley rats). The specific aim of the experiment was to determine if feeding orange pulp to orchidectomized rats dose-dependently reduces bone resorption as assessed by measurements of bone density, structure, and strength in fourth lumbar vertebra and left proximal tibias.

Hypothesis

Literature suggests an interaction between antioxidants and bone remodeling. Therefore, we hypothesize that feeding orange pulp to year-old orchidectomized rats will dose-dependently improve bone structure and strength.

Limitations

There are certain limitations in using an animal model of aging male osteoporosis to study a disease seen in the human male population. For example, although rats have similar growth periods, they are not identical in relation to their respective life spans. Total blood and tissue availability for analysis is reduced due to smaller body size in rats. Physiologically, the Haversian remodeling system is minimized in rats, thus limiting ability to estimate cortical remodeling. Sudden depletion of hormones via orchidectomy may have more dramatic effects on bone compared to the gradual reduction of hormones

seen in aging. The findings of this study hence cannot be extrapolated directly to humans due to species-specific differences between rats and humans.

Format of Thesis

The PubMed database and Reference Manager software were utilized as primary search tools to locate pertinent research articles and reviews. Search terms and major themes highlighted in this review include the following: bone, biology, osteoporosis, androgens, antioxidants, flavonoids, hesperitin, naringenin, orange, DXA, orchidectomy, micro-computed tomography (µCT), and finite element (FE) analysis. Retrieved article reference lists were also reviewed to identify additional citations.

The thesis was organized based on the Oklahoma State University thesis guidelines. Chapter IV is formatted as a manuscript for submission to Journal of Nutrition using their journal guidelines.

CHAPTER II

REVIEW OF LITERATURE

Ageing Male Osteoporosis

The dynamic process of ageing often results in a decline of function. In men and women alike, age is related to an increased rate of fractures. There is an increased rate of bone resorption in elderly populations, and the processes of bone resorption and bone formation are no longer balanced (9, 10). Biomarker indices of bone formation are inconsistent among elderly individuals. Elevation in some markers (i.e. serum osteocalcin, bone-specific alkaline phosphatase, procollagen peptides) may indicate an increase in bone turnover, not necessarily bone formation (11, 12).

Multiple factors contribute to the likelihood of fractures with ageing. In addition to an average loss of muscle and tissue strength, nutritional deficiencies, hormonal changes, environmental and genetic influences exacerbate tendencies to experience fractures. Loss of ability to balance properly and lack of shock absorption qualities associated with impact surfaces contribute to falling incidents with resulting injury (13). Compared to the force a bone can withstand prior to tissue fatigue from impact, the force of expected impact must be close to or greater than one before fracture is likely. In the elderly, a ratio of 0.3 exists when merely standing. When climbing stairs, the ratio approaches 0.6. Ratios resulting from falls can range from one to over 70.

Illnesses are common in elderly individuals and often result in medication usage, fatigue, and loss of equilibrium associated with falls (14).

Osteoporosis is not a sex-specific disease. Although typically recognized as a condition affecting women, osteoporosis affects men as well. Males who experience severely low gonadal function over time are at increased risk for developing osteoporosis. Deterioration of trabecular bone mass is associated with low testosterone levels in males (15). Mortality rates in men post hip fractures are twice as high as rates in women (16, 17). In men whose fractures result in hospitalization, almost one in ten dies (8% mortality rate), while only about one in 30 women die during hospitalization due to fracture (18).

As men age, a sharp decline in bone formation compared to bone resorption occurs, although approximately five to ten years later than that of women. Men typically experience secondary osteoporosis based on conditions resulting from medical complications. In addition to hypogonadism, alcohol abuse and excessive use of glucocorticoids increase risk of fractures. Only one-third to one-half of males with osteoporosis experience primary osteoporosis (related to aging or idiopathic) (19, 20).

Men have a decreased risk of fracture compared with women. This advantage is related to a greater accumulation of bone mass, including increased periosteal bone formation and expansion, as well as a delayed onset of loss of bone mass (21). Bone development in adolescent males attains a higher peak mass than that of females. Furthermore, thicker cortices and larger bones are usually associated with the male gender. Other risk factors typically associated with the female gender include low body weight, decreased physical activity, and menopausal decrease in estrogen.

Hormones and Bone Metabolism

Growth factors, cytokines, and systemic hormones exert influence over the balance of bone resorption and formation through positive and negative feedback loops and proliferation and activation of osteoblasts and/or osteoclasts and their subsequent local mediators (22). Both androgen-receptors and estrogen-receptors are contained within bone cells (23).

Hormone levels also vary according to age. Estrogen is a primary hormone in maintaining bone mineral density (BMD) and acts as a stimulant of osteoprotegerin (OPG). Estrogen levels correlated strongly with elderly male BMD in a Framingham cohort (24). Reduction of osteoclastogenesis is enhanced by estrogen inhibition of Interleukin-1 (IL-1), Interleukin-6 (IL-6), Macrophage-Colony Stimulating Factor (M-CSF), Tumor Necrosis Factor-alpha (TNF-α), and prostaglandin E_2 (PGE_2) (25).

Estrogen is associated with higher bone mass. Insufficient estrogen results in greater secretion of cytokines and RANK ligand. Cytokines act to recruit and stimulate osteoclasts. IL-1, IL-6, TNF-α, and M-CSF are released at an increased rate from monocytes, marrow stromal cells and granulocytes resulting in increased resorptive activity when estrogen is low (26). Trabecular skeletal bone has greater surface area than cortical bone and is most affected by estrogen deficiency (27).

Androgens may also impact bone mass through promoting formation of bone, while estrogen seems to be more involved in preventing bone loss. Hormone levels and gonadal function are affected by the aging process. Testosterone levels in the serum are reduced in aging by about 1.2% per year. Furthermore, sex-hormone binding globulin (SHBG) levels rise with age (28).

Benito et al. studied testosterone replacement in hypogonadal men to determine effects on trabecular microarchitecture. Ten subjects were recruited based on below normal levels of testosterone documented on two occasions (mean 88 ± 51 ng/d [3.1 ± 1.8 nM]) resulting from known pituitary or hypothalamic disease. Eugonadal test volunteers were paired for race and age $\pm$ ten years to each hypogonadal man. Transdermal testosterone gel was self-administered daily beginning with a dose of 50 mg per day and increased to as much as 100 mg per day. Serum testosterone increased notably from subnormal levels to midnormal levels (656 ± 332 ng/dl [22.8 ± 11.5nM]) by the third month of treatment. Within hypogonadal men pre- and post- 24 month testosterone treatment, comparisons of anterior-posterior (L1-L4) and right hip DXA scans revealed significantly improved spine ($p <0.001$), total hip ($p =0.008$), trochanter ($p = 0.04$), and interchanteric BMD ($p =0.004$). μMRI assessment of the right distal tibia in these hypogonadal men revealed significantly improved architectural parameters, including bone volume fraction ($p <0.001$), trabecular thickness ($p <0.001$), surface-to-curve ratio ($p =0.004$), and topological erosion index ($p =0.004$) following the 24 month treatment period. Improved surface-to-curve ratio and topological erosion index measures indicate restoration of trabecular connectivity and reversal of deterioration of trabecular architecture due to low testosterone levels (29).

Over a period of ten years, growth hormone (GH) levels are decreased by about 14% (30). In older men and women, this decrease in GH subsequently reduces insulin-like growth factor concentrations. Adrenal androgen (dehydroepiandrosterone, DHEA; dehydroepiandrosterone sulfate, DHEA-S) levels are also known to decline with age (31).

The Orchidectomized Rat Model of Ageing Male Osteoporosis

Men are at an increased risk for osteoporosis as aging occurs. Due to complications and difficulty in maintaining homogenous experimental conditions for human volunteers, it is useful to choose an appropriate animal model to approximate biological effects of treatment. Rats have similar growth phases to humans and offer a convenient model with fewer ethical constraints versus humans and other nonprimate animal models. According to the ACE Animals Inc. website Sprague Dawley rats have a lifespan of 2.5-3.5 years (32). Thus, a one-year-old rat is middle aged and nearly beginning to enter the second half of life by the end of a four month study. This age allows time for bone analysis without complications from ageing male osteoporosis in the rats. Sprague-Dawley rats appear to be a consistent model for analyzing age-related bone loss in trabecular and cortical bone (33). Remodeling of trabecular bone is quite similar in activation, formation, and resorption mechanisms (34).

Bone Development, Skeletal Homeostasis, Bone Metabolism

The skeletal system includes bone and cartilaginous tissue. Bone is composed of protein fibers, primarily collagen type I proteins that are arranged in a lamellar structure. Glycoproteins and proteoglycans aid in attaching hydroxyapatite crystals to the collagen matrix. Functionally, bone is a specific type of connective tissue that provides protection for vital organs, supports movement, and operates as a reservoir for minerals in order to maintain homeostasis. Osteoblasts, osteoclasts, bone lining cells, and osteocytes are the four types of bone cells. Osteocytes are found inside the bone, while the other cell types are located on the bone surface. Trabecular bone is known as spongy or cancellous bone,

while cortical bone forms the outer compact bone. The extracellular matrix is composed of organic and inorganic substances (35, 36).

Bone formation involves manipulation of inorganic and organic layers and interaction between multiple biological, environmental, and genetic influences. The role of osteoblasts is to build bone; they synthesize the organic matrix of proteins and polysaccharides. Bone turnover involves the formation of bone by osteoblasts and the resorption of bone by osteoclasts. Both cell types are affected by numerous activating and inhibiting factors. Osteoblasts build bone as cells mature and become fixed and calcified within the protein matrix. As this process continues, osteoblasts become known as osteocytes or flat lining cells.

Bone Formation

Osteoblast presence and activity within the bone regulates bone formation. Osteoblasts and bone protective factors are stimulated by a variety of endogenous and environmental factors (37). Adequate nutrition, weight-bearing exercise, and controlled hormone levels are important in promoting bone health (38). Osteoblasts originate from undifferentiated mesenchymal cells from the marrow, endosteum, periosteum, and bone canals (39). Complex signaling pathways function to modulate osteoblastogenesis and subsequent bone forming capabilities (37).

The collagen matrix is further constructed outside the osteoblast following its synthesis. It is primarily composed of type I collagen, although types V, VI, VIII, and XII are also involved. Fibrils of collagen overlap in a particular manner that leaves space between fibrils and forms the porous foundation linked with intermolecular structures to provide a functional framework and strength. Bone mineralization begins at a rapid rate

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and greater than half of localized mineralization is complete within a few hours. However, final completion proceeds at a gradual rate. Apatite and calcium-phosphate crystals deposit within and between collagen fibrils. As mineralization progresses, the bone becomes more hard and dense if appropriately supported (39).

This reservoir of minerals aids the body in achieving and monitoring mineral homeostasis. Bone serves as the primary source of the body's mineral salts, comprising approximately 99% of the calcium, 85% of the phosphorus, and about half of the sodium and magnesium found in the average individual (39).

Bone Remodeling

Bone remodeling requires a coupling of osteoclast cells with osteoblast cells. Initially, osteoclasts are stimulated by osteoblast precursors. Osteoclast cells function to break down bone, while osteoblast cells form new bone. Ideally, bone formation proceeds at a rate balanced with resorption processes. Remodeling is constantly utilizing a variety of factors which function to maintain skeletal homeostasis (25).

During the growth period, the epiphyseal cartilage region of the bone elongates prior to age-related ossification. Calcification of the epiphyseal cartilage into bone results in cessation of elongating growth. Remodeling, however, continues throughout the life cycle. The external cortex of bone, also known as compact bone, surrounds the inner marrow responsible for hematopoiesis. Near the ends of the bone, epiphysis and metaphysis, internal networks of calcified matrices are formed as trabecular bone (cancellous or spongy). Trabeculae are more conducive to metabolic functions, as opposed to protection and mechanical utility provided by the cortical bone (34).

Sex steroids, i.e. estrogen and androgens, affect bone development and maintenance in both males and females (40). Estrogen reduces osteoclast action through inhibiting the production of membrane bound M-CSF, receptor activator of NFκB ligand (RANKL), IL-1, IL-6, and TNF-α and initiating synthesis of osteoprotegerin (OPG), and transforming growth factor (TGF-β) (40).

M-CSF is involved in the differentiation and proliferation of preosteoclast cells through expression of receptor activator of NFκB (RANK). The interaction of RANK and RANKL induces the differentiation and activity of osteoclasts.

Osteoclasts produce superoxide dismutase and other reactive oxygen species (ROS) which in turn speed bone demineralization and remodeling (41). Some ROS act to stimulate osteoclastogenesis. Previous research documents the strong positive relation between oxidative stress and bone loss (41). Sixty days post ovariectomy (OVX), three month old female Wistar rats were killed and their antioxidant enzyme levels from homogenized femora samples were compared to controls. Hydrogen peroxide and lipid peroxidation, as indicators of stress, were significantly elevated in OVX rats. Glutathione S- transferase, superoxide dismutase and glutathione peroxidase levels were significantly reduced in OVX, suggesting an impairment in bone antioxidant enzyme activity (42).

In sex-steroid deficient mice, antioxidant levels in bone are lowered. This reduction results in an increase in ROS-mediated reactions, bone degradation, and resorption factors. TNF-α expression is stimulated and consequently increases bone loss (43). Interleukins 1, 6, 11, 15, and 17, in addition to TNF-α, Parathyroid Hormone (PTH), and 1, 25-OH vitamin D_3, excite cytokines which alter osteoclast activity (25). Upon

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initial binding of RANKL to RANK, the TNF receptor activating factor (TRAF) family is stimulated resulting in key pairing of critical signals that serve to regulate osteoclast synthesis and action (25). Additional factors such as prostaglandins, calcitriol, lipopolysaccharide, glucocorticoids, histamine, lack of weight-bearing activity, and genetic expression are important in osteoclastogenesis and activation (37).

In contrast, other factors are critical to suppression of osteoclast formation and activation. OPG inhibits osteoclast differentiation (25). Transforming growth factor-B (TGF-B), interferon-γ, calcitonin and interleukins 4, 10, 12, 13, and 18 also inhibit osteoclasts through increasing OPG synthesis and reducing expression of RANKL (25, 44).

Hormone Deficiency and Osteoporosis

Excessive bone resorption, inadequate bone formation, and a failure to reach peak bone mass during growth and development increase the likelihood of experiencing osteoporosis. Osteoporosis in men has become a more significant threat to health as life-span has increased. Similar to a postmenopausal lack of estrogen, androgen loss has a negative effect on bone maintenance. Compared to sham-operated Fisher 344 rats 13 months of age, orchidectomized (ORX) equivalents experienced a 7% loss of bone mineral density (BMD) over 180 days (45).

Male hormone deficiency appears to act similarly to female hormone deficiency, although at a slower pace. Male Wistar rats were evaluated at six months of age, 90 days following castration. Animals were killed and left mandibles evaluated to assess differences in periodontal ligament and alveolar bone thickness compared to non-castrated controls. While periodontal ligament fibers appeared disorganized and the

alveolar bone had increased numbers of osteoclasts compared to controls, these trends were not significant. This morphometric analysis suggests that males experience bone loss at a less rapid rate, although characteristic changes associated with hormone loss were evident (46).

Androgens, such as testosterone, dihydrotestosterone, and dehydroepiandrosterone, can have a direct effect through bone cell receptors or an indirect effect through conversion to estrogens through aromatization. Osteoblast cells are particularly sensitive to androgens and respond through increasing cell proliferation, cell differentiation, protein production, and mineralization (47).

Orchidectomy contributes to low plasma levels of estradiol (estrogens) (48, 49). The terminal enzyme necessary for irreversible conversion of androgens into estrogens is the cytochrome P450 aromatase (50). Deficiency of this enzyme in case reports of four young men have consistently shown elevated bone remodeling markers with critically osteopenic skeletal phenotypes. Administration of testosterone did not restore bone health, while estrogen improved bone mass (51-54).

In elderly males, testosterone levels decline. The ratio of free plasma testosterone concentration to free plasma estradiol concentration also declines, suggesting that aromatase activity is increased in elderly males. This allows for plasma estradiol levels in males (2-3 ng/dl) to exceed those of postmenopausal women (2.01 pmol/L, (55)), partially explaining bone retention in males compared to females. Estradiol has a protective effect on bone and is vital in regulating skeletal homeostasis in males as well as females (56). Fracture status was inversely associated with total (64.1 vs 75.4 pmol/L, p=0.012) and bioavailable (43.0 vs 51.4 pmol/L, p=0.008) estradiol levels in men (57).

Nutrition and Bone Health

Dietary Guidelines for Americans, 2005, recommends a selection of balanced and varied foods, including a high intake of nutrient dense fruits and vegetables (58). Vitamins and minerals are important in maintaining optimal skeletal health. Adequate levels of intake of calcium, vitamin A, vitamin C, vitamin D, vitamin K, potassium, iron, magnesium, and molybdenum have been implicated as beneficial in some aspects of bone health. Elderly populations are at a higher risk for inadequacy of many nutrients (59).

A diet high in fruits and vegetables is associated with increased bone mineral density. Effects of onions and other vegetables have been examined by Muhlbauer et al. Bone loss in seven week old male rats labeled with [^{3}H]-Tetracycline, was evaluated following a four week administration of onions and 13 other vegetables. Varied vegetables accrued an additive prevention of bone resorption, with onions being most potent. An ovariectomized (OVX) model was also used to assess dose-dependent effects of feeding graded doses of onion to female rats for 10 days post surgery. One and a half grams of onion inhibited bone resorption at a similar level (26±4% vs. 24±3%, respectively) to that of 17β-estradiol (27µg/kg body weight) (60). Recent research indicates that positive bone effects are not due to excess base (increased pH) related to concentrations of potassium, but rather to a bioactive compound. Despite being added to a vegetarian diet, onion maintains its ability to inhibit bone resorptive activity. Furthermore, buffering of metabolic acid by adding potassium citrate failed to inhibit bone resorption. This further supports an independent mechanism (61).

Fruits also provide important nutrients for bone health. Males between the ages of 31-50 years would need an estimated 150% increase in consumption above current

fruit intakes, or an actual increase of about 1.2 cups daily, to meet recommendations of the Dietary Guidelines 2005 for fruit intake (62). As an alternative to pharmaceutical management of osteoporosis, previous studies have demonstrated the effectiveness of functional foods, including fruit, in bone health.

Arjmandi et al. reported daily consumption of 100 g dried plums was effective in increasing mean serum levels of insulin-like growth factor-I and bone-specific alkaline phosphatase activity in a sample 58 postmenopausal women over a three month period, indicating higher rates of bone formation without affecting bone resorption markers. Seventy-five grams of dried apples comparatively provided similar amount of calories, fat, carbohydrate, and fiber per day (63).

In a later study, Deyhim et al. reported dried plum's (Prunus domestica L.) efficacy in restoring bone loss in 90 day old ovariectomized (OVX) Sprague-Dawley rats over a two month period. Rats were fed standard diets for 40 days to allow for bone loss effects to be seen post surgery prior to beginning experimental diets. Experimental groups included SHAM (sham-operated), OVX_0%, OVX_17β-estradiol (10 µg/kg body weight), OVX_5% dried plum, OVX_15% dried plum, OVX_25% dried plum. Groups received standard diet plus respective treatments. In all dried plum groups, femoral and tibial bone density was restored. OVX_25% dried plum also increased lumbar bone density significantly. Interestingly, dried plum fed in varying amounts improved trabecular microarchitectural properties, including bone volume and connectivity, in comparison with OVX after bone loss occurred (64).

Another functional food component, soy, has shown both antiestrogenic and estrogenic qualities. In a study of nine month old ovariectomized (OVX) rats, 7% added

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dietary soy increased bone formation and bone density in some bones over a nine week period (65). Genistein, a main bioactive compound present in soy, increased BMD only. No other estrogen agonistic effects were seen. Results showed inhibited uterine weight and increased frequency of B lymphopoietic bone marrow cells, which are antiestrogenic effects (66).

Antioxidants and Bone

Free radical damage at the cellular level and subsequent signaling pathway activation has been indicated in the process of senescence. Reactive oxygen species (ROS), (i.e. hydrogen peroxide, superoxide anions, and hydroxyl radicals), continuously impact vital biological components. Proteins, lipids, and DNA can be damaged by ROS with serious consequences (67). In a study of 48 women and 53 men, the association between in vivo oxidative stress biomarker 8-iso-PGF and bone mineral density was examined. Urinary samples of isoprostane 8-iso-PGF indicated a negative relation to BMD. Higher levels were correlated with lower BMD of total body ($p < 0.001$) and lumbar spine ($p < 0.04$) (68).

Animal studies have shown a relation between oxidative stress and bone resorption. Free radicals are related to osteoclastogenesis. Bone resorption is affected by nuclear factor-κB (NF-κB). NF-κB is a vital transcription factor in osteoclastogenesis. Activation of NF-κB can occur during oxidative stress in a cell-specific way (67). In mice lacking NF-κB1 and NF-κB2 gene expression proteins, osteopetrosis unexpectedly occurred. This implicates the importance of NF-κB in osteoclastogenesis and differentiation (69). Altered bone resorption may be related to imbalances in oxidative activation of NF-κB. DXA analyses of 31 male patients with primary osteoporosis,

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yielded a significant negative correlation between lumbar and femoral neck BMD

evaluation and superoxide dismutase activity and nitric oxide levels (70).

Orange Pulp Components and Their Effects

Citrus antioxidant studies appear to confirm the role of an overall health-

conscious diet meeting the USDA Dietary Guidelines as important for bone health (71).

Due to benefits from multiple assorted foods, it is important to honor balance and variety

in the diet.

Orange production in the United States (US) is assessed at 8.4 million tons for

2005/06. Adjusted for weight of inconsumable waste, from 1970 to 2004 per capita

consumption of fresh oranges has decreased from an average of 7.3 pounds per year (9.1

g per day) to 4.9 pounds per year (6.1 g per day). Juice consumption remained

approximately the same from 3.2 gallons per year (32 g per day) in 1970 to 3.2 gallons

per year (31.6 g per day) (72). The majority of Florida oranges are processed for juice,

contributing to the overall 68% of total citrus crop expected to be utilized for juice this

year. Exported oranges totaled 550,000 oranges for 2005/06 (73).

Citrus fruits contain many flavonoids in addition to important nutrients such as

vitamin C and potassium. Vitamin C, or ascorbic acid, levels correlate with total

antioxidant capacity (74). Well-known antioxidant capacity of vitamin C suggests

promotion of bone formation and reduction of resorption. Ascorbic acid is important in

the prevention of scurvy and in collagen maintenance and formation. Collagen forms the

backbone matrix for bone mineralization. Ascorbic acid has been shown to induce the

formation of osteoblasts from embryonic stem cells. In vitro stimulation of pre-

osteoblast gene mouse-calvaria derived cell lines provides evidence of ascorbic acid

promotion of bone formation through promotion of osteoblast differention (75). Phenolic antioxidant compounds present in orange juice, primarily hesperidin and narirutin, spare vitamin C from oxidation, thus enhancing total antioxidant capacity (76).

DXA assessments suggested an association between dietary fruit and vitamin C intakes in adolescent boys in spine size-adjusted bone mineral content (77). Alternate studies indicate alkaline-producing dietary components such as fruits, vegetables, magnesium, and potassium appear to be associated with increased BMD. Furthermore, magnesium and potassium intakes were significantly associated with higher bone mineral density in hip and forearm assessments ($p < 0.05$) (78).

Bioactive Components of Orange Pulp: Citrus Bioflavonoids and Limonoids

Flavonoids and limonoids are primary classes of bioactive components in citrus fruits. Substances isolated from Citrus sinensis (*C. sinensis)* included nobiletin, sinensetin, tetramethylscutellarein, heptamethoxyflavone, tangeretin, 3,5,6,7,3 ,4 -hexamethoxyflavone, 5-Hydroxy-6,7,3 ,4 -tetramethoxyflavone, and 3,5,7,8,3 ,4 -hexamethoxyflavone, listed in order of declining concentrations. Hesperidin and ferulic acid were also isolated from the orange peel. Following isolation, components were evaluated for capability to inhibit 15-lipoxygenase. Radical scavenging capabilities were also assessed using diphenylpicrylhydrazyl radical. Researchers found that their ability to inhibit catalyzation of lipid peroxidation in vivo was more notable than ability to scavenge radicals (79).

Flavonoids are polyphenolic and differentiated by a C6-C3-C6 carbon backbone of the low-molecular weight parent flavone compound (2-phenyl chromone or 2 –phenyl benzopyrone). Hydrophilic character and number of hydroxyl groups are important

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factors in protecting membrane integrity through flavonoid interaction. Researchers

found hydrophilic flavonoids prevent lipid oxidation, modulate membrane fluidity, and

protect membranes from disruption by detergent. Chemically flavonoids offer a structure

useful for quenching free radicals due to the 3'-4' hydroxyl groups and the 2,3 double

bond conjugated to a 4-ozo group in the C ring and hydroxyl groups in the 3 and 5

positions. Polar characteristics of membrane head groups increase ability of hydrophilic

flavonoids to interact with membranes possibly due to hydrogen bonding. Affinity for

liposomes was correlated with number of hydroxyl groups. The flavonones naringenin

and hesperitin are more tilted in configuration compared to flavonols with less potential

for membrane surface interaction (80). The differences in flavonoids, flavonones, and

flavonols are shown in the following figure copied from Erlejman, 2004, (80):

Structural formula	Compound	3	5	6	7	2'	3'	4'	5'
Flavanones	Flavanone	H	H	H	H	H	H	H	H
	Naringenin	H	OH	H	OH	H	H	OH	H
	Eriodictyol	H	OH	H	OH	H	OH	OH	H
	Hesperetin	H	OH	H	OH	H	OH	OMe	H
	Taxifolin	OH	OH	H	OH	H	OH	OH	H
Flavanols	Catechin	OH	OH	H	OH	H	OH	OH	H
	Epicatechin	OH	OH	H	OH	H	OH	OH	H
	Epigallocatechin	OH	OH	H	OH	H	OH	OH	OH
	Epicatechin gallate	GA*	OH	H	OH	H	OH	OH	H
	Epigallocatechin gallate	GA*	OH	H	OH	H	OH	OH	OH
Flavone/ols	Flavone	H	H	H	H	H	H	H	H
	Flavonol	OH	H	H	H	H	H	H	H
	Galangin	OH	OH	H	OH	H	H	H	H
	Kaempferol	OH	OH	H	OH	H	H	OH	H
	Morin	OH	OH	H	OH	OH	H	OH	H
	Quercetin	OH	OH	H	OH	H	OH	OH	H
	Myricetin	OH	OH	H	OH	H	OH	OH	OH
Isoflavones	Daidzein		H	H	OH	H	H	OH	H
	Genistein		OH	H	OH	H	H	OH	H
	7,3',4'THIF		H	H	OH	H	OH	OH	H
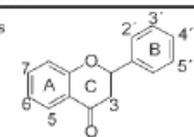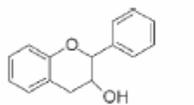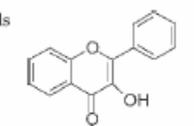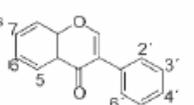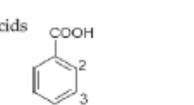
		2	3	4
Phenolic acids	Vanillic acid	H	OMe	OH
	Protocatechuic acid	H	OH	OH
Phenylpropanones	trans-Cinnamic acid	H	H	H
	p-Coumaric acid	H	H	OH
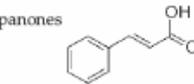
*GA: gallic group.

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Antioxidant capacity varies with structure and the existence of adjacent hydroxy groups within the structure. Based on experimentation with free-radical scavenging system 1, 1-diphenyl-2-picrylhydrazyl and the methyl linoleate oxidation system, fermentation produced hydroxyflavone forms (8-hydroxyhesperitin, 6-hydroxynaringenin, and 8-hydroxynaringenin) are more potent free-radical scavengers with greater antioxidant activity than the aglycones hesperitin and naringenin. Aglycone derivatives are, however, more successful antioxidants than flavonone glycosides hesperidin and naringin (81).

Flavonoids: Hesperidin and Naringin

Due to their phenolic properties, flavonoids interact uniquely within membranes and offer protection from the harmful effects of lipid peroxidation and free radicals (82). The citrus flavonoid hesperidin inhibits bone loss through reduction of osteoclast number and through improved mineralization of calcium, phosphorus, and zinc (83). Naringin, another citrus flavonoid, increases bone formation when bone grafts are incorporated with naringin in collagen matrix (84). Both hesperidin and naringin inhibit the activity of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, similar to the action of a statin drug (83, 84).

Endocrine effects of narigenin and hesperitin may partially account for benefits to bone health. Even in men, estrogen is important for maintaining bone and preventing resorption (57). Naringenin, in contrast to glucoside derivative naringin, was identified as a ligand for sex-hormone binding globulin (SHBG). Xenoestrogens, such as naringenin and genistein, act as ligands to increase free plasma levels of endogenous testosterone and estradiol through displacement from human plasma SHBG (85).

Hesperidin

An early clinical study in 94 women found that hesperidin aided in reducing menopausal symptoms and in the regulation of oestrogen (86). In a recent study, Chiba et al. ovariectomized (OVX) female mice to induce bone loss. Mice were fed 5 g/kg body weight hesperidin, 7 g/kg body weight α-glucosylhesperidin, or administered 0.3 μg/day 17β-estradiol. In OVX mice, proximal, middle, and distal femur regions showed significant bone loss. This loss was corrected and calcium, phosphorus and zinc concentrations were higher in all treatment groups other than OVX. In histomorphometric evaluation, trabecular bone volume fraction, thickness and separation were negatively impacted in the femoral metaphysis of OVX mice. In mice fed α-glucosylhesperidin, trabecular separation was significantly improved compared to OVX, but not restored to the level of SHAM. Estradiol treatment prevented all OVX-induced changes. Both forms of hesperidin treatments reduced osteoclast number. While estrogen-receptor (ER) effects could be possible, it seems unlikely in lieu of the methoxy group in the 4' B ring flavan nucleus of hesperidin. In order to gain estrogen potency, Chiba et al. hypothesize that a single hydroxyl group is required (83).

Estrogen elimination is inhibited by flavonones hesperitin and naringenin. In rat liver microsomes hesperitin and naringenin strongly inhibit glucuronidase, an enzyme that aids in glucuronidation and subsequent elimination of estrogen (IC_{50} value at 25 μM). Inhibition of glucuronidase reduces liver metabolism and elimination of estrogen. At 10μM and 50 μM, flavonones have competitive and noncompetitive inhibition strategies. This reduction in estrogen metabolism should translate to reduced bone resorption (87).

Fig. 1. Major citrus flavanone ions and the common daughter ion *m/z* 153, used in searching for flavanones, in the positive chemical ionization–collisionally activated dissociation tandem mass spectrometry (PCI-CAD MS/MS) analyses.
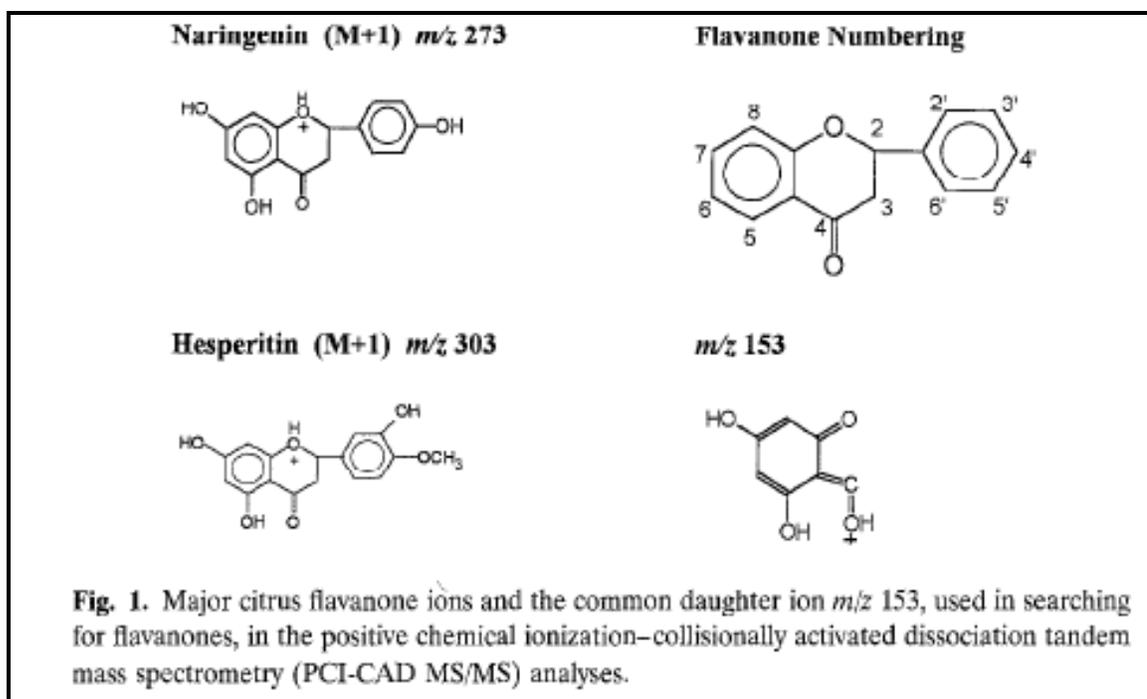
These structures of naringenin and hesperitin are adapted from Ameer, 1996, (88).

Naringin and Naringenin

As a phytoestrogen, relatively low concentrations of naringenin succeeded in displacing testosterone and estradiol from SHBG, whereas high concentrations of naringin were required. The amount of substance (mmol.1^{-1}) needed to displace 50% of estradiol from SHBG was 1.9 for naringenin versus 0.0031 for estradiol and 4.25 for genistein. The concentration (nmol.1^{-1}) needed to displace 50% of testosterone was 1.69 for naringenin versus 1.25 for genistin (85). Northern analysis of cells treated with 10 mmol naringenin revealed increased activity of the activator function 2 (AF2) of estrogen receptors. This tendency to act as an estrogen agonist was equipotent with endosulfan isomers, yet less potent than genistein and coumestrol in myometrial rat cells in increasing proliferation and progesterone receptor communication signals (89).

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Absorption

Previously, significant dietary flavonol absorption was doubted. Due to β-glycosidic bonds, it was presumed that gut absorption of bound flavonoids would be minimal. However, a study in ileostomy patients provided evidence for greater absorption of bound quercetin flavonoid versus both aglycone quercetin and pure quercetin rutinosides (90). A study conducted with a 25-year old male volunteer to determine absorption of flavonone compounds after administration found that the citrus flavonones naringin and hesperidin had low bioavailability (<25%) measured through cumulative urinary recovery. Urinary and plasma levels of narigenin and hesperitin were measured in post oral challenges. These aglycone compounds appeared to be absorbed and incorporated into red blood cells. Narirutin detected in oranges (*Citrus sinensis*) hydrolyzes to the aglycone narigenin. The primary flavonone in oranges is hesperidin, which hydrolyzes to the aglycone hesperitin (88).

Absorption of the flavonones hesperitin and narigenin allowed for a 5% urine recovery from pure compounds. Higher recovery (24%) of hesperitin from orange juice supports the idea that although gastrointestinal absorption of flavonone compound occurs, the compound medium might affect degree of absorption (88).

Not all studies find higher antioxidant capacity in flavonoid aglycone forms. An earlier study reflects this inconsistency. Comparisons of freeze-dried orange, lemon, and grapefruit peel extracts and autooxidation tests demonstrated superior antioxidant capacity of orange peel. Flavonone glycosides hesperidin and naringin appeared to account for more effective antioxidation than respective aglycones hesperitin and naringenin (91).

Despite popular research support for the antioxidant properties of polyphenolic compounds, certain polyphenols also pose a risk for damage. Hydrogen peroxide production and generalized oxidative stress is experienced by heavy polyphenol beverage drinkers, i.e. coffee, black tea, and green tea. It is important to recognize properties that allow for unexpected outcomes. Evidence suggests pro-oxidant actions may result from interactions of polyphenols and quasi-in vivo environmental conditions (92).

Research indicates that flavonoids naringin and hesperidin exert both estrogenic and antiestrogenic effects. For example, in vitro estrone sulfatase activity in human liver microsomes is inhibited by narigenin, and to a lesser extent hesperitin. This suggests an independent action by these flavonoids to reduce estradiol synthesis may aid in breast cancer prevention and offer alternative benefits. This antiestrogenic activity may seem contradictory toward bone health due to estrogen's role in maintaining bone. However, further study is warranted to delineate complex mechanisms and consequences of flavonoid interactions as phytoestrogens (93).

Estrogenic actions in flavonones hesperidin and naringenin are complex. In 11 cases of meningiomas, estrogen binding sites were examined. Hesperidin did not bind to estrogen receptors type II (94). Estrogen receptor subtype beta interacts with phytoestrogen naringenin which competes with estradiol for binding based on weakly estrogenic tendencies (95-97). Consistently contradictory results imply that naringenin exerts both antiestrogenic and estrogenic effects dependent upon various factors, such as cell context and expression of estrogen receptor alpha gene types. Agonist vs antagonist action depends greatly on cell context and the expressed gene-variant estrogen receptor.

Some natural and synthetic estrogens have variable estrogenic or antiestrogenic actions (98).

Bioavailability

Healthy non-obese adults (n=37, mean age 43 years) participated in a randomly assigned, cross-over study to determine bioavailability and plasma biomarkers for hesperitin, naringenin, and other bioactive food components. Designed from a hospital menu, a high vegetable diet with 211 g orange juice, one-half orange and one-half mandarin each day contained 132 mg hesperitin and 29 mg naringenin daily. Several portions of vegetables including cabbage, cauliflower, broccoli, red pepper, carrots, peas, broccoli, kiwi, strawberries or black currants were served daily. This treatment was compared to a low vegetable diet (hesperitin and naringenin, combined <46 mg per day) with no citrus fruits or juices added. After extraction, dietary flavonoids were quantified by high-performance liquid chromatography. Due to methodical limits of the quantification, estimated dietary naringenin concentrations were determined by multiplying hesperitin content by a factor of 0.22. Following a baseline period, two five-week treatment periods were separated by a three week wash-out interlude. Analysis of flavonoid content was performed through blood collection following an overnight fast, extraction, and hydrolysis from plasma proteins. After the wash-out period, flavonone concentrations were below 73 nmol for naringenin and 33 nmol for hesperitin. Low vegetable diet concentrations of average plasma hesperitin and naringenin (12.2 nmol and < 1 nmol) increased significantly to 325 nmol and 112.9 nmol following intake of the high vegetable diet ($p < 0.001$ and $p < 0.01$). Partially due to a relatively short half-life (1.3-2.2 hours), plasma concentrations of naringenin and hesperitin varied among

individuals. Results support the conclusion that hesperitin and naringenin are bioavailable in vivo, yet plasma biomarkers are not reliable indicators of intake (99).

Limonoids

Although less research has been completed on the antioxidant actions of limonoids, these compounds also have the capacity to quench superoxide radicals. Limonoids have been shown to stimulate cell apoptosis in cancer cells (100). Antioxidant ability is much less effective in limonoids than other citrus compounds, such as flavonoids. Limonin (Lim) and limonin 17-β-D-glucopyranoside (LG) inhibited the β-carotene-linoleate model system less than 7% compared to 44.4-51.3% by scutellarein, kaempferol, and rutin trihydrate. Free radical scavenging capability assessed by 1,1-diphenyl-2-picryl hydrazyl showed 0.5% and 2.5% respectively for Lim and LG, while formation of superoxide radicals was inhibited by 2.5-10%. Lim increased lag time of hamster low-density-lipoprotein oxidation by three-fold. Fewer hydroxyl groups are free to quench oxygen radicals in these highly oxygenated triterpenoids, hence they are weaker antioxidants (82).

Bone Microarchitecture and Micro-Computed Tomography

Audran et al. examined trabecular microarchitecture in 108 men with an osteopenic T-score equal or greater than 2.5 SDs below the mean. Correlations with fracture risk indicated a positive relation to trabecular separation and negative relation with trabecular number. Trabecular microarchitecture appears to be an important risk factor for fracture in men with osteoporosis (101). Bone quality involves more than just bone mineral density. Bone strength is affected by the morphometric and material properties of bone. Increased trabecular number and decreased separation contribute

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greatly to strength through improved structure. Stiffness of bone refers to the bone's initial reaction to a load. Stiffness relates to the bending of a bone to avoid fracture or the failure that occurs in fracture, known as buckling (7).

Bone volume fraction, expressed as a percentage, refers to the number of voxels defined as bone in the volume of interest (VOI) region analyzed. Trabecular thickness approximates trabecular thinning. The number of trabeculae that may be removed before separating the network completely refers to connectivity density. Connections are important for providing an adequate framework for cancellous mineralization. During the progression of osteoporosis, connectivity is reduced. Structural Model Index (SMI) refers to the plate-like or rod-like characteristics of bone (102). The index ranges from zero to three. An SMI of zero appears to be purely plate-like, while an SMI of three is purely rod-like (103). Microcomputed tomography and biopsy histomorphometry of 10 normal premenopausal Korean women correlated in bone volume and SMI parameters, although some other parameters are not correlated well. Caution would dictate that micro-CT be used as a complementary analysis tool to ensure accuracy in bone quality studies (104).

Bone Strength and Finite Element Analysis

Finite Element Analyses (FEA) seek to describe stress and strain components of bone (102). High-resolution images are acquired by utilizing micro-computed tomography (μCT) system evaluations to create a voxel model in the 3-D finite element analysis. FEA allows for determination of fracture failure risk based on this computer simulation. Parameters of interest include force for compression, average strain, rigidity or stiffness, cross-sectional area, size-independent stiffness, and Von Mises stress. Force

implies the amount of force necessary to completely crush the bone (105, 106). Load per

unit area and fractional change in length are known as stress and strain parameters,

respectively (107). The internal stress a bone experiences during a load is approximated

by the Von Mises stress measurement (105).

CHAPTER III

MATERIALS AND METHODS

Animals

One-year-old retired breeder Sprague-Dawley rats (n=40) served as a model of male

osteoporosis induced by orchidectomy procedures (ORX). Rats were acclimated to 12

hour light/dark cycles in an environmentally controlled laboratory and fed an established

laboratory diet (Table 1, AIN-93M, Teklad, Madison, WI) two days prior to surgery.

Similar weight animals were randomly assigned to five groups. After surgery, rats were

stabilized with the same diet for three days preceding initiation of their treatment diets for

a period of four months. Rats were pair-fed based on the mean food intake of SHAM

(surgery performed without the removal of testes) animals (design shown in appendix

Figure 1).

Surgical Procedures

Rats were orchidectomized (ORX) to induce bone loss through a loss of gonadal

function. Using aseptic procedures, animals were anesthetized using a mixture of

ketamine (100 mg/kg body weight) and xylazine (5 mg/kg body weight). While lying on

their back, the scrotal skin was carefully cleaned using 70% ethanol and 0.1% betadine

and a 1 cm incision at the tip of the scrotum was made. Following clearing of

subcutaneous connective tissue and slicing a 5 mm opening in the tip of the scrotal sac,

gonadal tissue was pulled through and removed. In addition to removal of the testes,

other structures were pulled through the incision including the caudal epididymis, caput epididymis, vas deferens, and spermatic blood vessels. Removal of the testes and epididymis was continued as a single ligature was placed around the blood vessels and the vas deferens. Remaining vas deferens and fat were replaced in the sac. Surgical wounds were closed with a single suture and tissue adhesive. Sham-operated (SHAM) rats were treated the same as the ORX groups without the removal of testes and epididymis. Animals were monitored on a daily basis.

Diet Composition

Rats were fed isonitrogenous, isocaloric, semi-purified, powdered casein-based treatment diets (modified AIN-93M, Teklad, Madison, WI, Table 1). Naval oranges (*Citrus sinensis*) were freeze-dried to obtain a sample of powdered orange pulp (OP) to add to treatment groups. Analysis of orange pulp nutrient composition was performed (Midwest Laboratories Inc., Omaha, Nebraska). Basal dietary components were manipulated as OP was added to maintain similar contents of fiber, calcium, and phosphorus. Deionized water was provided *ad libitum*.

Experimental Design

Rats were assigned to the following treatments for four months: SHAM, ORX_0_OP, ORX_2.5_OP, ORX_5_OP, and ORX_10_OP. OP is the amount of powdered orange pulp as a percentage of total diet weight. Throughout the study, the ethical care and treatment of animals guidelines from the Animal Care and Use Committee of Texas A&M University-Kingsville were followed.

Necropsy and Tissue Collection

Following the four month treatment period, rats were necropsied. A 100:5 mg/kg body weight ketamine:xylazine mix was utilized to anesthetize the rats. Blood samples were collected from the abdominal aorta. Samples were centrifuged (4°C) at 1500 x *g* for 15 minutes to separate sera for analysis (within four hours of necropsy) of alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP). Remaining sera were stored for further analyses. Fourth lumbar vertebrae and left tibias were isolated and frozen at -20°C until analyzed.

Analytical Procedures:

Serum Antioxidants, ALP, and TRAP

These analyses were conducted at Texas A&M University – Kingsville. Total antioxidant concentrations in plasma samples were quantified using a commercially available kit (Calbiochem, San Diego, CA). Total antioxidant status was determined spectrophotometrically by measuring at 600 nm the degree of inhibition of the oxidation of ABTS (2,2'-Azino-di-[3-ethylbenzthiazolinesulphonate]) to ABTS+ by metmyoglobin (a peroxidase). The amount of ABTS+ formed reflects the concentration of antioxidants (Miller NJ, 1993).

Indices of bone formation (alkaline phosphatase activity) and bone resorption (tartrate-resistant acid phosphatase activity) in plasma also were determined using commercially available kits (Thermo Electron, Louisville, CO).

Bone Analyses

Bone density and quality assessments were conducted. Parameters were assessed using dual energy x-ray absorptiometry (DXA), micro-computed tomography (μCT) and finite element analyses (FEA).

Dual Energy X-Ray Absorptiometry (DXA)

Bone mineral area, bone mineral content, and bone mineral density were evaluated via dual-energy x-ray absorptiometry using high resolution software designed for analysis of small animal bones (DXA; QDR-4500A Elite; Hologic Inc., Waltham, MA). Prior to scanning, the fourth lumbar vertebrae were placed in deionized water in standard orientation in plastic weighing boats. Tibias were scanned dry and were also placed in a consistent orientation.

Micro-Computed Tomography (μCT) Analysis of 4th Lumbar Vertebra and Left Tibia

Analysis of treatment effects on bone microarchitecture was performed using micro-computed tomography (μCT) methods. μCT imaging of left tibial metaphysis and 4th lumbar vertebra was performed with a μCT 40 scanner (Scanco Medical AG, Zurich, Switzerland). Using an integration time of 150 milliseconds per projection, scanning was completed using medium resolution with 55kVP energy, 144μA current ending in an isotropic voxel resolution of 16 microns. The operator was blinded to the treatment for the analyses. Bones were scanned in a 16 mm plastic tube from the proximal growth plate in the distal direction (16μm/slice). Approximately 400 (16μm/slice) slices resulted from scanning 4th lumbar vertebra from the caudal to the dorsal end. Total contoured slices for L-4 vertebra averaged 249 slices. Volume of interest (VOI) evaluations for tibias were based on selected regions beginning 20 slices from the growth plate at the proximal end

and continuing for 100 slices. The VOI for the midshaft evaluations of cortical bone was based on 30 slices.

Bone morphometric parameters were acquired through VOI evaluations. Parameters of interest focused on bone volume fraction (BV/TV), trabecular number (Tb. N.), trabecular separation (Tb. Sp.), trabecular thickness (Tb. Th.), connectivity density (Conn. D.), structural model index (SMI), and cortical thickness (Cort. Th.). Trabecular regions were selected to include secondary spongiosa located 330 µm and 165 µm from the growth plate for L-4 and tibia scans, respectively. Lumbar vertebra and tibia evaluations were segmented using a Gaussian filter (sigma 0.7, support 1) to remove noise and a fixed threshold level of 240 to obtain a 3-D bone structure. Tibia midshaft evaluations used a fixed threshold level of 280.

Finite Element Analysis

Following micro-architecture assessment of 4th lumbar vertebra and left tibias by microcomputed tomography, finite element analyses were performed. Parameters of interest included average strain (Av strain), von Mises stress (VM Stress), force for compression (physiological force), stiffness (Stiff), cross-sectional area (XSec Area), and size-independent stiffness (Ind Stiff).

Statistical Methods

DXA, microarchitecture, and FEA measurements were compiled in Excel and data were analyzed using the Statistical Analysis System (SAS version 9.1). The Generalized Linear Model (GLM) procedure was followed by Fisher's Least Significant Difference test for means separation. Significance level was set at $p<0.05$.

CHAPTER IV

Feeding orange pulp improved bone quality in a rat model of male osteoporosis[1]

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Word Count: 3212 words; 21838 characters (with spaces)

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1 **ABSTRACT**

2   This study evaluated the effects of feeding orange pulp (OP) on bone quality in a

3 rat model of male osteoporosis. One-year-old retired breeder rats were randomly assigned

4 to orchidectomy (ORX) [5] or sham-surgery (SHAM) treatments. Three days post-surgery,

5 ORX rats were assigned to the following treatments: ORX, ORX_2.5_OP, ORX _5_OP

6 and ORX _10_OP. Diets were isonitrogenous, isocaloric modified AIN-93M diets with

7 equal fiber content. All ORX rats were pair fed for four months to the mean food intake

8 of SHAM. At the end of the study, blood and bone samples were collected. Plasma

9 antioxidant capacity was determined spectrophotometrically. Bone density, structure, and

10 strength were assessed using dual energy X-ray absorptiometry, microcomputed

11 tomography, and finite element analyses. Least square means procedure was used to

12 separate treatment differences ($p<0.05$). Orchidectomy decreased ($p<0.05$) antioxidant

13 status while OP as low as 2.5% maintained antioxidant capacity of ORX rats to that of

14 SHAM. In 4th lumbar trabecular cores, ORX rats had significantly reduced bone volume

15 fraction, connectivity density, and trabecular number and increased trabecular separation.

16 Ten percent OP tended ($p<0.07$) to increase bone volume fraction and trabecular number,

17 as well as to decrease trabecular separation compared to ORX. ORX consistently had

18 negative impacts on bone density, structure, and strength parameters. Improvements in

19 microarchitectural properties of L-4 vertebral and tibial bones due to orange pulp are

20 evidenced by trends toward increased bone volume fraction, connectivity density and

21 decreased trabecular separation in ORX_10_OP rats compared to ORX rats. Dietary

22 orange pulp induced subtle favorable changes in bone.

23 KEY WORDS: *orange • osteoporosis • orchidectomy • microcomputed tomography*

24 *(μCT) • finite element analysis (FEA)*

INTRODUCTION

According to the National Institutes of Health's Osteoporosis and Related Bone Diseases Resource Center, approximately ten million people are affected by osteoporosis each year and twenty percent of these are men (1). This condition of porous bone is distinguished by a loss of bone mass, structure, and strength. Excessive bone resorption, inadequate bone formation, and a failure to reach peak bone mass during growth and development increase the likelihood of experiencing osteoporosis.

Osteoporosis in men has become a more significant threat to health as life-span has increased. Male hormone deficiency appears to have similar consequences as female hormone deficiency, although it often occurs later in life (2). Males who experience severely low gonadal function over time are at increased risk for developing osteoporosis. Deterioration of trabecular bone mass is associated with low testosterone levels in males (3). Similar to a postmenopausal lack of estrogen, androgen loss has a negative effect on bone maintenance. Compared to sham-operated Fisher 344 rats 13 months of age, orchidectomized (ORX) equivalents experienced a 7% loss of bone mineral density (BMD) over 180 days (4).

Mortality rates in men post hip fractures are twice as high as rates in women (5-6). In men whose fractures result in hospitalization, almost one in ten dies (8% mortality rate), while only about one in 30 women die during hospitalization for fracture (7).

Prevention of osteoporosis focuses on increasing peak bone mass at an early age and maintaining bone mass later in life. Treatments may include lifestyle modifications (including dietary habits) and pharmaceuticals (8). Vitamins and minerals are important in maintaining optimal skeletal health (9). Citrus fruits contain many flavonoids in addition to important nutrients and other food components, including Vitamin C and

49   potassium. Due to their phenolic properties, flavonoids interact uniquely within

50   membranes and offer protection from the harmful effects of lipid peroxidation and free

51   radicals (10). The citrus flavonoid hesperidin inhibits bone loss through reduction of

52   osteoclast number and through improved mineralization of calcium, phosphorus, and zinc

53   (11). Naringin, another citrus flavonoid, increases bone formation when bone grafts are

54   incorporated with naringin in collagen matrix (12). Citrus antioxidant studies appear to

55   confirm the role for bone health of an overall health-conscious diet similar to the USDA

56   Dietary Guidelines recommendations (13). The aim of this study was to examine the

57   effects on bone of orange pulp incorporated into a diet fed to orchidectomized rats.

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MATERIALS AND METHODS

Animals and diets

One-year-old retired breeder Sprague-Dawley rats (n=40) served as a model of male osteoporosis induced by orchidectomy procedures (ORX). Rats were acclimated to 12 hour light/dark cycles in an environmentally controlled laboratory and fed a semi-purified diet (Table 1, AIN-93M, Teklad, Madison, WI) for two days prior to surgery. Similar weight animals were randomly assigned to five groups. Following surgery, animals were stabilized with the same diet for three days preceding initiation of experimental diet treatments.

Surgery

Rats were orchidectomized to induce bone loss. Using aseptic procedures, animals within ORX groups were anesthetized for surgical removal of the testes and epididymis using a combination of ketamine (100 mg/kg body weight) and xylazine (5 mg/kg body weight). Sham-operated (SHAM) rats were treated the same as the ORX groups without the removal of testes and epididymis.

Experimental Design

Rats were assigned to the following treatments for four months: SHAM, ORX_0_orange pulp (OP), ORX_2.5_OP, ORX_5_OP, and ORX_10_OP. OP is the amount of powdered orange pulp as a percentage of total diet weight. Throughout the study, the ethical care and treatment of animals guidelines from the Animal Care and Use Committee of Texas A&M University-Kingsville were followed.

Diet Composition

Rats were fed the experimental diets three days post-surgery. Experimental diets were isonitrogenous, isocaloric, casein-based diets modified from the American Institute

97   of Nutrition's (AIN-93M) maintenance formulation (Table 1). Naval oranges (*Citrus*

98   *sinensis*) were freeze-dried to obtain powdered orange pulp to add to treatment groups.

99   Analysis of orange pulp nutrient composition was performed (Midwest Laboratories Inc.,

100   Omaha, Nebraska) and basal dietary components were manipulated as orange pulp was

101   added to maintain similar concentrations of fiber, calcium, and phosphorus. Deionized

102   water was provided *ad libitum*.

103   *Necropsy and tissue collection*

104   Following the four month treatment period, rats were necropsied. A 100:5 mg/kg

105   body weight ketamine:xylazine mix was utilized to anesthetize the rats. Blood samples

106   were collected from the abdominal aorta. Samples were centrifuged (4°C) at 1500 x *g* for

107   15 minutes to separate sera for analysis (within four hours of necropsy) for activities of

108   alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP). Remaining

109   sera were stored for further analyses. Fourth lumbar vertebrae and left tibias were isolated

110   and frozen at -20°C until analyzed.

111   *Analytical Procedures: Serum Antioxidants, ALP, and TRAP*

112   These analyses were conducted at Texas A&M University – Kingsville. Total

113   antioxidant concentrations in plasma samples were quantified spectrophotometrically

114   using a commercially available kit (catalogue # 615700, Calbiochem, San Diego, CA).

115   Indices of bone formation (alkaline phosphatase activity) and bone resorption (tartrate-

116   resistant acid phosphatase activity) in plasma also were determined using commercially

117   available kits (Thermo Electron, Louisville, CO).

118   *Bone Analyses: Dual Energy X-Ray Absorptiometry (DXA)*

119   Bone mineral area, bone mineral content, and bone mineral density were

120   evaluated via dual-energy x-ray absorptiometry using high resolution software designed

121   for analysis of small animal bones (DXA; QDR-4500A Elite; Hologic Inc., Waltham,

122   MA). Prior to scanning, the fourth lumbar vertebrae were placed in deionized water in

123   standard orientation in plastic weighing boats. Tibias were scanned dry and were also

124   placed in a consistent orientation.

125   *Microcomputed Tomography (µCT) Analysis of 4th Lumbar Vertebra and Left Tibia*

126   Micro-computed tomography (µCT) imaging of left tibial metaphysis and 4th lumbar

127   vertebra was performed with a µCT 40 scanner (Scanco Medical AG, Zurich,

128   Switzerland). Using an integration time of 150 milliseconds per projection, scanning was

129   completed using medium resolution with 55kVP energy, 144µA current with an isotropic

130   voxel resolution of 16 microns. The operator was blinded to the treatment for the

131   analyses. Bones were scanned in a 16 mm plastic tube from the proximal growth plate in

132   the distal direction (16µm/slice). Approximately 400 (16µm/slice) slices resulted from

133   scanning 4th lumbar vertebra from the caudal to the dorsal end. Total contoured slices for

134   L-4 vertebra averaged 250 slices, with volume of interest evaluations (VOI) based on

135   selected regions of secondary spongiosa beginning 10 slices from the growth plate at the

136   proximal end. VOI evaluations for tibias were also based on selected regions of

137   secondary spongiosa beginning 35 slices from the growth plate bridge at the proximal

138   end and continuing for 100 slices. The VOI for the midshaft evaluations of cortical bone

139   was based on 30 slices.

140   Bone morphometric parameters were acquired through VOI evaluations.

141   Parameters of interest focused on bone volume fraction (BV/TV), trabecular number (Tb.

142   N.), trabecular separation (Tb. Sp.), trabecular thickness (Tb. Th.), connectivity density

143   (Conn. D.), structural model index (SMI), and cortical thickness (Cort. Th.). Lumbar

144   vertebra and tibia evaluations were segmented using a Gaussian filter (sigma 0.7, support

145 1) to remove noise and a fixed threshold level of 240 to obtain a 3-D bone structure.

146 Tibial midshafts were analyzed using a fixed threshold level of 280.

147 *Finite Element Analysis*

148   Following micro-architecture assessment of 4th lumbar vertebra and left tibias by

149 microcomputed tomography, finite element analyses were performed. Parameters of

150 interest included average strain, Von Mises stress, force for compression, stiffness, cross-

151 sectional area, and size-independent stiffness.

152 *Statistical Methods*

153   Data were compiled in Excel and analyzed using the Statistical Analysis System

154 (SAS version 9.1, Cary, NC). The Generalized Linear Model (GLM) procedure was

155 followed by Fisher's Least Significant Difference test for means separation. Significance

156 level was set at $p<0.05$.

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169   **RESULTS**

170   *Serum Antioxidants, ALP and TRAP*

171   Antioxidant capacity was significantly decreased in ORX rats receiving the

172   standard diet only. In all ORX groups fed orange pulp levels of total antioxidant capacity

173   was restored to the level of SHAM. ALP activity was not significantly affected by

174   treatment. TRAP activity was significantly increased in the ORX_10_OP group

175   compared to the SHAM, ORX, and ORX_2.5_OP groups (Table 2).

176   *Bone Mineral Area, Content, and Density*

177   In 4th lumbar vertebrae (L-4) and left tibia DXA scans, ORX rats had

178   significantly reduced bone mineral area and content regardless of treatment (Table 3,

179   Figs. 1-4). Bone mineral density (BMD) was not significantly affected by ORX or dietary

180   orange pulp levels in either bone (Figs. 5-6).

181   *µCT Analyses*

182   Within L-4 vertebral trabecular cores, ORX significantly decreased bone volume

183   fraction, connectivity density, and trabecular number (Figs. 7-9), and increased trabecular

184   separation (Table 4, Fig. 10) (p=0.01) compared to SHAM. In ORX_10_OP rats, mean

185   measurements of the above parameters were not significantly different from SHAM. Ten

186   percent orange pulp tended (p<0.07) to improve the same parameters excluding

187   connectivity density compared to ORX.

188   In left proximal tibia trabecular cores, all ORX rats regardless of treatment had

189   significantly reduced total volume, bone volume (not shown), connectivity density and

190   trabecular number and tendencies toward increased trabecular separation (p=0.06)

191   (Figs.11-14). ORX_10_OP showed tendencies to improve bone volume fraction

192   compared to other ORX groups (Fig. 15). Decreases in total area at midshaft and cortical

193   bone area at midshaft in all ORX groups were significant (Figs. 16-17). Tibial midshaft

194   bone area was reduced in ORX_0_OP, ORX_2.5_OP, and ORX_5_OP groups compared

195   to SHAM ($p= 0.006$, Table 5). ORX_10_OP restored bone area to be statistically

196   equivalent to the size of SHAM and tended to increase bone area compared to

197   ORX_0_OP ($p=0.08$).

198   *FE Analyses*

199   Strength assessments in 4th lumbar vertebrae and left proximal tibias, yielded

200   some significantly reduced parameters in ORX compared to SHAM (Tables 6, 7). L-4

201   ORX_0_OP showed more than 50% decrease in average strain (not shown), force for

202   compression (Figs. 18-19), stiffness (not shown), and size-independent stiffness (Figs.

203   20-21) measurements compared to SHAM. Tibial ORX_0_OP showed decreases in

204   average strain (19%, not shown), force for compression (43%, Figs. 18-19), stiffness

205   (43%, not shown), cross-sectional area (10%, Fig. 22), size-independent stiffness (36%,

206   Figs. 20-21), and Von Mises Stress (29%) measurements compared to SHAM ($p<0.04$).

207   No significant differences in tibial FE analyses were seen between ORX and all

208   ORX groups fed orange pulp with the exception of cross-sectional area which decreased

209   with OP. However, the 10% dietary orange group tended to show improvements in many

210   vertebral bone parameters including force for compression, average strain, and size-

211   independent stiffness compared to ORX. Tibial bones in the 10% orange pulp group

212   tended to show increases for size-independent stiffness compared to ORX, and the mean

213   was similar to SHAM, possibly due to a reduced cross-sectional area in all ORX_OP

214   groups.

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217   **DISCUSSION**

218   This study assessed bone quality in orchidectomized male rats fed orange pulp for

219   four months. The improvements in some measures of bone structure suggest potential

220   benefits for orange in improving bone health. One mechanism by which orange exerts

221   beneficial effect is through relief of oxidative stress. Antioxidant status of ORX rats was

222   restored to levels of the SHAM ($p<0.05$) by feeding orange pulp, even at the lowest

223   concentration.

224   Orchidectomy results in a loss of gonadal function in males. Literature suggests

225   this loss of function negatively impacts bone health and increases risk of fracture (18).

226   μCT analysis showed a significant decline in bone structure in orchidectomized rats. As a

227   model for inducing bone loss in males, orchidectomy successfully reduced measures of

228   bone density, structure, and strength. This confirms efficacy as an animal model for

229   studying male osteoporosis.

230   Feeding orange pulp to orchidectomized rats improved some bone characteristics;

231   however, small sample size or weight variations limit sensitivity of results. In DXA

232   analyses, significant changes were seen in bone mineral area and bone mineral content.

233   While trends showed a positive effect on bone mineral density, means were not

234   significantly different. Results support a possible threshold effect in that ten percent

235   dietary orange pulp consistently tended to improve μCT measures of bone parameters

236   compared to ORX animals fed the standard diet. Finite element analyses of fourth lumbar

237   vertebra suggested possible improvements in bone parameters in ORX_10_OP animals

238   compared to ORX_0_OP animals but these were not significant differences.

239   In the present study, despite trends to improve some characteristics of bone

240   structure, effects of feeding orange pulp to orchidectomized rats were not statistically

241 significant. Components of citrus likely to produce the subtle beneficial effect observed

242 include flavonoids, (specifically hesperidin and narigenin), limonoids, and vitamin C.

243   Flavonoids (i.e. hesperitin, naringenin), limonoids, vitamin C, and potassium are

244 bioactive components within oranges that may improve bone health through increasing

245 antioxidant status, promoting collagen formation, and maintaining an alkaline

246 environment (9, 11-12, 15-16). Benefits likely related to antioxidants include bone

247 resorption protection and bone formation mechanism activation (10, 13, 17). Flavonoids

248 act to spare vitamin C from oxidation, in addition to independent antioxidant capabilities

249 (18). Through direct and indirect antioxidant action, components may reduce oxidative

250 stress in vivo and alleviate stimulation of bone resorption (19). However, in the present

251 study, high levels of TRAP observed in all ORX groups suggest feeding orange pulp did

252 not inhibit TRAP activity likely due to the rigorous negative effects of orchidectomy on

253 bone.

254   Endocrine effects of narigenin and hesperitin may partially account for benefits to

255 bone health. Even in men, estrogen is important for maintaining bone and preventing

256 resorption (20). Estrogenic and antiestrogenic actions by hesperidin and naringin are

257 complex. As phytoestrogens glycone flavonoids, hesperitin and naringenin, are important

258 in preventing bone loss.

259   Flavonones contribute to increased plasma levels of free estradiol. Xenoestrogens,

260 such as naringenin and genistein, act as ligands to increase free plasma levels of

261 endogenous testosterone and estradiol through displacement from human plasma sex-

262 hormone binding globulin (21). Furthermore, estrogen elimination is inhibited by

263 flavonones hesperitin and naringenin. Inhibition of glucuronidase reduces liver

264 metabolism and elimination of estrogen. This reduction in estrogen metabolism should

46

265   translate to reduced bone resorption (22). Hesperidin has been shown to inhibit bone loss

266   in ovariectomized mice through reduction in osteoclast number (11).

267   Increasing dietary consumption of functional foods, including oranges, provides

268   an inexpensive strategy for maintaining bone health. Literature suggests bioactive

269   components benefit bone structure through antioxidant and estrogenic actions. Although

270   improvements are subtle, this study supports the USDA recommendation to consume a

271   wide variety of fruits and vegetables daily (13).

272   *Conclusion*

273   Data showed that orchidectomy significantly reduced parameters of bone density,

274   structure, and strength in year-old rats over a four-month period. ORX_10_OP tended to

275   positively affect some parameters of bone density, structure, and strength. Effects of

276   lower dietary intakes of orange pulp are not easily discerned from our results.

277   Further study is warranted to elucidate effects of feeding orange pulp to orchidectomized

278   Sprague-Dawley rats.

[5]Abbreviations used: OP, orange pulp; ORX, orchidectomy; ORX_0_OP, ORX + 0% OP; ORX_2.5_OP, ORX + 2.5% OP; ORX_5_OP, ORX + 5.0% OP; ORX_10_OP, ORX + 10.0% OP; SHAM, sham-operated; BMC, bone mineral content; BMA, bone mineral area; BMD, bone mineral density; μCT, microcomputed tomography; FEA, finite element analysis; ALP, alkaline phosphatase; TRAP, tartrate-resistant acid phosphatase.

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TABLE 1

Composition of Diets [1,2,3]

Ingredients (g/100g diet)	Control diet	Orange pulp (%)		
		2.5	5	10
Corn starch	45.5	43.47	41.5	37.40
Maltodextrin	15.5	15.5	15.5	15.5
Sucrose	10.73	10.73	10.73	10.73
Orange pulp[1]	-----	2.5	5.0	10.0
Casein	14.45	14.23	14.02	13.58
Cystine	0.2	0.2	0.2	0.2
Soybean oil	4.6	4.5	4.31	4.08
Fiber	5.0	4.89	4.79	4.58
Vitamin premix[2]	1.0	1.0	1.0	1.0
Trace mineral[3]	1.34	1.34	1.34	1.34
Choline chloride	0.20	0.20	0.20	0.20
Calcium carbonate	0.61	0.60	0.59	0.57
Dicalcium phosphate	0.873	0.86	0.85	0.82
Total calorie/Kg diet	3,675	3,675	3,675	3,677
Protein (%)	12.62	12.62	12.62	12.62

[1]Orange Pulp Composition (total calorie per gram 3,854; crude protein 7.62%; crude fat 1.61%; acid detergent fiber 4.23%; phosphorous 0.18%; magnesium 0.09%; calcium 0.31%; iron 11 ppm; manganese 2 ppm; copper 4 ppm; zinc 5 ppm

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

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

TABLE 2

Plasma Antioxidants and Indices of Bone Remodeling [1,2,3]

Parameters:		ORX_Orange Pulp (%)			
	SHAM	0%	2.5%	5.0%	10.0%
Antioxidant capacity (nM)	1.23 ± 0.03[a]	1.09 ± 0.03[b]	1.25 ± 0.03[a]	1.25 ± 0.03[a]	1.26 ± 0.03[a]
ALP activity (U/L)	32 ± 6.0	43 ± 6.0	34 ± 8.0	31 ± 7.0	41 ± 6.0
TRAP activity (U/L)	4.7 ± 0.62[b]	6.3 ± 0.82[b]	5.9 ± 0.73[b]	7.1 ± 0.94[ab]	8.4 ± 0.62[a]

[1]Measurements based on serum analysis by collaborators at Texas A&M University-Kingsville.
[2]Means $\pm$ SEM (n = 9).
[3]Values within a row not sharing a common letter differ significantly, ($p < 0.05$).

TABLE 3

Effects of orchidectomy and feeding orange pulp on DXA measurements of L4 and tibial bone mineral area, content and density in year-old Sprague-Dawley rats [1,2]

Parameters:	SHAM	ORX_Orange Pulp (%)				p-values
		0%	2.5%	5.0%	10.0%	
L4 Vertebra (n=39)						
Bone Mineral Area, cm^2	0.828 ± 0.033 [a]	0.725 ± 0.013 [b]	0.703 ± 0.013 [b]	0.705 ± 0.019 [b]	0.721 ± 0.026 [b]	0.0012
Bone Mineral Content, g	0.230 ± 0.008 [a]	0.194 ± 0.006 [b]	0.190 ± 0.007 [b]	0.191 ± 0.008 [b]	0.205 ± 0.009 [b]	0.0048
Bone Mineral Density, g/cm^2	0.278 ± 0.005	0.268 ± 0.008	0.269 ± 0.006	0.271 ± 0.006	0.284 ± 0.005	0.3377
Left Tibia (n=42)						
Bone Mineral Area, cm^2	2.30 ± 0.05 [a]	2.06 ± 0.03 [b]	2.01 ± 0.05 [b]	2.05 ± 0.05 [b]	2.06 ± 0.05 [b]	0.0005
Bone Mineral Content, g	0.600 ± 0.019 [a]	0.511 ± 0.014 [b]	0.498 ± 0.017 [b]	0.520 ± 0.019 [b]	0.528 ± 0.019 [b]	0.0024
Bone Mineral Density, *g/cm*^{2}	0.259 ± 0.005	0.247 ± 0.004	0.247 ± 0.004	0.253 ± 0.004	0.256 ± 0.004	0.1793

[1]Means ± SEM (n = 7-9).
[2]Values within a row not sharing a common letter differ significantly, ($p < 0.05$).

TABLE 4

Effects of orchidectomy and feeding orange pulp on μCT measurements of L4 vertebral bone volume fraction, connectivity density, trabecular number, and trabecular separation in year-old Sprague-Dawley rats [1,2]

Parameters:	SHAM	ORX_Orange Pulp (%)				*p*-values
		0%	2.5%	5.0%	10.0%	
L4 Vertebra Microarchitecture						
Total Volume, *mm³*	23.37 ± 1.75	22.56 ± 1.35	23.75 ± 0.54	22.50 ± 1.95	19.43 ± 1.46	0.3241
Bone Volume, *mm³*	5.40 ± 0.44	4.15 ± 0.36	4.37 ± 0.34	4.33 ± 0.50	4.24 ± 0.36	0.1963
Bone Volume/Total Volume, *%*	23.3 ± 1.5 [a]	18.5 ± 1.3 [bc]	18.3 ± 1.1 [c]	19.1 ± 1.0 [bc]	21.8 ± 0.7 [ab]	0.0135
Connectivity Density, *1/mm³*	40.2 ± 3.8 [a]	27.4 ± 2.8 [b]	28.0 ± 4.2 [b]	27.0 ± 2.2 [b]	32.9 ± 2.7 [ab]	0.0311
Structural Model Index	0.80 ± 0.13	1.11 ± 0.10	0.96 ± 0.08	1.03 ± 0.11	0.93 ± 0.06	0.2595
Trabecular Number, *1/mm*	3.170 ± 0.164 [a]	2.512 ± 0.153 [bc]	2.423 ± 0.206 [c]	2.587 ± 0.078 [bc]	2.925 ± 0.085 [ab]	0.0049
Trabecular Thickness, *mm*	0.086 ± 0.003	0.086 ± 0.002	0.085 ± 0.002	0.087 ± 0.002	0.087 ± 0.002	0.9774
Trabecular Separation, *mm*	0.311 ± 0.016 [c]	0.411 ± 0.029 [ab]	0.441 ± 0.043 [a]	0.385 ± 0.014 [abc]	0.337 ± 0.011 [bc]	0.0073

[1] Means ± SEM, (n = 7-8).
[2] Values within a row not sharing a common letter differ significantly, (*p* <0.05).

TABLE 5

Effects of orchidectomy and feeding orange pulp on μCT measurements of tibial bone volume, connectivity density, total area at midshaft, bone area, marrow area, and tibial length in year-old Sprague-Dawley rats [1,2]

| Parameters: | SHAM | ORX_OrangePulp (%) | | | | *p*-values |
		0%	2.5%	5.0%	10.0%	
Proximal Tibial Microarchitecture						<0.0001
Total Volume, *mm³*	34.43 ± 1.12 [a]	30.94 ± 1.12 [b]	27.65 ± 0.78 [c]	27.91 ± 1.06 [c]	25.14 ± 0.66 [c]	
Bone Volume, *mm³*	5.61 ± 0.54 [a]	4.01 ± 0.26 [b]	3.62 ± 0.38 [b]	3.66 ± 0.26 [b]	3.79 ± 0.27 [b]	0.0015
Bone Volume/Total Volume, *%*	16.2 ± 1.2	13.0 ± 0.8	13.1 ± 1.3	13.0 ± 0.5	15.1 ± 1.0	0.0916
Connectivity Density, *1/mm³*	32.2 ± 3.0 [a]	22.0 ± 2.9 [b]	20.2 ± 2.8 [b]	19.8 ± 1.6 [b]	23.2 ± 2.2 [b]	0.0098
Structural Model Index	2.01 ± 0.08	2.20 ± 0.06	2.11 ± 0.13	2.21 ± 0.04	2.07 ± 0.10	0.4017
Trabecular Number, *1/mm*	2.891 ± 0.169 [a]	2.34 ± 0.15 [b]	2.23 ± 0.13 [b]	2.20 ± 0.09 [b]	2.44 ± 0.14 [b]	0.0091
Trabecular Thickness, *mm*	0.085 ± 0.005	0.087 ± 0.002	0.087 ± 0.003	0.091 ± 0.003	0.090 ± 0.003	0.6271
Trabecular Separation, *mm*	0.344 ± 0.028	0.439 ± 0.036	0.458 ± 0.030	0.450 ± 0.022	0.406 ± 0.027	0.0594
Tibial Midshaft Microarchitecture						
Cortical Thickness, *mm*	0.669 ± 0.015	0.628 ± 0.009	0.645 ± 0.015	0.642 ± 0.011	0.669 ± 0.013	0.1144
Total Area at Midshaft, *mm²*	6.762 ± 0.146 [a]	5.843 ± 0.136 [b]	5.783 ± 0.204 [b]	5.873 ± 0.159 [b]	6.105 ± 0.166 [b]	0.0008
Bone Area, *mm*	4.755 ± 0.100 [a]	4.145 ± 0.098 [b]	4.196 ± 0.161 [b]	4.221 ± 0.112 [b]	4.452 ± 0.130 [ab]	0.0059
Marrow Area, *mm*	2.007 ± 0.112 [a]	1.698 ± 0.047 [b]	1.587 ± 0.056 [b]	1.652 ± 0.066 [b]	1.652 ± 0.055 [b]	0.0016
Tibia Length	46.95 ± 0.38	45.76 ± 0.35	45.61 ± 0.53	45.81 ± 0.36	45.73 ± 0.46	0.1793

[1]Means ± SEM, (n = 8-9).
[2]Values within a row not sharing a common letter differ significantly, (*p* <0.05).

TABLE 6

Effects of orchidectomy and feeding orange pulp on finite element analyses of L-4 trabecular bone strain, physiological force, stiffness, cross-sectional area, size-independent stiffness, and von Mises stress in Sprague-Dawley rats [1,2]

| Parameters: | SHAM | ORX_Orange Pulp (%) | | | | p-values |
		0%	2.5%	5.0%	10.0%	
Average Strain	0.2001 ± 0.0181[a]	0.0856 ± 0.0162[b]	0.1215 ± 0.0244[b]	0.0946 ± 0.0137[b]	0.1315 ± 0.0212[b]	0.0012
Force for Compression	6.546 ± 0.810[a]	2.109 ± 0.407[b]	3.251 ± 0.737[b]	2.472 ± 0.520[b]	3.431 ± 0.635[b]	0.0002
Stiffness	428403 ± 57621[a]	138139 ± 27735[b]	220272 ± 54012[b]	158779 ± 33085[b]	222563 ± 42560[b]	0.0004
Cross-Sectional Area	4.549 ± 0.368	4.331 ± 0.178	4.607 ± 0.161	4.314 ± 0.347	3.741 ± 0.230	0.2315
Size-Independent Stiffness	492.1 ± 69.6[a]	170.5 ± 36.6[b]	233.0 ± 51.2[b]	191.4 ± 36.4[b]	300.6 ± 51.6[b]	0.0004
Von Mises Stress	32.30 ± 4.33	52.70 ± 5.95	48.20 ± 6.27	49.34 ± 5.56	47.03 ± 6.77	0.1317

[1]Means ± SEM, (n = 7-8).
[2]Values within a row not sharing a common letter differ significantly, (p <0.05).

TABLE 7

Effects of orchidectomy and feeding orange pulp on finite element analyses of tibial trabecular bone strain, physiological force, stiffness, cross-sectional area, size-independent stiffness, and von Mises stress in Sprague-Dawley rats [1,2]

| Parameters: | SHAM | ORX_Orange Pulp (%) | | | | p-values |
		0%	2.5%	5.0%	10.0%	
Average Strain	0.3059 ± 0.0156 [a]	0.2481 ± 0.0120 [b]	0.2417 ± 0.0215 [b]	0.2189 ± 0.0177 [b]	0.2472 ± 0.0197 [b]	0.0191
Force for Compression	32.917 ± 5.008 [a]	18.772 ± 1.873 [b]	17.098 ± 3.411 [b]	15.320 ± 2.341 [b]	17.748 ± 2.359 [b]	0.0023
Stiffness	657544 ± 100034 [a]	374983 ± 37408 [b]	339476 ± 65472 [b]	307568 ± 47552 [b]	354531 ± 47121 [b]	0.0022
Cross-Sectional Area	57.461 ± 1.874 [a]	51.623 ± 1.869 [b]	46.166 ± 1.424 [c]	46.409 ± 1.723 [c]	41.944 ± 1.095 [c]	<0.0001
Size-Independent Stiffness	188.7 ± 24.5 [a]	121.0 ± 11.3 [b]	121.7 ± 21.9 [b]	107.9 ± 12.9 [b]	141.2 ± 18.6 [ab]	0.0292
Von Mises Stress	4.60 ± 0.36 [b]	6.51 ± 0.52 [a]	7.47 ± 0.73 [a]	7.46 ± 0.56 [a]	7.18 ± 0.87 [a]	0.0138

[1]Means ± SEM, (n = 8-9).
[2]Values within a row not sharing a common letter differ significantly, (p <0.05).

Effects of orchidectomy and feeding orange pulp on DXA measurements of L4 and tibial bone mineral area in year-old Sprague-Dawley rats [1,2]


L-4 Vertebra: Bone Mineral Area

Left Tibia: Bone Mineral Area
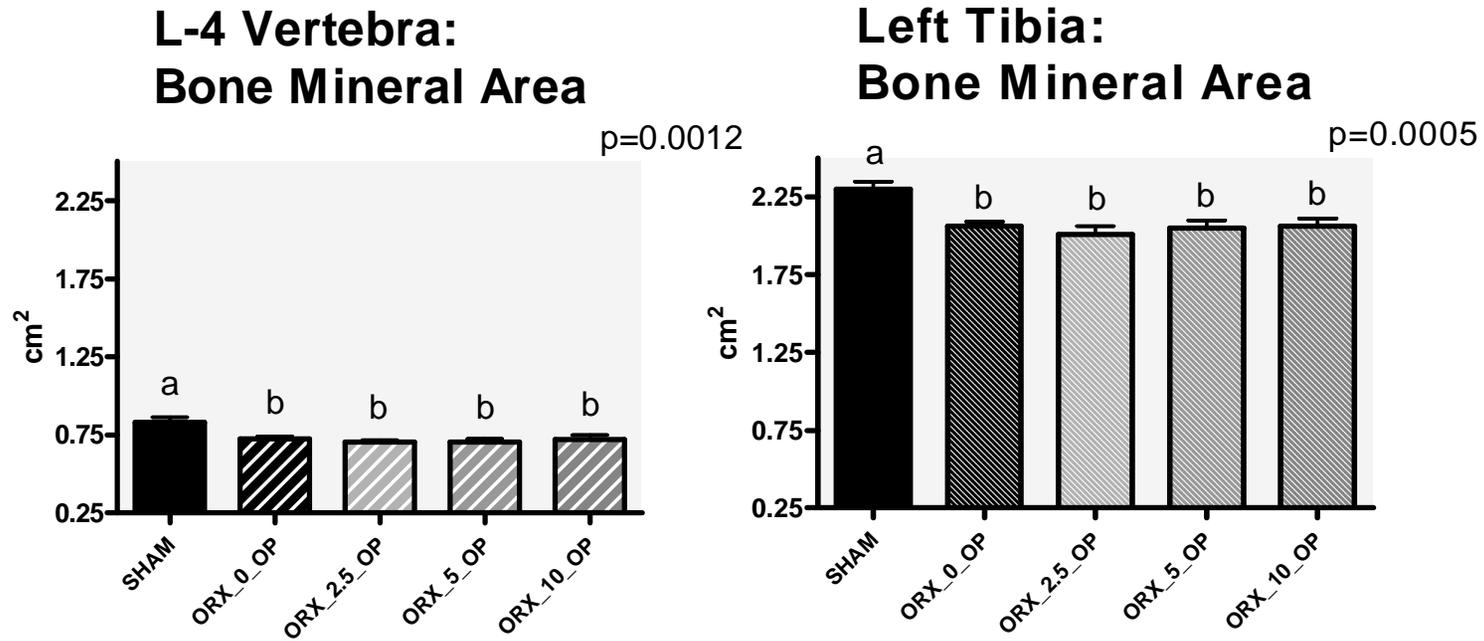
[1]Means ± SEM (n = 7-9).
[2]Graph bars not sharing a common letter differ significantly.

FIGURES 3-4.

Effects of orchidectomy and feeding orange pulp on DXA measurements of L-4 and tibial bone mineral content in year-old Sprague-Dawley rats [1,2]
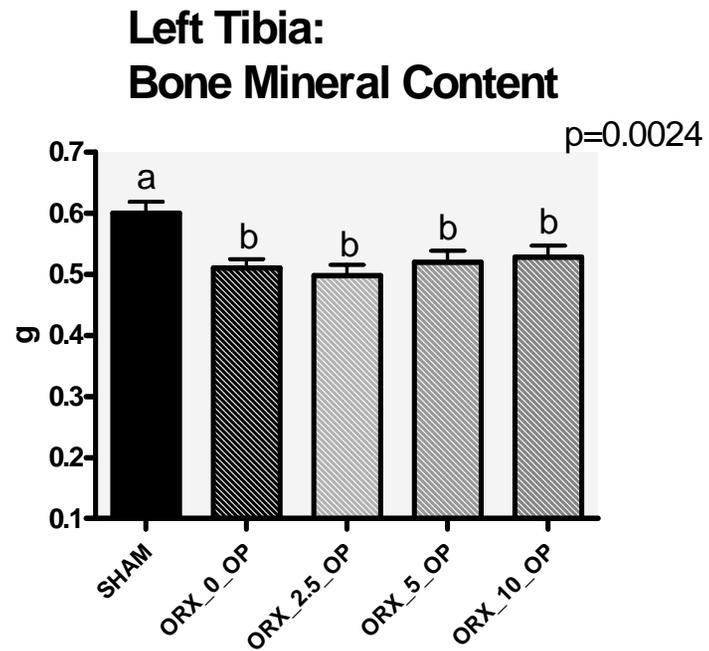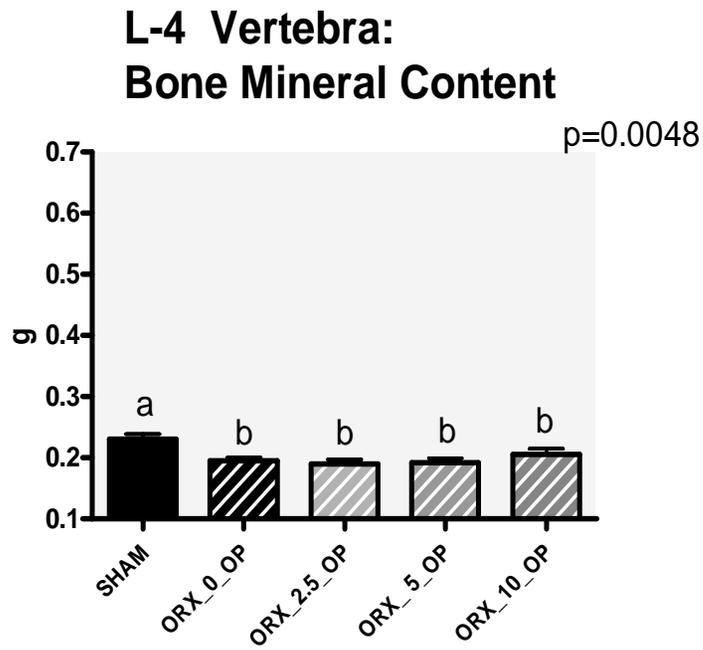

[1]Means ± SEM (n = 7-9).
[2]Graph bars not sharing a common letter differ significantly.

FIGURES 5-6.

Effects of orchidectomy and feeding orange pulp on DXA measurements of L4 and tibial bone mineral density in year-old Sprague-Dawley rats [1,2]
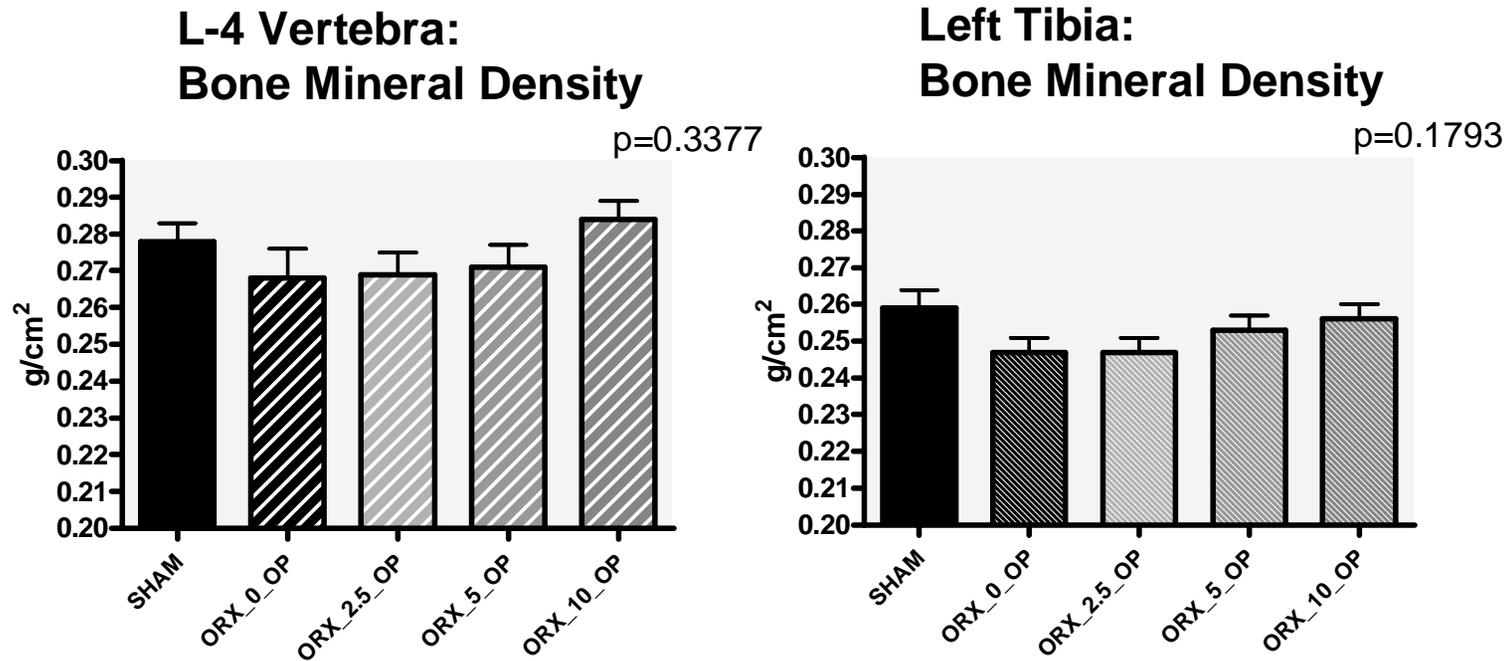
[1]Means ± SEM (n = 7-9).

[2]Graph bars not sharing a common letter differ significantly.

FIGURE 7.

Effects of orchidectomy and feeding orange pulp on μCT measurements of L4 vertebral bone volume fraction in year-old Sprague-Dawley rats [1,2]
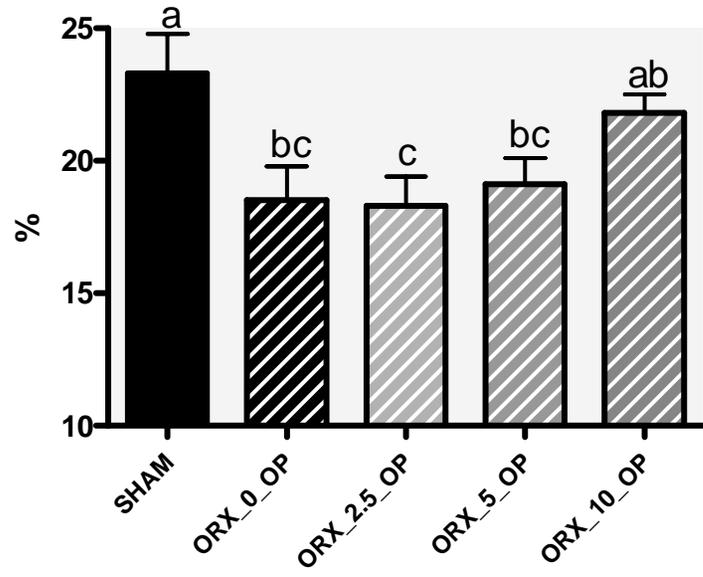
[1]Means $\pm$ SEM (n = 7-8).
[2]Graph bars not sharing a common letter differ significantly, (p = 0.0135).

FIGURE 8.

Effects of orchidectomy and feeding orange pulp on μCT measurements of L4 vertebral connectivity density in year-old Sprague-Dawley rats [1,2]
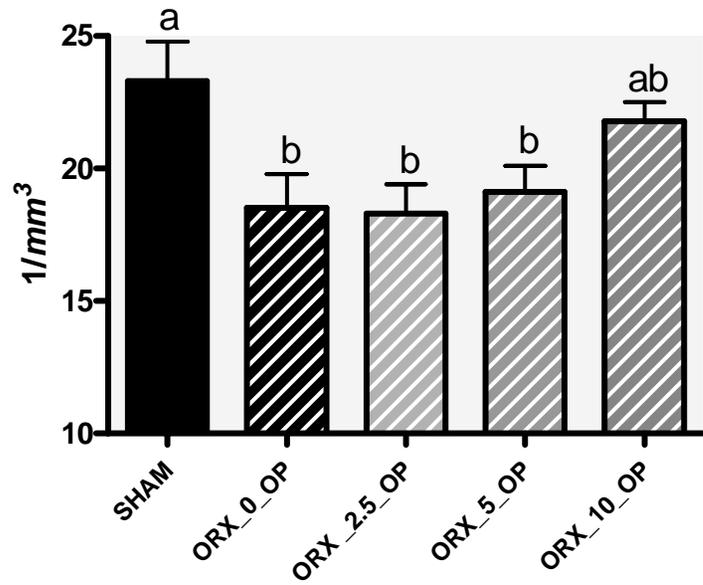
[1]Means $\pm$ SEM (n = 7-8).
[2]Graph bars not sharing a common letter differ significantly, (p = 0.0311).

FIGURE 9.

Effects of orchidectomy and feeding orange pulp on μCT measurements of L4 vertebral trabecular number in year-old Sprague-Dawley rats [1,2]
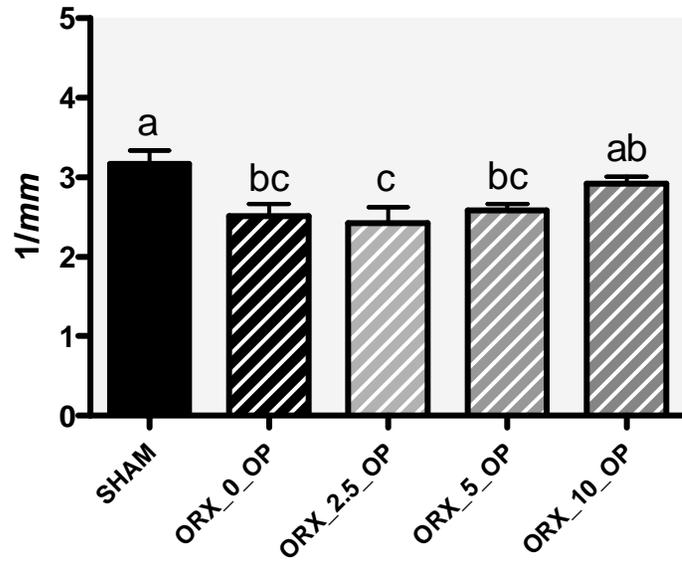
[1]Means ± SEM (n = 7-8).
[2]Graph bars not sharing a common letter differ significantly, (p =0.0049).

FIGURE 10.

Effects of orchidectomy and feeding orange pulp on μCT measurements of L4 vertebral trabecular separation in year-old Sprague-Dawley rats [1,2]
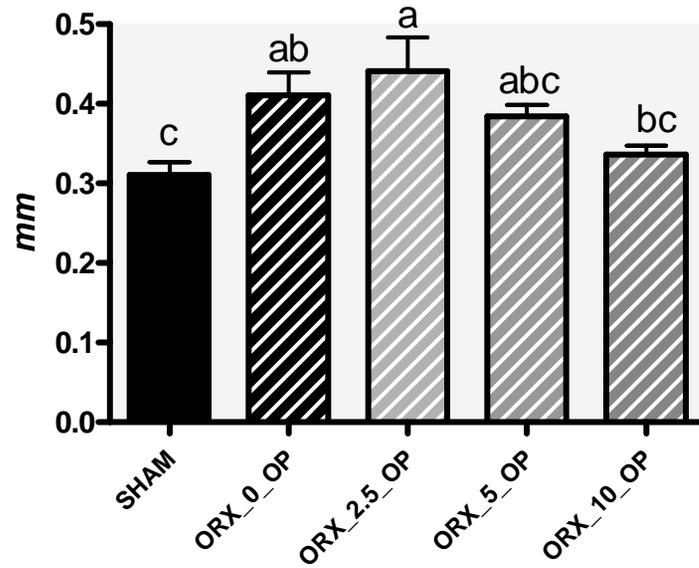
[1]Means ± SEM (n = 7-8).
[2]Graph bars not sharing a common letter differ significantly, (p =0.0073).

FIGURE 11.

Effects of orchidectomy and feeding orange pulp on μCT measurements of total tibial volume in year-old Sprague-Dawley rats [1,2]
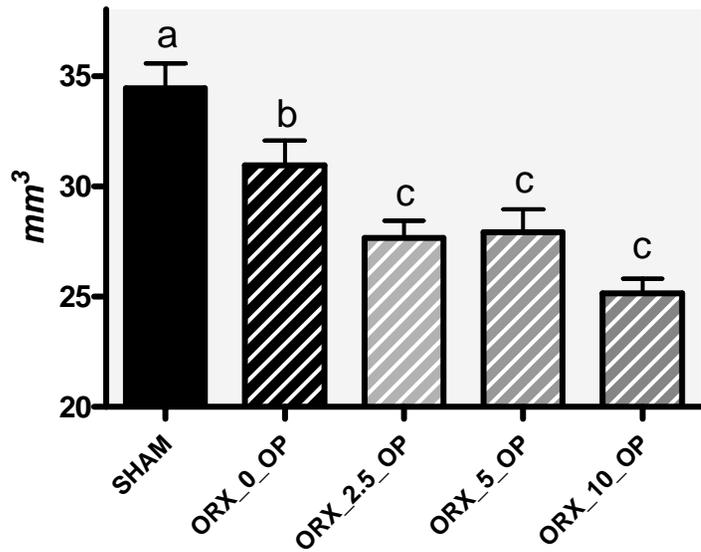
FIGURE 12.

Effects of orchidectomy and feeding orange pulp on μCT measurements of tibial connectivity density in year-old Sprague-Dawley rats [1,2]
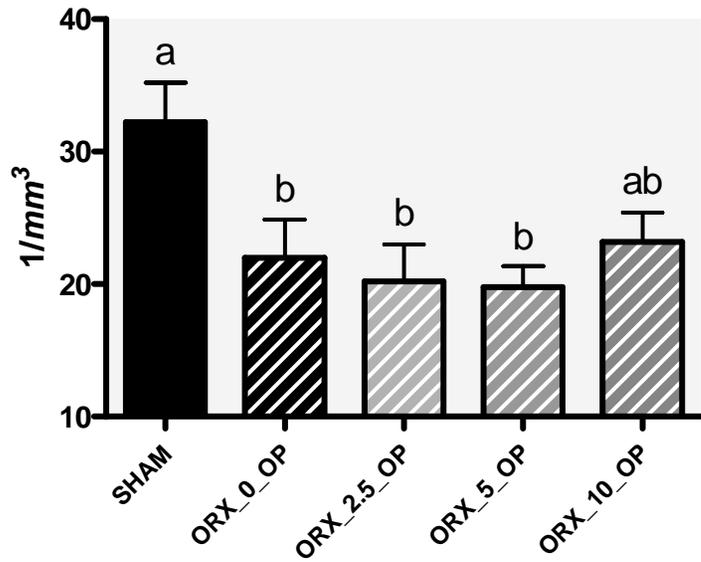
[1]Means ± SEM (n = 8-9).
[2]Graph bars not sharing a common letter differ significantly, (p =0.0098).

FIGURE 13.

Effects of orchidectomy and feeding orange pulp on µCT measurements of tibial trabecular number in year-old Sprague-Dawley rats [1,2]
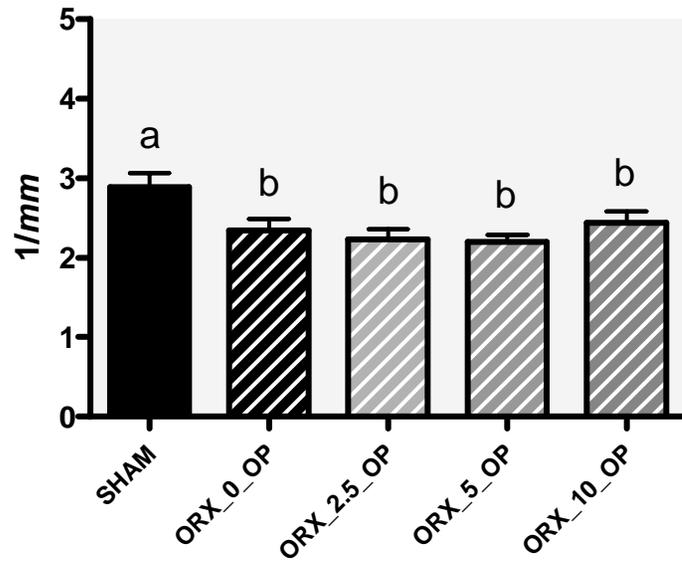
[1]Means ± SEM (n = 8-9).
[2]Graph bars not sharing a common letter differ significantly, (p =0.0091).

FIGURE 14.

Effects of orchidectomy and feeding orange pulp on μCT measurements of tibial trabecular separation in year-old Sprague-Dawley rats [1,2]
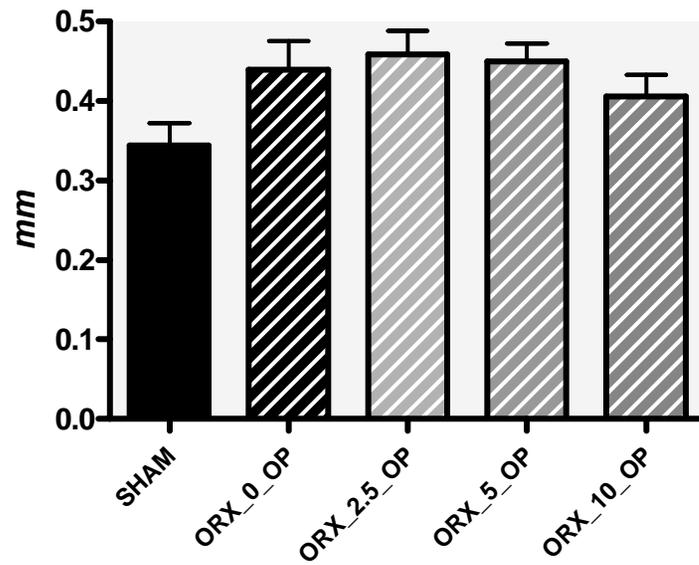
[1]Means ± SEM (n = 8-9).
[2]Graph bars not sharing a common letter differ significantly, (p =0.0594).

FIGURE 15.

Effects of orchidectomy and feeding orange pulp on μCT measurements of tibial bone volume fraction in year-old Sprague-Dawley rats [1,2]
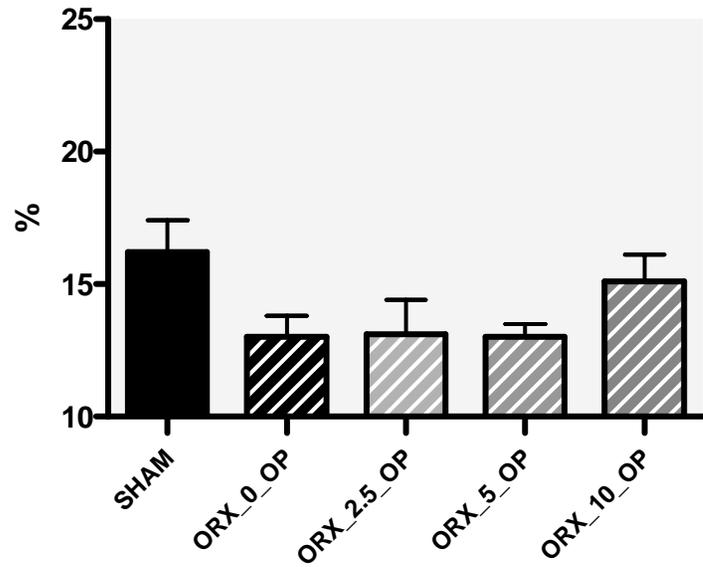
[1]Means ± SEM (n = 8-9).
[2]Graph bars not sharing a common letter differ significantly, (p =0.0916).

FIGURE 16.

Effects of orchidectomy and feeding orange pulp on μCT measurements of tibial total area at midshaft in year-old Sprague-Dawley rats [1,2]
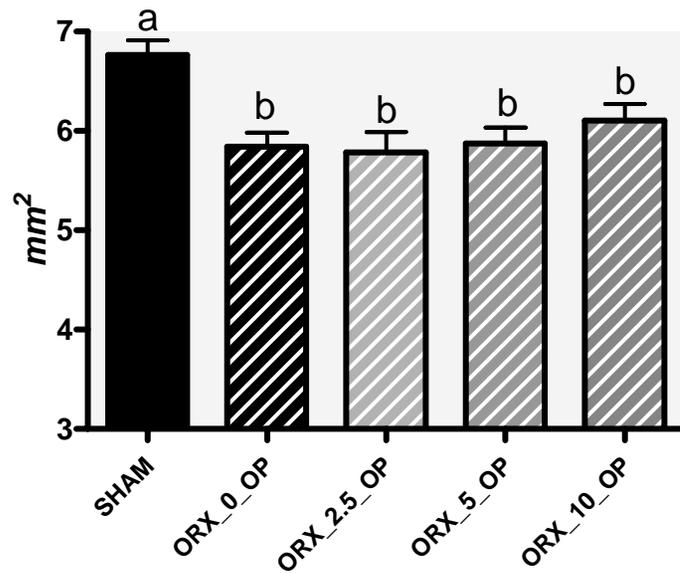
[1]Means ± SEM (n = 8-9).
[2]Graph bars not sharing a common letter differ significantly, *(p=0.0008).*

FIGURE 17.

Effects of orchidectomy and feeding orange pulp on µCT measurements of tibial cortical bone area at midshaft in year-old Sprague-Dawley rats [1,2]
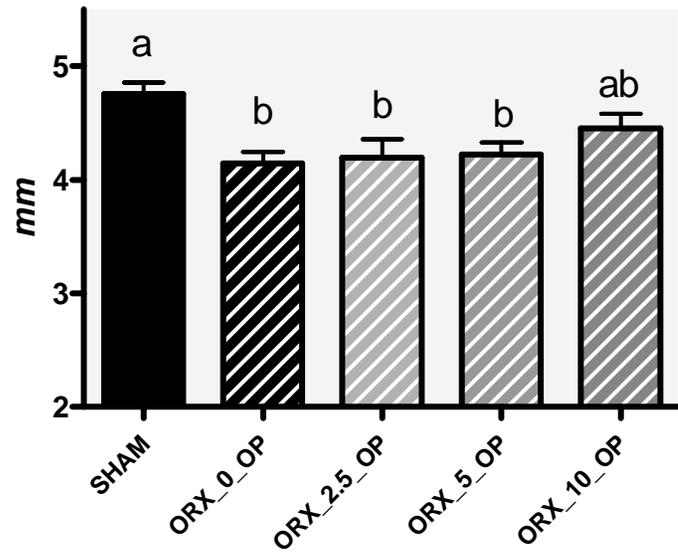
[1]Means ± SEM (n = 8-9).
[2]Graph bars not sharing a common letter differ significantly, *(p=0.0059)*.

FIGURES 18-19.

Effects of orchidectomy and feeding orange pulp on FEA measurements of L4 and tibial force for compression in year-old Sprague-Dawley rats [1,2]
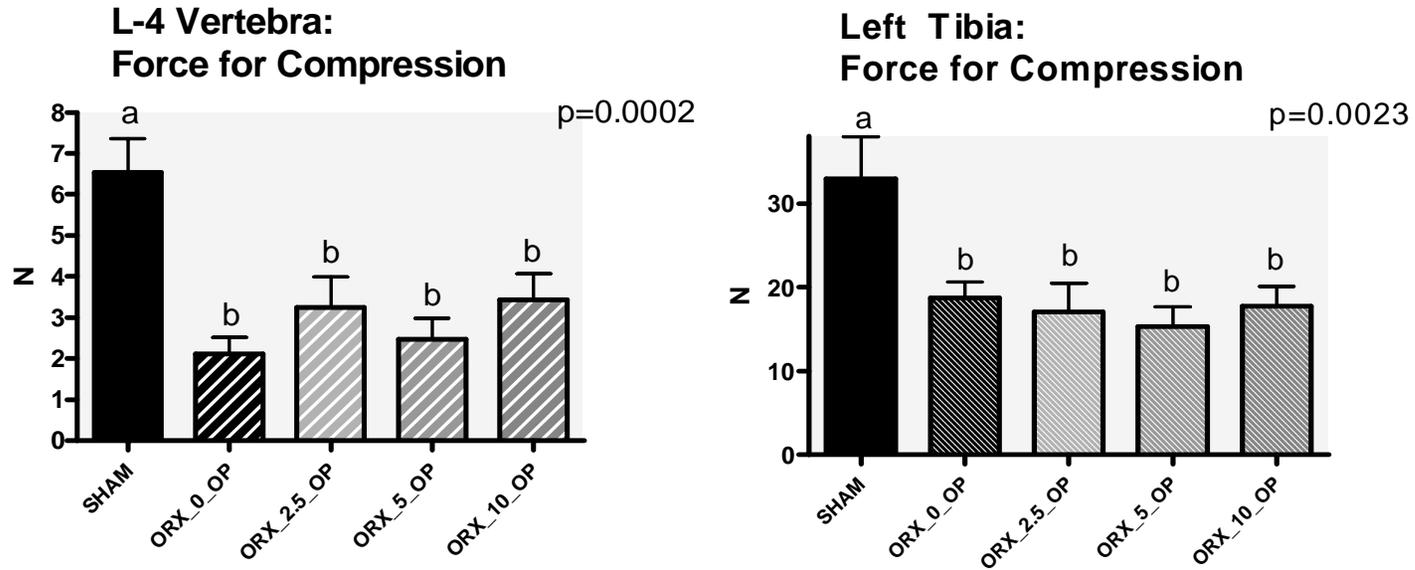
L-4 Vertebra:
Force for Compression

Left Tibia:
Force for Compression

[1]Means ± SEM (n = 7-9).
[2]Graph bars not sharing a common letter differ significantly.

Effects of orchidectomy and feeding orange pulp on FEA measurements of L4 and tibial size-independent stiffness in year-old Sprague-Dawley rats [1,2]

L-4 Vertebra:
Size-Independent Stiffness

Left Tibia:
Size-Independent Stiffness
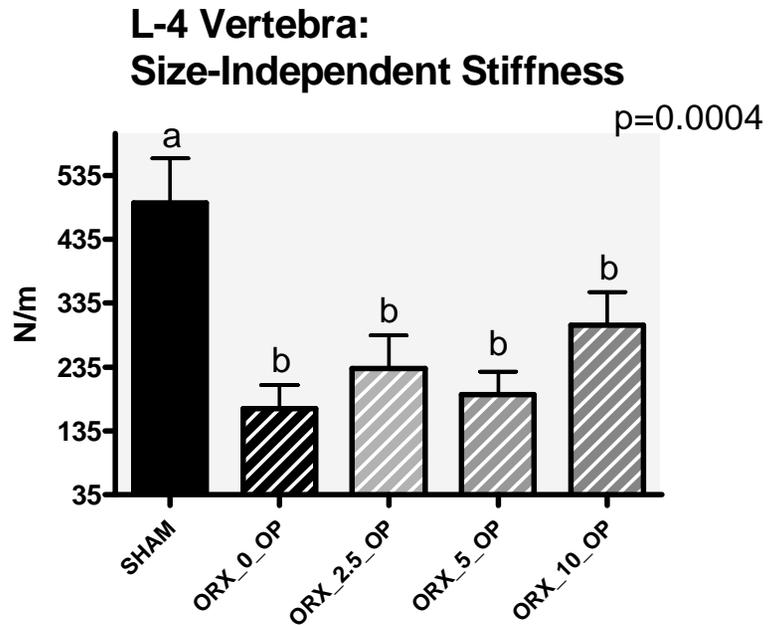
[1]Means ± SEM (n = 7-9).
[2]Graph bars not sharing a common letter differ significantly.

FIGURE 22.

Effects of orchidectomy and feeding orange pulp on μCT measurements of tibial cross-sectional area in year-old Sprague-Dawley rats [1,2]
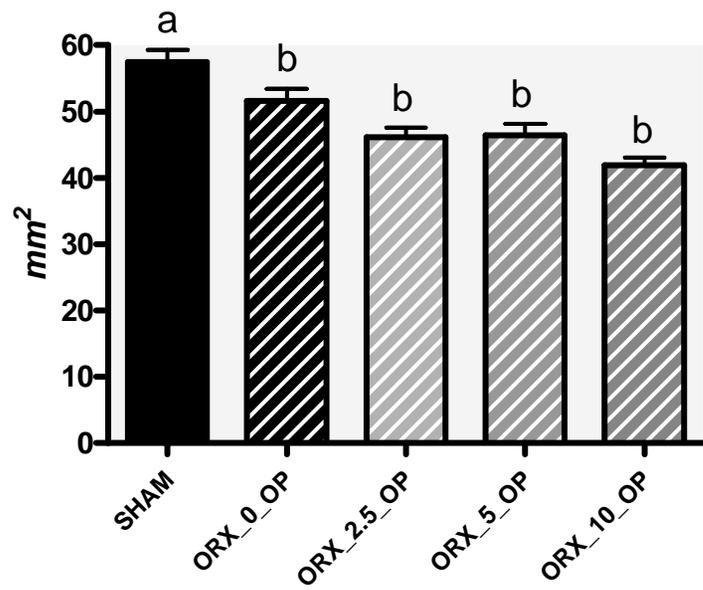
[1]Means ± SEM (n = 8-9).
[2]Graph bars not sharing a common letter differ significantly, *(p<0.0001)*.

CHAPTER V

CONCLUSION

Summary and Conclusions

As a model for inducing bone loss in males, orchidectomy successfully reduced measures of bone density, structure, and strength. In our experimental analyses, feeding orange pulp to orchidectomized rats appeared to improve some characteristics of bone structure, although most were not significantly greater than orchidectomized rats fed only the standard diet. Components of citrus likely to produce this effect include flavonoids, (specifically hesperidin and narigenin), limonoids, and vitamin C. Through direct and indirect antioxidant action, components may reduce oxidative stress in vivo and alleviate stimulation of bone resorption (110). Complex estrogenic and antiestrogenic actions by hesperidin and naringin contribute to bone maintenance. Dietary consumption of functional foods, including oranges, provide an inexpensive strategy for maintaining bone health. Although improvements are subtle, this study supports the USDA recommendation to consume a wide variety of fruits and vegetables daily.

Suggestions for Further Research

Data showed that orchidectomy significantly reduced parameters of bone density, structure, and strength in year-old rats over a four-month period. This confirms efficacy as an animal model for studying male osteoporosis. The ORX_10.0% orange pulp group showed positive tendencies toward improved parameters of bone density, structure, and

strength. However, at lower doses, effects are not easily discerned. Further study is warranted to elucidate effects of feeding orange pulp to Sprague-Dawley rats, mechanism of action, and ideal dose. Methodology considerations for future studies include determining effects of feeding orange pulp using higher percentages of orange pulp in the diet, longer time periods, fresh fruit instead of freeze-dried samples, and a larger sample size. Investigation using these methodology modifications may aid in delineating effects.

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APPENDICES

TABLE 1.

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-93M; 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.

FIGURE 1.
Experimental Design
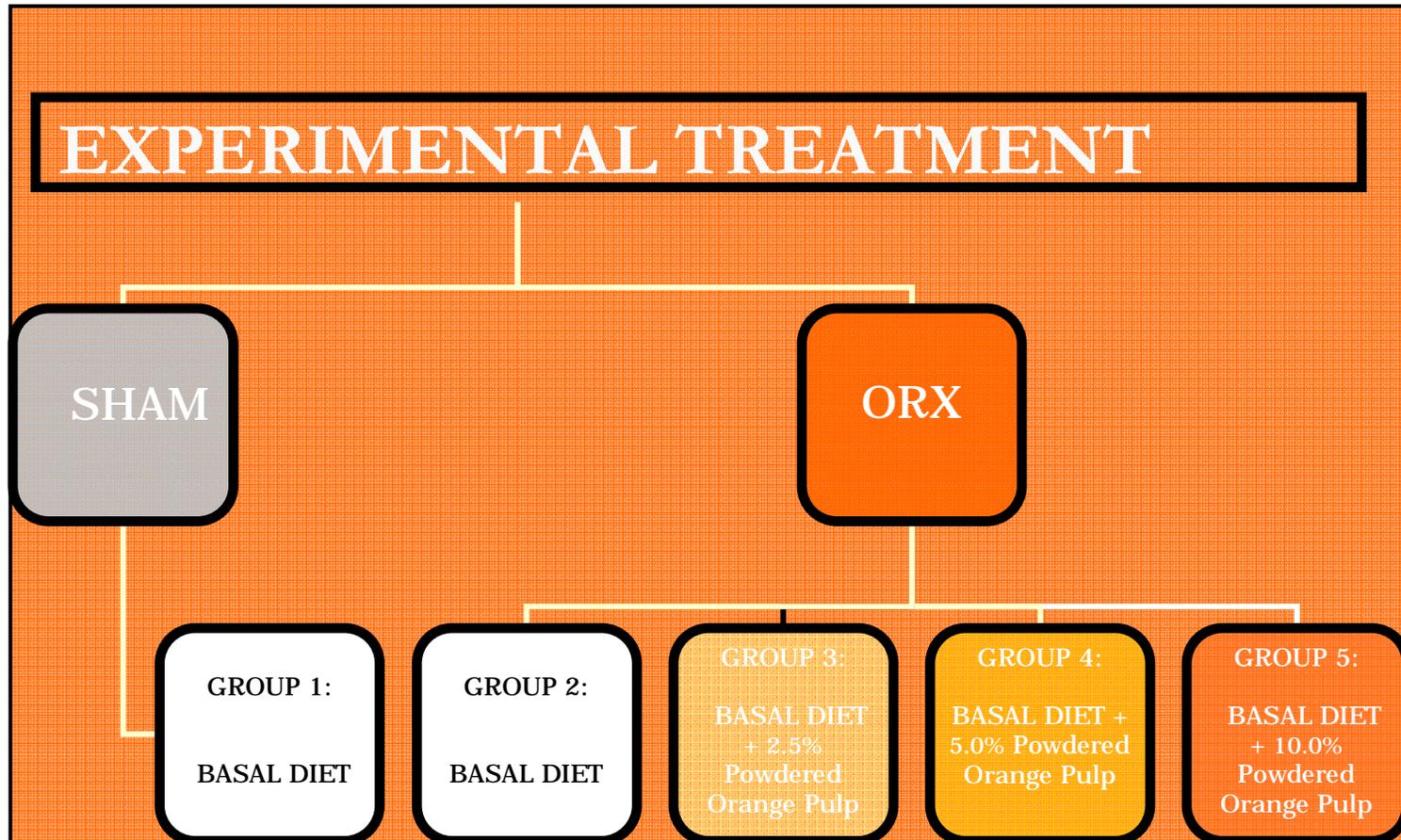
FIGURE 2.

Effects of orchidectomy and feeding orange pulp on μCT measurements of tibial bone length in year-old Sprague-Dawley rats [1,2,3]
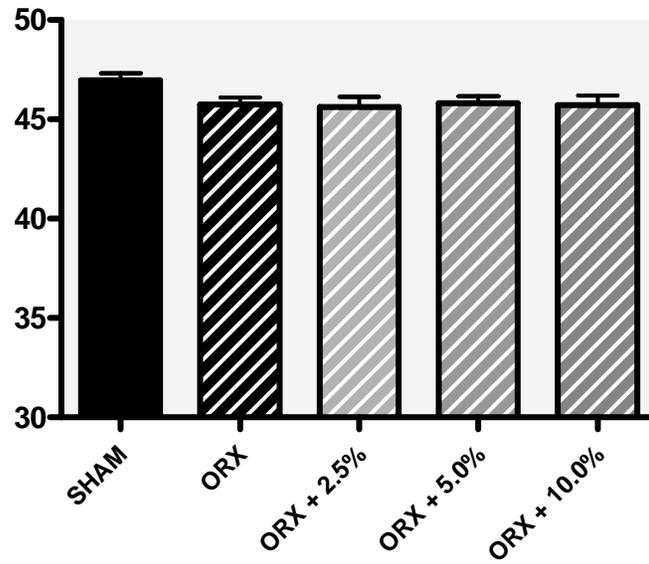
[1]Means ± SEM, (n = 8-9).
[2]Graph bars not sharing a common letter differ significantly, ($p = 0.1793$).

VITA

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Master of Science

Thesis: FEEDING ORANGE PULP IMPROVED BONE QUALITY IN A RAT
MODEL OF MALE OSTEOPOROSIS

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Scope and Method of Study: This collaborative study evaluated the effects of feeding orange pulp (OP) on bone quality in a rat model of male osteoporosis. One-year-old retired breeder rats were randomly assigned to the following orchidectomy (ORX) or sham-surgery (SHAM) treatments: SHAM, ORX, ORX_2.5_OP, ORX_5_OP and ORX _10_OP. Diets (isonitrogenous, isocaloric modified AIN-93M) were pair-fed to food intake of SHAM. Plasma antioxidant capacity and bone density, structure, and strength were assessed ($p<0.05$).

Findings and Conclusions: Orchidectomy decreased ($p<0.05$) antioxidant status while OP maintained antioxidant capacity of ORX rats to that of SHAM. ORX reduced bone volume fraction, connectivity density, and trabecular number and increased trabecular separation in L-4 vertebra, significantly. Ten percent OP tended ($p<0.07$) to improve some bone parameters compared to ORX.

ORX consistently had a negative impact on bone density, structure, and strength parameters. ORX_10_OP rats tended to have improved parameters compared to ORX rats. Dietary OP induced subtle favorable changes in bone.

- ADVISER'S APPROVAL: Dr. Barbara J. Stoecker