PREVENTING BONE LOSS AND WEIGHT GAIN WITH COMBINATIONS OF VITAMIN D AND PHYTOCHEMICALS

by

CHING-YI LAI

(Under the Direction of Clifton A. Baile)

ABSTRACT

This study examined if dietary supplementation with a combination of phytochemicals with vitamin D inhibits bone loss and decreases adiposity in ovariectomized female rats. Ovariectomized female rats were given control, vitamin D or vitamin D + resveratrol + quercetin + genistein for 8 week. The high dose combination treatment significantly reduced body weight gain (control: 80±5 g; vitamin D: 81.5±5 g; high: 62.0±3, $p<0.05$) and fat pads as a percentage of body weight (control: 7.5±0.2; vitamin D: 7.6±0.2; high: 6.4±0.2, $p<0.05$) compared to the control and vitamin D alone. Results from the DEXA showed that bone mineral density (BMD) and content (BMC) of femora were significantly increased by the high dose combination, although they weren't different from vitamin D alone. However, BMD corrected for body weight was significantly greater in the high dose treated group compared to both the control and vitamin D groups (control: 4.7±0.05; vitamin D: 5.0±0.2; high: 5.6±0.2, $p<0.05$). Histomorphometric analysis of sections from the right tibia indicates that rats receiving the high dose combination have fewer marrow adipocytes (control: 22.86±1.8; vitamin D: 17.25±2; high: 12.53±0.8, $p<0.001$) and significantly reduced osteoclast number in

trabecular bone compared to both the control and vitamin D groups (control: 4.89±0.5; vitamin D 5.51±0.7; high: 2.96±0.6 N.OC/B.Pm, $p<0.05$). We conclude that ovariectomized female rats supplemented with vitamin D and a combination of genistein, quercetin and resveratrol showed improved bone density and reduced body weight gain and marrow adipocytes compared to rats that received vitamin D alone. We predict that similar treatments in postmenopausal women would have similar effects.

INDEX WORDS: Bone loss, Obesity, Vitamin D, Phytochemicals

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B.S., Chung Shan Medical University, Taiwan (R.O.C.), Jun 9, 2006

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2010

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December, 2010

DEDICATION

This thesis is dedicated to my family and friends. To my parents, who supported me with their care, thoughtfulness, and finances while studying in U.S.; to Wen-Ting Tseng, who has been a source of motivation and strength during my moments of despair and discouragement; to my best friend, Jenny Hwang, who always expressed concern for me as a family member: thank you for giving me the strength to chase my dreams and always being there for me.

ACKNOWLEDGEMENTS

I would like to sincerely thank my major professor, Dr. Clifton A. Baile, for giving me an opportunity to pursue a Master degree and for his continuous support and guidance throughout this project, and especially for his confidence in me.

I would also like to thank my committee members, Drs. Mark Hamrick, Richard Lewis, and Michael Azain, for their invaluable advice; a special thank you to Dr. Hamrick who patiently guided me the literature and technologies related to bone measurements and assessments.

Gratitude is also expressed to all those who helped me to complete this thesis. I would like to thank Diane Hartzell for her plentiful assistance with my study, improving my language skills, and even helping with the details of living in Athens. I am grateful to Dr. MaryAnne Della-Fera for her comments and helping me to interpret some results presented in this thesis.

I would also like to thank Drs. Jeong-Yeh Yang, Srujana Rayalam, Suresh Ambati, and the rest of the Baile lab for their extended support. Your help made it was possible for me to complete this research project.

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CHAPTER 1

INTRODUCTION

Obesity and osteoporosis are major public health concerns due to their prevalence in the increasingly sedentary and aging society. In the United States today, an estimated 55% of people 50 and older are at risk for developing osteoporosis.[1, 2] An estimated 10 million Americans suffer from osteoporosis, and eighty percent of those are women. Because as much as 20% of bone mass can be lost in the first five to seven years following menopause, osteoporosis is a major health issue for aging women.[3] Previous evidence has shown that bone formation rate is inversely correlated with adipocyte numbers in bone tissue in both men and women,[4] and women with osteoporosis have been shown to have higher number of marrow adipocytes than women with healthy bone.[5-7] Studies have suggested that mesenchymal stem cells (MSC) are by default programmed to differentiate into adipocytes;[8] thus the presence of appropriate growth factors likely determines which developmental pathway is activated. Because adipocytes secrete osteoclastogenic cytokines such as interleukin 6 (IL-6),[9, 10] which can inhibit osteoblast activity in culture once a certain level of bone marrow adiposity is reached, conditions may promote further adipogenesis at the expense of osteogenesis.[10] Existing therapies for osteoporosis fall broadly into two categories: anti-resorptives, which inhibit osteoclast activity, and anabolic agents, which increase bone formation. Recent studies indicate that compounds regulating lipid metabolism may also have a significant effect on bone formation in ovariectomized,[11] estrogen-deficient rats,[12] and in postmenopausal

women.[13] Therefore, treatments that prevent or reverse the increase in bone marrow adipocytes could both increase new bone formation and inhibit bone destruction. The objective of this study was to examine the effects of vitamin D in combination with phytochemicals including genistein, quercetin, and resveratrol on both body weight and bone mass in ovariectomized female rats.

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CHAPTER 2

LITERATURE REVIEW

Biology of Bone

The skeleton is a vital organ that serves the structural function to provide mobility, support, and protection for the internal organs. To provide the body with a frame that is both light and strong, the outer dense shell is composed of compact or cortical bone, which is highly calcified comprising 80% of total skeletal mass and contributes to the mechanical, structural and protective functions; the inner of bone is cancellous or trabecular bone with a spongy-like appearance, making up the remaining 20% of the skeleton.[1] Particularly, trabecular bone has a larger surface area and hence carries out the relatively high metabolic activity compared to cortical bone.[2]

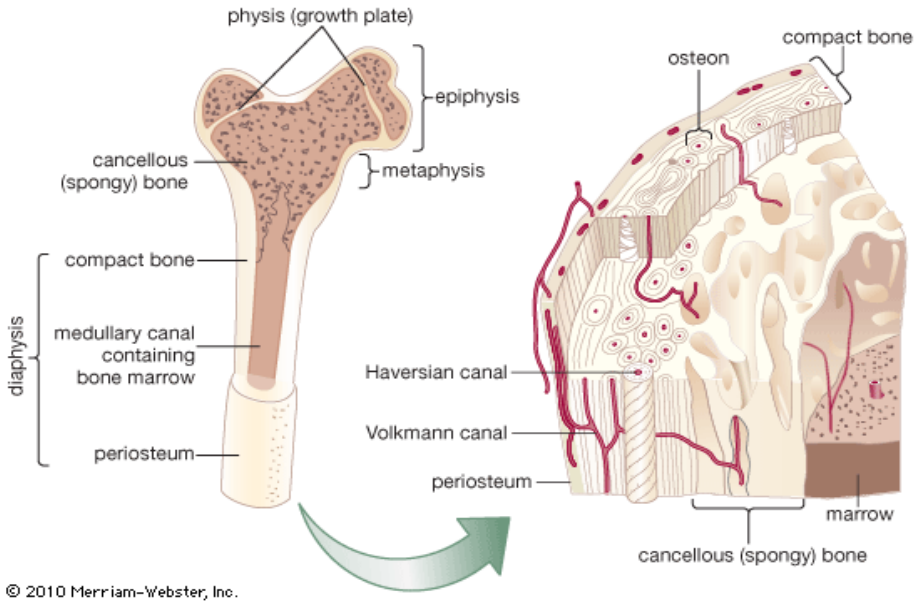

Figure 2.1 Structure of bone (Source: Encyclopedia Britannica Online)

There are different types of cells generated by the bone marrow that are involved in bone metabolism including the osteoclasts, or bone-resorption cells, and the osteoblasts, or bone-forming cells. Osteoclasts are derived from hemopoietic stem cells of the monocyte-macrophage lineage cells and are usually large multinucleated cells with numerous lysosomes.[3] Their most notable feature is bone resorption, which operates by attaching to the bone surface and secreting the specific enzymes to dissolve the calcified bone matrix with the ruffled border of osteoclasts.[4] On the other hand, osteoblasts, and the other related bone-forming cells including lining cells and osteocytes, are derived from local mesenchymal stem cells.[5] The fully differentiated osteoblasts are responsible for bone formation, producing the main component of bone matrix called type I collagen and several other proteins such as osteocalcin and osteonectin, which are involved in the formation of bone matrix.[6]

The function of osteoblasts and osteoclasts are intimately linked.[7] Throughout one's lifetime, especially during development and growth, the skeleton is constantly reconstructed by the removal of the old matrix by osteoclasts and the deposition of the new bone by osteoblasts to achieve skeletal formation and renewal; these processes are called modeling and remodeling, respectively.

Bone Modeling and Remodeling

The processes of bone modeling and remodeling are necessary. During growth, modeling allows individual bones to construct their size, shape and thickness; the purpose is to establish the skeleton's peak bone strength. It is well known that achieving a high peak bone mass in early life predicts a relatively higher bone mass, and hence greater protection from fracture in later life.[8, 9] Although the remodeling process does not

change the shape of the bone; nevertheless, it is crucial to maintain bone strength. In adults, remodeling replaces the damaged bone with a new bone to prevent accumulation of old bone, which causes brittle bone.[10]

The processes that drive bone remodeling are still not fully understood, but it is a tightly regulated process in a specific site for bone remodeling, known as the basic multicellular unit (BMU). Bone remodeling has been defined as a cycle consisting of activation of osteoclast formation, osteoclast-mediated resorption, a short period of reversal, and then a long period of bone matrix formation mediated by osteoblasts.[11, 12]

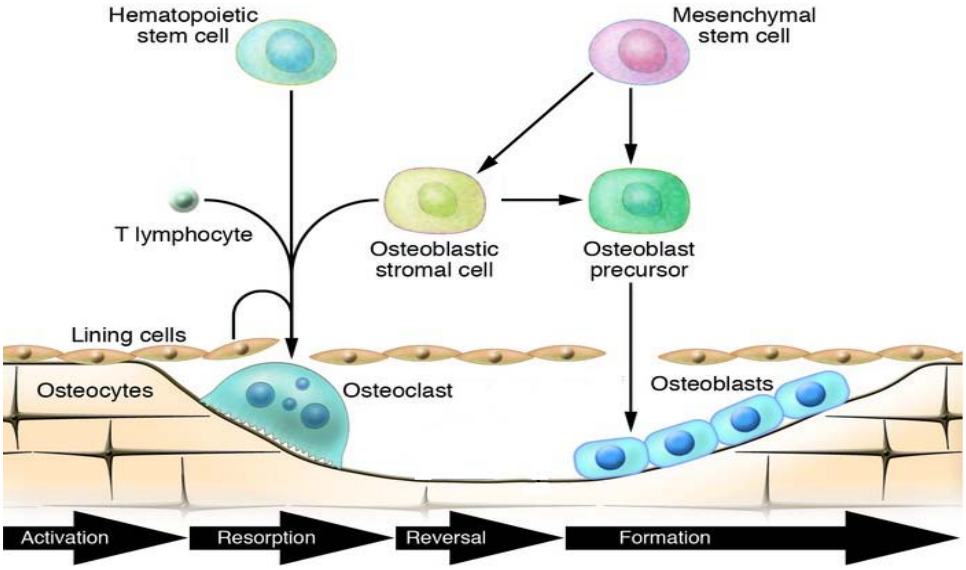

Figure 2.2 Bone remodeling cycle.[13]

Osteoclastogenesis is initiated by interaction between osteoclast precursors and cells in the osteoblast lineage which are under the control of stromal cells to ensure the normal coupling between processes.[14] In addition, some components are expressed at different stages of osteoblast differentiation to allow advanced bone remodeling. For example, osteoblasts produce macrophage colony stimulating factor (M-CSF), which

binds to its receptor on preosteoclastic cells, and contributes to the survival of cells in the macrophage-osteoclast lineage, but M-CSF on its own is unable to complete the process.[15] RANK ligand (RANKL), expressed on the surface of preosteoblastic, or stromal cells, binds to receptor activators of NF-κB (RANK) on the osteoclastic precursor cells, and is necessary for differentiation and activation of osteoclasts. Conversely, the appearance of osteoprotegerin (OPG) serves as a very effective inhibitor to block the activation by competitively binding RANKL.[16] Furthermore, a number of local cytokines, such as tumor necrosis factor α (TNF-α) and interleukin 1 (IL-1), and hormones including parathyroid hormone (PTH) and sex steroid hormones, modulate osteoclastogenesis by regulating stromal cells production of OPG and RANKL.[17-20]

As a consequence, osteoclasts must be synthesized and instructed where bone resorption is needed. Osteocytes, cells derived from osteoblasts spaced throughout the bone matrix to form a dendritic network, are an essential cellular component for bone remodeling. This arrangement suggests that osteocytes have the ability to detect the need for bone augmentation or reduction by contacting osteoblasts or bone lining cells.[8] It has been proposed that micro-damage and deformation in bone are first sensed by osteocytes, which then trigger osteoclast activity. Indeed, the death of osteocytes by apoptosis may secrete regulatory factors that define the location and stimulate osteoclast differentiation.[21] Therefore, osteocytes are involved in initiating remodeling, with osteoblast precursors directing osteoclastogenesis.

Simultaneously active lining cells, descendents of osteoblasts, partly form a canopy of the bone remodeling compartment once the signals are released by osteocytes. This compartment provides a micro-environment for attachment of osteoclasts and

osteoblasts.[22] The process of osteoclastic bone resorption lasts about three weeks in human bone. The reversal phase is a period when resorption is inhibited, osteoclasts undergo apoptosis, and osteoblasts are attracted and differentiated.[12] It has been suggested that coupling is mediated by growth factors released from the matrix during resorption, for example insulin-like growth factor 1 (IGF-I) or transforming growth factor β (TGF-β), are produced by osteoblasts and other bone cells and stimulate osteoblast proliferation and differentiation.[23-25] Bone formation is carried out by the recruited osteoblasts that then refill the unit of bone slowly and last much longer than resorption.[13] Therefore, it is a critical factor contributing to osteoporosis due to bone remodeling, and this process accelerates at menopause.

Biology of Fat

Adipose tissue has notable functions in physiological processes. First, adipocytes involve lipid metabolism including triacylglycerol storage in periods of energy excess and fatty acids release during energy deprivation. Second, adipocytes also release fatty acids and glycerol into the circulation because most organs for fuel use fatty acids when glucose is limited. Lastly, adipocytes synthesize and secrete peptides, which include hormones, cytokines and other proteins with specific biological roles involved in various physiological functions such as immune response, blood pressure control, bone mass and reproductive function.[26]

Most mammals have adipose depots, especially white adipose tissue (WAT), located throughout the body as a lipid reservoir to maintain energy homeostasis. Subcutaneous adipose tissue is located underneath the skin and also contributes to the

physical functions including temperature regulation and thermal isolation. Another type of adipose tissue known as visceral adipose tissue occupies the body cavity, surrounding the heart and other important organs to keep them in the appropriate position. However, it undergoes degradation and releases directly into the portal circulation that has been linked to many metabolic diseases associated with obesity, including type 2 diabetes and cardiovascular disease.[27]

In addition to the typical white adipose tissue, mammals have a second terminally differentiated adipose cell type that composes brown adipose tissue (BAT). The role of BAT is opposed to that of WAT, as it serves primarily to dissipate energy instead of storing it.[28] BAT contains a large number of mitochondria and expresses uncoupling protein-1 (UCP-1) which dissipates the proton gradient across the inner mitochondrial membrane during the passage of electrons along the respiratory chain. This generates heat at the expense of ATP.[29] Rodents have a distinct BAT located in the interscapular region. However, in humans, it surrounds the heart and great vessels only in newborns and regulates thermogenic processes. It tends to disappear as humans mature until it becomes so subtle that it is hard to recognize.

The Adipocyte Life Cycle

Adipose tissue contains different cell types including fibroblasts, macrophages, monocytes and preadipocytes. One third of the adipose tissue is composed of mature adipocytes, which are derived from mesenchymal stem cells.[30] In the adipocyte life cycle, the cell undergoes alteration of cell shape and growth arrest, clonal expansion and a complex sequence of changes in gene expression leading to storage of lipids and finally cell death.[31]

Committed preadipocytes resemble fibroblasts morphologically during growth phase but withdraw from the cell cycle before entering adipose conversion, a process known as growth arrest. Following growth arrest, preadipocytes undergo at least one round of DNA replication leading to the clonal amplification of committed cells and require adipogenic signals to continue through the subsequent differentiation processes.[32] The central stimulus of adipocyte differentiation is peroxisome proliferator-activated receptor (PPARγ), which is a member of the steroid/ retinoid nuclear receptors generated by PPARγ gene.[33] Multiple CCAAT/ enhancer-binding proteins (C/EBPs) also play a crucial role, with C/EBPβ and C/EBPδ driving PPARγ in the early stages of differentiation that in turn activate the expression of PPARγ and C/EBPα in later stages.[34] During the process of differentiation, cells are induced to the progressive modification of cell morphology as the cell converts from a fibroblastic to a spherical shape.

During the terminal stages of differentiation, activation of transcriptional cascade leads to increase the expression of protein and mRNA levels of enzymes related to triacylglycerol synthesis and degradation.[31] Expansion of WAT takes place rapidly after birth resulting in increased fat cell size as well as an increase in the number of fat cells, but the ability to generate new fat cells is limited at the adult stage. Nevertheless, although it was once thought that the total number of adipocytes did not change throughout one's life, it is now believed that new forming adipocytes can still be generated or removed by the process of apoptosis.[35]

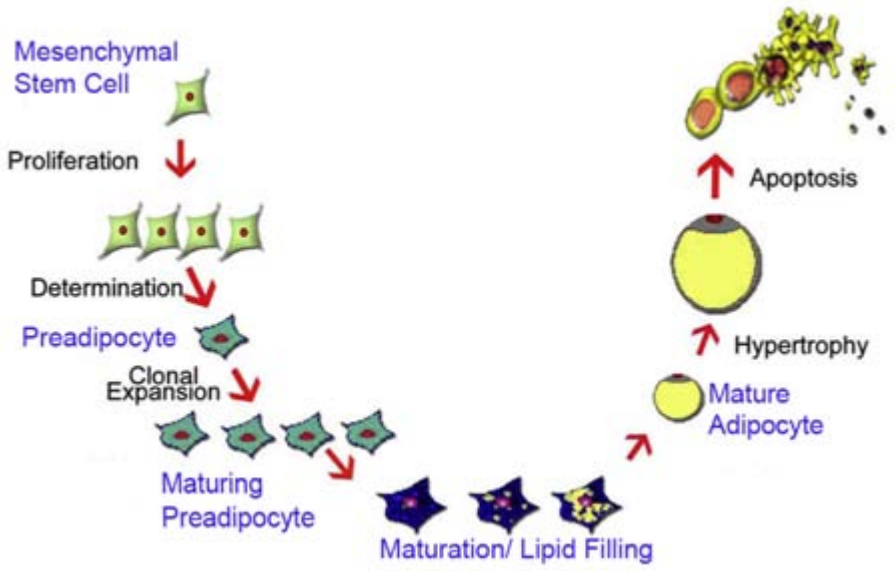

Figure 2.3 Adipocyte life cycle [36]

Roles of Estrogen in Menopause

The term *menopause* derives from the Greek words *meno* (month) and *pause* (to end). Thus, the literal definition is the end of the cycle of monthly menstrual bleeding. The age at which menopause occurs varies widely; ranging from the late thirties to the late fifties, with most women experiencing menopause between the age 48 and the age 55. The development of menopause results in decreased estrogen and the irregular hormonal cycle associated with ovulation and ultimately leading to termination of reproductive life.[37] Clinically, menopause is defined as the absence of menses for at least 6 months to a year.

Estrogen acts through two receptors located within the target cell nuclei including estrogen receptor α (ERα) and estrogen receptor β (ERβ). Once bound by estrogen, this nuclear estrogen-ER complex undergoes a conformational change resulting in a serial genomic response to regulate associated mRNA levels and protein production, as well as

a physiological response.[38] Conversely, estrogen also can act through the ER located in or adjacent to the plasma membrane. The consequences of the hormonal changes of menopause not only lead to cessation of reproduction and accompanying symptoms in women, but also dramatically impact health in later life. Estrogen deficiency is critical to the pathogenesis of osteoporosis, which is based on the fact that postmenopausal women, whose estrogen levels naturally decline, are at the highest risk for developing the disease. There are clinical and experimental data to support the protective effects of estrogen against neurodegenerative diseases and cardiovascular disease in humans.[39, 40] In addition, estrogen has been recognized as a major factor in regulating adipose development and deposition in females.[41] The accumulation of abdominal fat in premenopausal women appears to be suppressed by estrogen, whereas men do not show this hormonal protection, leading to deposition of abdominal fat. Nevertheless, the accumulation of abdominal fat tends to increase in postmenopausal women which correlates with the metabolic complications.[42]

Roles of Estrogen in Obesity and Osteoporosis

Obesity

Obesity continues to be a public concern in the United States and throughout the world. The definition of obesity is a body mass index (BMI) of 30 or greater, which is calculated from a person's weight and height and provides a reasonable indicator of body fatness and weight categories that may lead to health problems. In women, a waist circumference of >88cm (>35 inches) is considered as abdominal obesity, placing the individual at increased health risk.[43] The deleterious effects of estrogen deficiency in

increasing adipose mass in women are caused by the fact that loss of estrogen signaling produces a preferential increase in visceral fat. This is unfavorable because an increase in visceral fat stores is closely associated with increases in the risk of serious health problems such as cardiovascular disease and type 2 diabetes.

Although the mechanism responsible for the regulation of adipose development and deposition by estrogen remains unclear, human and rodent adipocytes express both ERα and ERβ, indicating that estrogen may modulate a certain level of adipose metabolism.[44, 45] Indeed, estrogen has been suggested that directly act on adipose tissue to decrease lipogenesis by reducing activity of lipoprotein lipase (LPL), a lipogenic enzyme that regulates the metabolism of plasma triglycerides to free fatty acids and that increases lipid uptake by adipocytes.[46] In addition to inhibiting lipogenesis, estrogens indirectly affect lipolysis by inducing the lipolytic enzyme hormone-sensitive lipase, which catalyzes the breakdown of stored triacylglycerol and the release of fatty acids.[47, 48] In particular, the finding suggested that estrogens preferentially induce subcutaneous adipose deposition in females because estrogen seems to inhibit lipolysis only in subcutaneous depots and results in shifting the fat from abdominal depots to subcutaneous depots.[49]

On the other hand, estrogens also affect adipose tissue by acting through other tissues that regulate appetite, energy expenditure or metabolism.[50] Changes in the various components of energy expenditure have also been believed to play an important role in the increased overall and central adiposity of women undergoing the menopause transition. Taken together, these findings suggest that hormonal changes of menopause may lead to body fat distribution changes by affecting regional adipose tissue metabolism.

Osteoporosis

Osteoporosis, characterized by low bone mass and micro-architectural deterioration, leads to susceptibility to fragility fractures. Hip fractures are the most common type of fractures that leads to hospitalization and causes serious disability and increased mortality, consequently.[51] Generally, there are two forms of osteoporosis, one related to estrogen deficiency at menopause and the other to calcium deficiency and aging of the skeleton.[52] Although adequate dietary calcium is well known for the prevention of osteoporosis, an acute decrease in ovarian hormones is associated with the loss of 20% of bone mass in the first 5-7 years of the postmenopausal period. Therefore, estrogen plays a central role in normal physiological remodeling.

There are multiple pathogenic mechanisms that cause loss of bone mass and micro-architectural deterioration of the skeletal structure. They can result from failure to produce a skeleton of optimal mass and strength during growth. In adult life, the skeleton is continually remodeled in an orderly sequence of bone resorption followed by bone formation. If excessive bone resorption and inadequate bone formation occurs, the remodeling imbalance eventually results in skeletal fragility. In particular, previous studies demonstrated that estrogen deficiency increases and estrogen treatment decreases the rate of bone remodeling, as well as the amount of bone lost with each remodeling cycle.[53, 54]

Estrogen receptors are mostly present in osteoblasts and have profound effects on osteoblasts.[55] For example, the interaction of estrogen and its receptor enhances the production of transforming growth factor-β (TGF-β), which inhibits resorption and accelerates osteoclast apoptosis.[56] Estrogen can also act on osteoblasts to inhibit

production of interleukin-6 (IL-6), which is a prominent cytokine involved in early stages of osteoclastogenesis, thus promoting osteoclast development. Therefore, the effect of estrogen on IL-6 suggests the pathogenic mechanism of the osteopenia caused by estrogen deficiency.[57] On the cellular basis, osteoblasts express RANKL on their surface; RANKL binds with RANK, which is its receptor expressed on osteoclast precursors. The interaction of RANKL and RANK promotes the differentiation of osteoclasts resulting in their activation and extended survival. Estrogen exerts its anti-resorptive effects on bone by stimulating the expression of osteoprotegerin (OPG), which is mainly secreted by osteoblasts and that blocks the interaction of RANKL and RANK and thus serve as a physiological regulator of bone turnover.[58]

Notably, there is a complex connection between adipose tissues and bone, suggesting that adiposity is likely involved in the development of osteoporosis, even though the relationship between bone and fat still remains controversial. As a person increases in age, adipocytes tend to accumulate in certain regions of the body such as bone marrow, liver, and muscle. Despite the increased risk of developing certain diseases, adult obesity seems to protect against bone loss at various skeletal sites.[59] Some researchers are proposing that an increase in body weight or BMI in adults correlates with an increase in bone mineral content (BMC) or bone mineral density (BMD).[60-62] Thus, body weight has previously been used as a positive predictor of bone mineral density.[63] Conversely, recent studies challenge that adipocytes do not protect against decreases in bone mass; however, adiposity contributes to reduction in bone mass.[64]

In the bone marrow, mesenchymal stem cells can differentiate into a variety of cell lineages including chondroblasts, myoblasts, osteoblasts and adipocytes, and of these

lineages, the osteogenic and adipogenic lineages are closely related.[65] Studies have demonstrated that women with osteoporosis have higher numbers of marrow adipocytes than women with healthy bone quality, and bone formation rate is negatively correlated with the number of adipocytes in bone marrow.[64, 66] In addition, adipocytes secrete osteoclastogenic cytokines such as IL-6, which can inhibit osteoblast activity and ultimately promote osteoclast development. Once a certain level of bone marrow adiposity is reached, conditions may promote further adipogenesis at the expense of osteogenesis.[57] Thus, it is now well documented that the common progenitors are directed towards the adipocyte lineage rather than the osteoblast lineage, increasing accumulation of adipocytes within bone marrow, which leads to an increase in the risk of osteoporosis and fractures.[67]

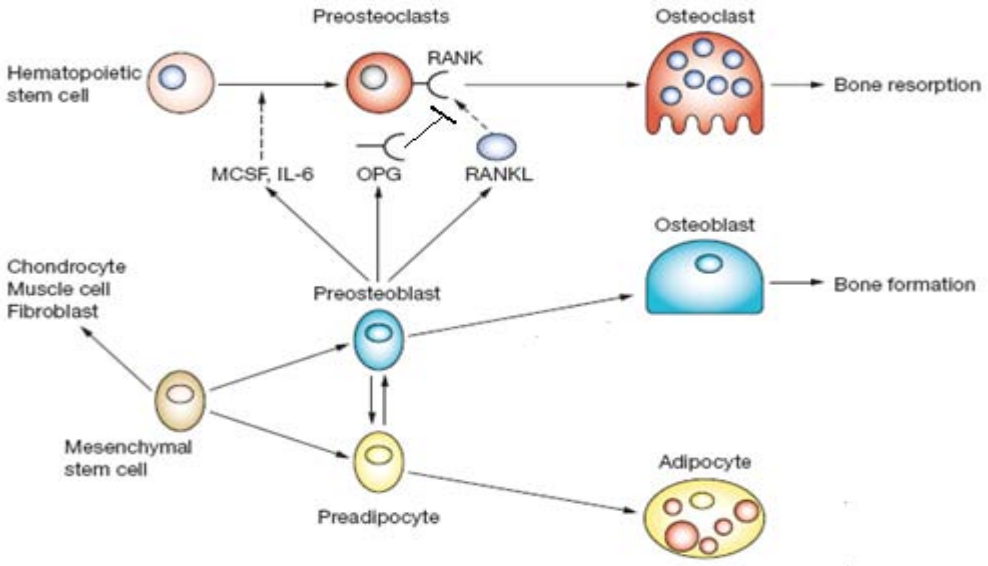

Figure 2.4 Overview of the differentiation of osteoclasts, osteoblast and adipocytes[68]

Vitamin D

In the 17th century, rickets, a childhood disease characterized by impeded growth and deformity of the long bones, was first identified as a disorder. Edward Mellanby discovered vitamin D and its role in preventing rickets in the early 20th century. By altering the diets of dogs raised in the absence of sunlight, Mellanby concluded that rickets was linked with a deficiency of a trace component present in the diet. Furthermore, he established that cod liver oil was an excellent anti-rachitic agent in dogs as the ability is identical with fat-soluble vitamin A.[69] In 1921, McCollum and colleagues observed that bubbling oxygen through a preparation resulted in the retention of vitamin D but inactivated vitamin A. Consequently, they explored the idea that the anti-rachitic agent found in certain fats was distinct from vitamin A.[69] The chemical structure of vitamin D was determined in the 1930s by Windaus et al.[70] Simultaneously, vitamin D was discovered with many other vitamins and rapidly classified as a vitamin. However, recent studies have demonstrated that vitamin D is more specifically a prohormone, not only a vitamin.

Vitamin D consists of a group of fat-soluble sterols that are rarely found in natural foods. Vitamin D_3 (Cholecalciferol) also can be produced in the skin through ultraviolet irradiation of 7-dehydrocholesterol, a precursor of cholesterol. It is biologically inert and must be converted to $25(OH)D_3$ in the liver, which acts as a prohormone for the renal production of the $1,25(OH)_2D_3$ hormone, also known as calcitriol. Despite the ability to synthesize vitamin D in the skin and the availability of vitamin D in some dietary sources, vitamin deficiency is indeed a widespread problem. Vitamin D deficiency is highly prevalent in adults over the age of 65 and in patients with osteoporosis. In particular, numerous studies have reported deficiency is common in postmenopausal women.[71]

Vitamin D hormone along with the actions of parathyroid hormone (PTH) and calcitonin is the basic system maintaining plasma calcium. The hormonal metabolite of vitamin D, $1,25(OH)_2D_3$ induces the proteins involved in active intestinal calcium absorption and further stimulates active intestinal absorption of phosphate. In addition, both vitamin D and PTH are involved in the mobilization of calcium from bone when it is absent from the diet. Simultaneously, vitamin D enhances the resorption of distal renal tubules for calcium to achieve an adequate level. Under normal circumstances, environmental calcium is used at the beginning and then the internal stores are gradually depleted if environmental sources are absent. [71]

The bioactive vitamin D metabolite functions through binding to the cellular vitamin D receptor (VDR). It has the high affinity binding to the cellular VDR that forms a heterodimer with the retinoid X receptor (RXR). In turn, RXR is capable of binding to vitamin D response elements in various genes, and causes the transactivation or repression of vitamin D response genes in a variety of tissues. Moreover, bone is one of

the major target organs of vitamin D, and VDR ligands regulate both bone resorption and formation. $1,25(OH)_2D_3$ has been considered to stimulate bone resorption because it enhances production of RANKL in bone marrow by osteoblasts, resulting in osteoclastogenesis.[72] However, VDR ligands decrease bone resorption and increase bone formation in ovariectomized animals and osteoporotic women. A number of studies have shown that $1,25(OH)_2D_3$ increases the expression of osteocalcin in osteoblasts and total body and spine bone mineral density in osteoporotic individual.[73] Physiological doses of $1,25(OH)_2D_3$ preferentially stimulate the intestinal absorption of calcium without inducing bone resorption, which then stimulates bone mineralization.[72] Therefore, appropriate $1,25(OH)_2D_3$ levels enable the normal mineralization of bone, which is associated with bone growth and bone remodeling by osteoblasts and osteoclasts, protecting older adults from osteoporosis.[74]

Interestingly, accumulating evidence indicates that VDR exist in a large number of different cells throughout the body other than those of the intestine, bone, kidney and parathyroid gland resulting in the reorganization of non-calcemic actions of VDR ligands. Recent studies suggest that vitamin D may play an important role in the regulation of body fat content.[75, 76] Adipogenesis is mediated at the molecular level through VDR-dependent inhibition of C/EBPα and PPARγ expression and decrease in PPARγ transactivation activity. A growing body of evidence suggests that the expression of adipocyte specific transcription factors like C/EBPβ and PPARγ was markedly suppressed by $1,25(OH)_2D_3$ in mouse epididymal fat tissue cultures, and in addition to inhibiting adipogenesis, $1,25(OH)_2D_3$ also induced apoptosis in 3T3-L1 preadipocytes.[77, 78] Furthermore, osteogenic differentiation of adipose tissue-derived mesenchymal stem

cells and bone marrow-derived mesenchymal stem cells can be induced by $1,25(OH)_2D_3$, which acts directly on stem cells to alter the developmental path towards osteogenesis.[79, 80] The observations showed the significantly negative association between serum $25(OH)D_3$, body fat mass and BMI, indicating the potential role of vitamin D in adiposity.[81, 82] Consequently, several investigations have found that serum concentrations of $25(OH)D_3$ are inversely related to the prevalence of diabetes and to blood glucose concentrations.[83] In women over 40 years old with vitamin D deficiency, the prevalence of metabolic syndrome was significantly higher than in women with normal vitamin D levels of a similar age.[84, 85] Hence, the possible importance of vitamin D status has gained more attention because of the rising prevalence of obesity-related diseases.

Genistein

Because of the protective effects of estrogen, hormone replacement therapy (HRT) administered in a dose dependent manner effectively prevents bone loss in postmenopausal women.[86] Unfortunately, it has been also reported that long term HRT results in a 26% increase in breast cancer and, surprisingly, an increase in the risk of heart disease and stroke among those taking the hormone therapy, compared with women taking a placebo.[86] On the other hand, Asian populations, such as those in Japan, Taiwan and Korea, are estimated to consume 20-150 mg/day of isoflavones, with a mean of about 40mg from tofu and miso, showing reduced rates of hip fractures in the epidemiologic evidence.[87] Hence, of all the natural alternatives currently under investigation, phytoestrogen supplementation may benefit some women by alleviating menopausal symptoms.

The majority of phytoestrogens found in typical human diets are classified as two primary classes: isoflavones and lignans.[89] Specifically, genistein (4,5,7-trihydroxyisoflavone) is the most extensively studied isoflavone at present. Most isoflavones, genistein and daidzein, exist in plants in the bound form as glycosides and are biologically inactive.[88] Genistein is also formed from the precursor, biochanin A, after breakdown by intestinal glucosidases. The metabolism of phytoestrogens is likely facilitated by colon bacteria, which remove the sugar moiety producing active compounds.[89]

Foods containing isoflavones are found in a variety of plants including fruits and vegetables, but they are predominantly found in leguminous plants and are especially abundant in soy. The concentration of genistein in most soy food products ranges from 1-2 mg/g protein. Generally, many Asian populations consume 20-80 mg/day genistein, almost entirely derived from soy, and have low rates of breast and prostate cancer, whereas the dietary intake of genistein in the United States has been estimated at only 1-3 mg/day.[90]

In addition, the content of isoflavones in different soy products varies due to a wide diversity of processing steps.[91] Once ingested, isoflavones are subject to several factors that play an important role in determining the preferred pathways of metabolism and the bioavailability. The complex metabolic reactions occur in the gastrointestinal tract after consumption of plant lignans and isoflavones, resulting in the formation of heterocyclic phenols whose structure shares many similarities with endogenous estrogen.[92] Because of this property, isoflavones have been investigated as estrogen substitutes for treatment of symptoms associated with the reduction of natural estrogens

during menopause. Of primary interest has been their use for treatment of bone loss and weight gain resulting from the withdrawal of natural estrogens.

Estrogen receptors ERα and ERβ are found in the main cell types in human bone including osteoblasts, osteoclasts, and osteocytes, although the expression of the ERs varies considerably during differentiation.[93] Studies indicate that genistein, which is structurally similar to estrogen, has agonist activity for both estrogen receptors, but has greater affinity for ERβ (8.4 nM) than ERα(145 nM).[94, 95] Genistein exerts its ability to mediate the signals by interacting with nuclear ERs to either activate or inhibit transcription of specific genes.[96]

There is considerable evidence from *in vitro* studies and *in vivo* studies in rodents, as well as from some clinical trials in women that isoflavones such as genistein, and other phytoestrogens, have a beneficial effect in reducing bone loss and in some cases stimulating new bone growth.[97-103] Studies indicated that genistein has been shown to suppress the production of IL-6 and to stimulate the production of OPG in human osteoblast-derived cell lines.[104, 105] It is well documented that an increase in bone turnover accompanied by a dramatic shift toward the direction of adipogenesis is the major cause of osteoporosis. Furthermore, Heim et al.[106] reported that genistein decreased the adipogenic differentiation via TGF-β signaling and enhanced the commitment and differentiation of bone marrow stromal cells to the osteoblast lineage, providing a further mechanism for the bone effects of soy isoflavones.

Although *in vitro* studies provide useful insight into the possible actions of genistein on bone cell lines, it is important to take account of their bioavailability and biological activity by digestion. Blair et al. tested the effects of pure genistein in

ovariectomized rats and found that it increased BMD by 12% with a month of treatment.[107] In postmenopausal women, dietary isoflavone intake has been associated with decreased BMI, body weight, waist circumference, and total body fat mass.[108] Morabito et al. reported a randomized double-blind, placebo-controlled clinical study of supplementation with genistein (54 mg/d), a standard HRT combination (17β-estradiol/norethisterone acetate), or placebo; effects were monitored at 6 and 12 months.[99] They showed that genistein significantly increased BMD in the femur and lumbar spine without exerting adverse effects on uterus and breast, suggesting the positive effect of genistein on reducing bone resorption and enhancing new bone formation. While the mechanisms of action for isoflavones remain elusive, the presence of estrogen receptors in bone and the wide-ranging biological properties of genistein should play a role in bone remodeling.

Quercetin

Flavonoids have attracted attention in relation to their ability to act against oxidative stress-related chronic diseases. Most flavonoids share a common three-ring structure and are categorized as flavonol, flavanol, flavanone, flavone, isoflavone and anthocyanidin.[109] Quercetin (3,5,7,3',4'-pentahydorxyflavone) is the typical flavonol present in the plant kingdom. In contrast to genistein, quercetin is a fairly common component of Western diets, as it is found in high amounts in apples, tea, wine, and is especially abundant in onion extracts (200-600mg/quercetin/kg onion).[110] In particular, the intake of quercetin was estimated to range between 3 and 38 mg/day in the Seven Countries Study.[111]

The chemical activities of quercetin can be attributed to its reducing activity, which results from the presence of a phenolic hydroxyl group. This property contributes to the anti-oxidant activity by scavenging free radicals, such as superoxide anions, perhydroxyl radicals and chain-propagating lipid peroxyl radicals.[112] Quercetin and similar flavonols have been particularly studied for their ability to inhibit cancer cell growth and induce apoptosis of tumor cells. Their cancer-protective effects have been attributed to various mechanism including their anti-oxidative activity, the inhibition of enzymes that activate carcinogens, the modification of signal transduction pathways, and interactions with receptors and other proteins.[112] One study showed that the ability of quercetin and other flavonoids to inhibit growth and induce apoptosis paralleled their ability to inhibit fatty acid synthesis in tumor cells.[113]

On the other hand, there has been a focus on several vegetables for their potential beneficial effects for prevention of bone resorption. Among them, onions have been observed to have the additive effect to regulate bone metabolism.[114] In addition, a glycosidic quercetin, rutin (quercetin-3-*O*-glucose rhamonse), inhibits trabecular bone loss in ovariectomized rats, both by suppressing resorption and increasing osteoblast activity.[115] Therefore, a growing body of evidence suggests that quercetin has inhibitory effects on osteoclasts [116-119], promoting the differentiation of human stromal cells to osteoblasts, inhibiting differentiation to adipocytes [120, 121] and reducing bone resorption.[115]

Resveratrol

Resveratrol (3,5,4' trihydroxystilbene) is a naturally occurring phytoalexin, used by the plant to defend itself against fungal and other attacks such as pathogens, injuries,

stresses, UV irradiation, chemicals, and climatic conditions.[122] Its structure belongs to a class of polyphenolic compounds called stilbenes, and both *cis* and *trans* isomers can be found in grapes and grape products such as red wine, with the significantly higher concentrations found in the latter.[123, 124]

Resveratrol has been used extensively in traditional Asian medications for treatment of fungal, inflammatory, hypertensive, allergic, and heart diseases.[125] In 1992, Siemann et al. suggested that resveratrol might be the biologically active compound of red wine [126]. This finding might help explain the "French Paradox" which is the observation that populations with moderate drinking of red wine (i.e. consuming the equivalent of 1 glass of wine, 1 to 6 times per week) over a long period of time appear to have a lower incidence of heart disease.[127] The incidence of coronary heart disease in the French population is low, even though their diet is rich in fat. Since then, scientists became interested in investigating potential health benefits of resveratrol.

There is growing evidence to indicate that resveratrol is a potent inhibitor of platelet aggregation because of its activity to suppress the oxidation of polyunsaturated fatty acids (PUFA) found in LDL that plays a major role in atherosclerosis.[128, 129] In addition to the cardiovascular effects, resveratrol has also been shown to have antioxidant, anti-inflammatory, antiproliferative, and pro-apoptotic activities that contribute to anti-cancer activity.[130-133] Interestingly, in the last five years, the data from lower organisms have provoked intense research, indicating that resveratrol activates sirtuin enzymatic activity (Sir2) to retard the aging process.[134, 135] Furthermore, activation of the mammalian Sir2 homologue SIRT1 by resveratrol has been identified that appear to mimic some aspects of caloric restriction in multiple organisms.[136] Indeed, there is

growing evidence that resveratrol can prevent or delay the progression of a wide variety of illnesses.[135]

Moreover, the similarity in structure between trans-resveratrol and the synthetic estrogen diethylstilbestrol (DES) indicates the potential phytoestrogenic activity. Like genistein, resveratrol has a higher affinity for the estrogen receptor β (ERβ) than α and transcriptionally activates ERβ at low concentrations.[137] Resveratrol has been shown to stimulate proliferation and differentiation of osteoblasts and to inhibit activity of osteoclasts because of the ability to bind ERβ, which has been shown to be expressed in higher levels in bone marrow.[138, 139] This may account for the activity of phytoestrogens in preventing bone loss. Furthermore, resveratrol (0.7 mg/kg) was shown to increase bone mineral density and to inhibit bone loss in ovariectomized rats, suggesting that it could play a role in protecting against bone loss induced by estrogen deficiency.[140].

Conclusion

Treatments that prevent or reverse the increase in bone marrow adipocytes could both increase new bone formation and inhibit bone destruction. Likewise, vitamin D and certain phytoestrogens have been shown to act directly to inhibit activities of adipocyte and osteoclast and promote differentiation of osteoblasts. Moreover, growing evidence suggested that resveratrol can prevent or delay the progression of a wide variety of illnesses by mediating the SIRT1 activity. Therefore, the objective of this study was to examine the effects of vitamin D in combination with genistein, quercetin, and resveratrol on body weight and bone mass in ovariectomized female rats.

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CHAPTER 3

Preventing Bone Loss and Weight Gain with Combinations of Vitamin D and Phytochemicals[1]

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Preventing Bone Loss and Weight Gain with Combinations of Vitamin D and Phytochemicals

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Running Title: Effects of vitamin D plus phytochemicals on bone

Abstract

Vitamin D and certain natural compounds have been shown to regulate both lipid metabolism and bone formation. Treatments that prevent or reverse age-related increase in bone marrow adipocytes could both increase new bone formation and inhibit bone destruction. In this study, we tested the hypothesis that dietary supplementation with combinations of vitamin D and phytochemicals inhibit bone loss and decrease adiposity to a greater extent than control or vitamin D alone diets. Aged ovariectomized female rats (12 mo old, N=10, initial BW=240g) were given control (AIN-93M diet alone), vitamin D (AIN-93M with 2400 IU/kg diet), or vitamin D with resveratrol (R: 16, 80, or 400 mg/kg diet), quercetin (Q: 80, 400 or 2000 mg/kg diet), and genistein (G: 64, 256 or 1040 mg/kg diet). Animals were sacrificed after an eight-week treatment period. The high dose combination treatment (Vit. D+R400+Q2000+G1040) significantly reduced body weight gain ($p<0.05$), and the fat pads as a percentage of body weight ($p<0.05$). Treatment with the high dose combination also significantly increased the serum concentration of IGF-I compared to control ($p<0.05$). Moreover, densitometry results showed that bone mineral content (BMD) of femur was significantly increased by the high dose combination, although they were not different from vitamin D alone. However, BMC corrected for body weight was significantly greater in the high dose treated group compared to both control and vitamin D groups ($p<0.05$). The results obtained from microCT analysis indicated that the high dose combination treated rats significantly preserved trabecular bone mass in the distal femur ($p<0.05$). Additionally, histomorphometric analysis from the right tibia indicated that rats receiving the high dose combination had fewer marrow adipocytes ($p<0.001$) and significantly reduced osteoclast

number in trabecular bone ($p<0.05$). We conclude that aged ovariectomized female rats supplemented with vitamin D combined with genistein, quercetin and resveratrol had improved bone density and reduced body weight gain and marrow adipocytes. The synergistic effects of a combination of phytochemicals with vitamin D may be effective in reducing bone loss and weight gain after menopause.

Key words*: *Osteoporosis, Bone loss, Obesity, Vitamin D, Genistein, Phytochemicals

Introduction

Obesity and osteoporosis are major public health concerns due to their prevalence in the increasingly sedentary and aging society. In the United States today, an estimated 55% of people 50 and older are at risk for developing osteoporosis.[1, 2] An estimated 10 million Americans suffer from osteoporosis, and eighty percent of those are women. Because as much as 20% of bone mass can be lost in the first five to seven years following menopause, osteoporosis is a major health issue for aging women. [3]

It is well known that mesenchymal stem cells (MSC) in bone marrow can differentiate to form a variety of cell types including osteoblasts and adipocytes. As a person increases in age, adipocytes tend to accumulate in certain regions of the body such as bone marrow, liver, and muscle. Studies have demonstrated that women with osteoporosis have higher numbers of marrow adipocytes than women with healthy bone quality, and bone formation rate is inversely correlated with the number of adipocytes in bone marrow.[4, 5] Therefore, treatments that direct MSC towards the osteoblast lineage rather than adipocyte lineage, should prevent the accumulation of adipocytes within bone marrow, decreasing the risk of osteoporosis and fractures in the aging population.[6]

Studies have shown that estrogen and vitamin D can be effective in the management of postmenopausal osteoporosis. Likewise, the isoflavone genistein (4,5,7-trihydroxyisoflavone), which shares a structural similarity with estrogen and has a high affinity for estrogen receptor-β (ER-β) has been shown to improve bone parameters in several studies.[7, 8] For example, Morabito et al. have shown that supplementation with genistein (54 mg/d) significantly increased BMD in the femur and lumbar spine in postmenopausal women without exerting adverse effects on uterus and breast.[9] Genistein has also been shown to decrease food intake, body weight, and fat pad weight and induce apoptosis of adipose tissue in ovariectomized mice.[10]

Quercetin (3,5,7,3',4'-pentahydroxyflavone) is a flavonol present in a wide variety of plants, and especially predominately found in onion.[11] Interestingly, onions have been observed to exert a particularly strong effect in regulating bone metabolism in ovariectomized rats.[12] Quercetin alone has been shown to have inhibitory effects on osteoclasts and to promote the differentiation of human stromal cells to osteoblasts, thus inhibiting differentiation to adipocytes. [12, 13]

Resveratrol (*trans*-3,5,4'-trihydroxystilbene), a naturally occurring phytoalexin found in red wines, is also believed to stimulate proliferation and differentiation of osteoblasts and inhibit activity of osteoclasts.[14] Interestingly, in the last five years, the data from lower organisms have provoked intense study, indicating that resveratrol has ability to retard the aging process by stimulating sirtuin enzymatic activity (Sir2).[15, 16] Furthermore, activation of the mammalian Sir2 homologue SIRT1 by resveratrol has been demonstrated and appears to mimic some aspects of caloric restriction in multiple organisms, indicating that resveratrol can prevent or delay the progression of a wide

variety of illnesses.[17] Like genistein, resveratrol has been shown to stimulate proliferation and differentiation of osteoblasts and to inhibit activity of osteoclasts because of the ability to bind ER-β, which has been shown to be expressed in higher levels in bone marrow.[14, 18] Furthermore, resveratrol (0.7 mg/kg) was shown to increase bone mineral density and to inhibit bone loss in ovariectomized rats, suggesting that it could play a role in protecting against bone loss induced by estrogen deficiency.[19] While the mechanisms of action for phytoestrogens remain elusive, the presence of estrogen receptors in bone and the wide-ranging biological properties of phytoestrogens indicate that they likely play a role in bone remodeling.

In addition to regulating the concentration of plasma calcium, vitamin D can act directly on stem cells to alter the developmental path towards osteogenesis to increase bone formation and alter adipogenesis.[20] Interestingly, accumulating evidence indicates that vitamin D receptor (VDR) exists in a variety of cell types, in addition to intestine, bone, kidney and parathyroid gland, resulting in non-calcemic actions of VDR ligands. A growing body of evidence suggests that vitamin D has direct effects on adipocytes. For example, the expression of adipocyte specific transcription factors like C/EBPβ and PPARγ was markedly suppressed by $1,25(OH)_2D_3$ in mouse epididymal fat tissue cultures, and in addition to inhibiting adipogenesis, $1,25(OH)_2D_3$ also induced apoptosis in 3T3-L1 preadipocytes.[20, 21]

Furthermore, osteogenic differentiation of adipose tissue-derived mesenchymal stem cells and bone marrow-derived mesenchymal stem cells can be induced by $1,25(OH)_2D_3$, which acts directly on stem cells to alter the developmental path towards osteogenesis.[22, 23] A significantly negative association between serum $25(OH)D_3$

concentration and body fat mass or BMI, indicates the potential role of vitamin D in modulation of adiposity.[24, 25]

Numerous studies have investigated the effects of individual natural compounds to determine underlying mechanisms of action on bone and adiposity. However, relatively few studies have investigated the effects of vitamin D combined with natural compounds, although flavonoids occur naturally as combinations in low concentration. Previously, we found that the combination of genistein and $1,25(OH)_2D_3$ cause significant increases in the expression of VDR in maturing 3T3-L1 adipocytes, and results in an enhanced inhibition of lipid accumulation and induction of apoptosis.[26]

We have proposed that the molecular mechanisms involved in the synergistic enhancement of activity with a combination of specific natural compounds and vitamin D *in vitro* may lead to a new strategy for preventing the increase in bone loss and adiposity that occurs with the onset of menopause.[27-30] The objective of this study was to examine the effects of vitamin D in combination with genistein, quercetin, and resveratrol on body weight and bone mass in ovariectomized female rats.

Materials and Methods

Animals and design

Fischer 344 rats (12 months old, N=55) were obtained from the National Institute on Aging (NIA). Upon arrival the rats were housed in individual cages with food and water available ad libitum. They were adapted to a semipurified phytoestrogen-free casein-based diet with 4% safflower oil replacing the soybean oil for 5-7 days (Mod AIN-93M® 5SQV, TestDiet, Richmond, IN), after which they were weighed and randomly assigned to one of five treatment groups (N=10), as shown in the Table 3.1. Two rats from each treatment group were ovariectomized each day over a 5-day period. All rats were allowed to recover from surgery for 3 days before the test diets were provided. Compounds were thoroughly mixed with the diets and provided for the next 8 weeks. Food containing the test compounds were prepared weekly in the AIN-93M diet containing 2.4 IU/g Vit D (Mod AIN-93M® 5SQU, TestDiet, Richmond, IN), and stored in plastic bags within airtight containers at 4°C. Food intake was monitored on a daily basis and body weights were measured weekly. Rats were fasted for 2 hours, weighed and then killed by CO_2 asphyxiation followed by decapitation.

Tissue collection

After rats were sacrificed, trunk blood was collected into a tube containing DPP4 protease inhibitor (10 µl/ml of blood) (Millipore, Billerica, MA) and glucose concentration was determined by using the FreeStyle Blood Glucose monitoring system (Abbott, Alameda, CA) that required 0.3 ml of whole blood. The remaining blood was allowed to clot on ice, and then centrifuged (2,000 g) for 20 min to obtain the serum. The serum samples were stored at -80 °C for measurements. Inguinal (I), parametrial (P) and

retroperitoneal (Rp) fat pads were removed bilaterally, weighed and fixed in 4% paraformaldehyde. Intrascapular brown adipose tissue, and gastrocnemius and soleus muscles were removed, weighed and frozen immediately in liquid nitrogen. The right hindlimb was fixed for 24 hours in 10% neutral buffered formalin and then stored in 70% ETOH prior to densitometry. Uteri were removed, and sectioned so that the length from the junction and each horn was of equal distance. It was then weighed and stored in 10% neutral buffer formalin. All surgical and experimental procedures proposed in this study were conducted in accordance with the NIH Guidelines and was approved by the Animal Care and Use Committee for The University of Georgia prior to initiating the study.

Serum concentration of biomarkers

Serum concentrations were determined using the Luminex100™ instrumentation and following manufacturer's instructions for the single and multi-plex kits (Millipore, Billerica, MA). Insulin, leptin, glucagon, and GLP-1 were determined using the Rat Endo Multiplex Kit (RENDO-85K-04). Rat bone single-plex kits were used to determine the serum concentrations of RANKL (RBN-31K-1RANKL), OPG (RBN-31K-1OPG), osteocalcin (RBN-31K-1OC), and IGF-I (RMIGF187K).

Bone densitometry

Muscle, skin and major tendons were removed from the right femur before testing. The whole-bone mineral content (BMC) and bone mineral density (BMD) of the right femur was determined by using Dual-energy X-ray absorptiometry (DEXA) densitometry (PIXImus system, GE Lunar Corp., Waukesha, WI).

Microcomputed tomography analysis

The trabecular bone microstructures of the right distal femur were measured with a high-resolution microcomputed tomograph scanner (μCT; Skyscan 1072; Skyscan, Aartselaar, Belgium). Four samples were selected from each of the following groups: Control, Vit D, and High. The X-ray source was set at 60 kV and 165 μA, with a pixel size at 26 μm. The image slides were reconstructed using NRecon software (Skyscan). After images were reconstructed, the region for analysis was 1 mm below the growth plate and extended toward midshift for 3 mm, approximately 267 slides established the volume of interest. Trabecular bone was separated from cortical bone by free drawing the regions of interest using CT analyzer software (Skyscan). A fixed threshold value of 250 was used to analyze the three-dimensional (3-D) parameters and to obtain a 3-D image of the original trabecular bone. The tissue volume (TV, mm^3), the bone volume (BV, mm^3), and bone surface (BS, mm^2) were directly measured from the original 3-D images. The trabecular bone volume (BV/TV; %) and bone surface density (BS/TV) were normalized to compare samples of different sizes. The parameters of trabecular microstructures were trabecular thickness (Tb. Th, μm), trabecular number (mm^{-1}), and trabecular separation (Tb. Sp; mm). The structure model index (SMI) is a parameter to quantify the characteristic in terms of the amount of plates and rods. The degree of anisotropy (DA) defines the magnitude of the preferred orientation of the trabeculae.

Bone histomorphometry

Muscle, skin and major tendons were removed from the right tibia, which was then fixed for 24 hours in 10% neutral buffered formalin and then stored in 70% ETOH. Consequently, the bones were decalcified in 4% EDTA for 3 weeks, changing the buffer

daily and keeping the samples refrigerated. The specimen was checked with a 26-G needle to determine the end point of decalcification. Once the needle could penetrate the bone with ease, the tibia was cut across the proximal third of the shaft. It was then embedded in paraffin followed by dehydration, cleaning, and infiltration. Transverse cross sections were cut 5 μm thick. Some sections were stained with hematoxylin and eosin (H&E) for measuring the number of adipocyte. The number of adipocytes was counted over a 0.10 mm^2 area. Other sections were stained for tartrate-resistant acid phosphatase (TRAP) (Sigma Kit 386, Sigma, St. Louis, MO) as a marker of active osteoclasts. For TRAP staining, slides were deparaffinized, decalcified, and incubated for 1.5 hour (37ºC) in the dark in a solution of 44 ml water, 2 ml acetate solution, 2 ml napthol AS-BI phosphoric solution, 2 ml tartrate solution and contents of 1 capsule of Fast Garnet GBC Salt to develop bright red TRAP localization. Osteoclasts were counted on the endosteal surface and expressed as a number of osteoclasts per bone perimeter (N.Oc/B.Pm).

Statistical analysis

STATISTICA Software (version 7.0, Tulsa, OK) was used for all statistical analysis. Significance of treatment effects was determined by one or two way ANOVA (treatment x block). Significance of differences among treatment means was determined by Fisher's LSD. Significance was established at $p<0.05$.

Results

Food intake and body weight

During 8 weeks of treatment, average daily food intake was not significantly affected by treatments (Control: 14.39±0.2 g; Vit D: 14.96±0. 3 g; High: 14.22±0.3g). According to the approximate amount of average daily food intake (15 g) in each treatment group, the ovariectomized rats daily consumed the treatments with the combined phytochemicals in low dose (G: 4 mg/kg BW + Q: 5 mg/kg BW + R: 1 mg/kg BW), medium dose (G: 16 mg/kg BW + Q: 25 mg/kg BW + R: 5 mg/kg BW), and high dose (G: 65 mg/kg BW + Q: 125 mg/kg BW + R: 25 mg/kg BW). The final body weight was not affected significantly by treatments (Table 3.2). However, the high dose combination significantly reduced total weight gain by 22.5% after 8 weeks of treatment (Control: 80±5 g; Vit D: 81.5±5 g; High: 62.0±3g; $p<0.05$) (Fig. 3.1).

Tissues and fat pad weights

Inguinal fat pads significantly decreased by 9.2% in rats treated with the high dose combination ($p<0.05$) (Table 3.2). Additionally, the high dose combination significantly reduced retroperitoneal plus inguinal fat pads as a percentage of body weight by 14.6% compared to control ($p<0.05$) (Fig. 3.2). The administration of phytoestrogen treatments increases the uterine weight, and the high dose combination was associated with a trend towards a higher uterine mass, reflecting the estrogenic effects on this organ (Table 3.2).

Serum concentration of biomarkers

Rat treated with high dose combination significantly increased serum IGF-I levels by 32% when compared to rats treated with control ($p<0.05$). However, the other serum biomarkers such as insulin, leptin, osteocalcin were not affected by any of treatments (Table 3.3).

Bone densitometry

Results from the DEXA analysis showed that the high dose combination was associated with the greater BMD in the femur, although the results were not different from those found with vitamin D alone. In addition, there was no significant difference in BMC between treatments (Fig. 3.3). However, BMC corrected for body weight was 17.1% and 10.4% greater in the high dose combination treatment compared to both the control and vitamin D alone, respectively ($p<0.05$) (Fig.3.4).

Microcomputed tomography analysis

After eight weeks of treatment, the trabecular bone in distal femur was markedly diminished in the control group after ovariectomy. Diet containing enhanced vitamin D could partially prevent trabecular bone loss and administration of high dose combination to the ovariectomy rats efficiently retained most of trabecular bone as revealed by the 3-D pictures (Fig. 3.5). The indices of trabecular bone microarchitecture, including BV/TV, BS/TV, Tb. Th and Tb. N in the rats treated with the high dose combination were significantly higher than those of control and vitamin D groups. As shown in Table 4, the high dose combination significantly increased BV/TV and BS/TV by 108% and 45% ($p<0.01$ for both). Furthermore, Tb. Th ($p<0.05$) was 25% higher in the rats treated with

the high dose combination and led to a 40.4% reduction in Tb. Sp ($p<0.05$). On the other hand, SMI and DA were suppressed in the rats treated with high dose combination ($p<0.001$ for SMI, $p<0.002$ for DA). Taken together, the results suggest that the high dose combined treatment was able to prevent the trabecular bone loss after loss of natural estrogen.

Bone histomorphometry

Histomorphometry data from the right tibia showed that the high dose combination treatment significantly decreased the bone marrow adipocyte number by 40.9% and 27.7% as compared to the control and vitamin D alone ($p<0.05$) (Fig. 3.6). TRAP-stained sections indicated that osteoclasts along the endosteal surface were significantly decreased by 43% and 47.7% in rats receiving the medium and high dose combination as compared to the control, respectively ($p<0.05$) (Fig. 3.7).

Discussion

The findings with a decrease in fat pad are consistent with a recent study demonstrating that genistein has anti-lipogenic effects in ovariectomized mice which primarily reflect decreases in adipose tissue size.[31] Naaz et al. have been suggested that 500-1500 ppm dietary genistein produce dose-dependent decreases in adipose tissues of 37-57% after a 12-day treatment and that the anti-lipogenic effect of genistein was similar in subcutaneous (inguinal) as well as in visceral (parametrial and retroperitoneal) fat pads. However, in the current study, we only found a significant decrease in weight of the inguinal fat pad. It could be due to the difference in the age of rodents used in those two studies. In our study, we used the 12-month-old rats instead of juvenile mice (25-27 days old), which were used in the study by Naaz et al. The effect of the treatment is more prominent in the juvenile model than in the aging ovariectomized model, possibly associated with the sensitivity of estrogen receptor. Additionally, a small part of parametrial fat pad was removed during the process of ovariectomy, which along with the age of the rats might have contributed to the bias in the final result. Nevertheless, the high dose combination treatment significantly reduced the percentage of body fat as corrected by their final body weight.

Our data demonstrated that high dose combination treated rats tended to gain less weight as compared to control and vitamin D alone groups. The beneficial effect of treatment on body weight and fat mass were not resulted from decreased food intake, indicating that the treatment increased energy expenditure and modulated metabolic homeostasis. Among the phytochemicals used in the study, 22.4 mg/kg/day of resveratrol has been demonstrated to increase mitochondrial number, which consequently

affects energy expenditure, by activating SIRT1 and this effect is reflected significantly in the mice with a high fat diet.[32] Hence, we hypothesized that decrease in body weight gain and fat mass in rats daily treated with 25 mg/kg of resveratrol might be correlated to changes in energy expenditure.

The current data also indicate that bone mass was significantly increased by the high dose combination, although the result was not different from those produced by vitamin D alone. Simultaneously, serum IGF-I concentration was elevated which stimulates proliferation and differentiation of the osteoblast, as evidenced by the increase in BMD in our study. In this regard, Arjmandi et al. have reported that administration of daily supplement with 40 g soy protein for 3 months may positively affect bone mass by promoting IGF-I production in postmenopausal women, supporting the beneficial effect of the treatment on bone loss in our study.[33]

Furthermore, histochemical results show loss of bone marrow adipocytes coupled with a decrease in osteoclast number with the high dose combination treatment in ovariectomized rats. These findings are consistent with the recent report that a treatment with 10 mg/kg/day of flavonoids in the ovariectomized rats for 4 months significantly increased the values of dynamic histomorphometric indices for bone formation, decreased bone resorption as measured by the low osteoclast formation, and inhibited the expression of adipogenic genes via down-regulating the activity of PPARγ2 in bone marrow stromal cells.[34] The existence of an inverse relationship between osteogenic and adipogenic differentiation of the mesenchymal stem cells supports the hypothesis that the common progenitors are directed towards the osteoblast lineage, reducing the accumulation of adipocyte lineage. This has important implications for use of

phytochemical combinations as a therapeutic strategy in bone disorders such as osteoporosis.

Synergistic interactions with combinations of phytochemicals for the treatment of cancer have been investigated, indicating that vitamin D can synergize with genistein to inhibit the growth of prostatic or breast cancer cell.[35, 36] Similarly, we have reported that the combination of genistein and vitamin D caused a significant increase in VDR protein levels in maturing preadipocytes, and these results indicate the potentiality of both the increase in apoptosis and suppression of adipogenesis. Moreover, other studies also demonstrated that resveratrol stimulates vitamin D receptor expression in bone marrow osteoblast precursors and synergizes with $1,25(OH)_2D_3$ to induce the expression of osteocalcin and osteopontin.[14] Such findings provide information that the phytochemicals contribute to synergistic activities with $1,25(OH)_2D_3$ through up-regulation of VDR signaling pathway.

Of all the natural alternatives currently under investigation, phytoestrogens appear to provide the most potential with few adverse effects. The study regarding breast safety suggested that daily consumption of 54 mg genistein exhibited a promising safety profile with positive effects on bone formation in postmenopausal women with three year of treatment.[37] Nevertheless, the result of uterine weight in our study showed that a mild uterotropic activity was indeed observed in three doses of combined phytoestrogens, but the change did not differ from that ovariectomized rats received a diet with vitamin D alone. Notably, a chronic safety study in rats indicated that there were no treatment related histopathologic changes in several organs including uterus after 13 weeks of treatment with genistein at dose up to 500 mg/kg/d, even though it was seen an increase

in the uterine weight.[38] One of the limitations in the current study is that we were not able to design shamed-operated group due to the limited capacity of animal facility which serve as a reference to compare the relative uterine weight of the ovariectomized rats treated with control diet and further determine the estogenic effect on uterine weight. It is commonly used to access the proliferation of uterine endometrial by weighing the uterus. Since there are few literatures on potential toxicities of the combined phytochemicals, further study is needed to determine the optimal amount of combinations required for estrogenic-like activity, and most importantly, excreting a promising safety profile with positive effects on both adiposity and bone.

There are some limitations present in this study that may need further studies to address. Firstly, though the static histomorphometric evidence showed the anti-resorptive effects based on decrease in bone marrow adipocytes and osteoclasts, whereas the dynamic histomorphometric indices and activity of osteoblasts related to the effect of bone formation were not confirmed in current study. Secondly, the effect of treatments on intact bone was not accessed as sham-operated rats were only treated with regular diet. Generally, the efficacy on intact bone should be less detectable as ovariectomized rats resemble postmenopausal osteoporosis in human. Even so, the anabolic effect on intact bone should be included in future studies with larger sample size. Thirdly, the activities observed in the rodent study may not be more than predictive of the action/synergies of the components in humans. It is also important to consider the bioavailability and the potential interaction of the compounds. Therefore, application of this research to a clinic trial still requires further investigation to determine safety and effectiveness in human. Although results from rodent experiments cannot be directly extrapolated to human

clinical effects, this study revealed the synergistic effects of vitamin D combined with genistein, quercetin, and resveratrol which may help in developing a new strategy for the prevention of osteoporosis and obesity.

We conclude that supplementation of vitamin D combined with genistein, quercetin, and resveratrol improved bone density and reduced body weight gain in ovariectomized female rats. Most likely, multiple phytochemicals acting on numerous targets on adipocytes and osteoblasts simultaneously can achieve synergistic beneficial effects at a dose known to be well within safe ranges in humans. These synergistic effects of the combination observed in this rat study remain to be tested for efficacy for treatment of obesity and in preventing osteoporosis in menopausal women. Thus, we propose that the synergistic effects of a combination of phytochemicals with vitamin D would be effective in preventing osteoporosis after menopause.

Acknowledgments

This work was supported in part by a grant from the Georgia Research Alliance and by the Georgia Research Alliance Eminent Scholar endowment held by CAB.

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Figure Legends

Figure 3.1 Treatment effect on total body weight gain

Total body weight gain after 8 weeks of treatment in ovariectomized female rats (n=10). Graphs show means ± SEM. a,b: columns without a common letter are different, $p<0.05$

Figure 3.2 Treatment effect on percentage of fat pad weights

Weights of retroperitoneal and inguinal fat pads as a percentage of final body weight (n=10). Graphs show means ± SEM. a,b: columns without a common letter are different, $p<0.05$

Figure 3.3 Treatment effect on bone mineral density (BMD) and bone mineral content (BMC)

Measurement of BMD and BMC in the right femur by DEXA (PIXImus) (n=10). Graphs show means ± SEM. a,b: columns without a common letter are different, $p<0.05$

Figure 3.4 Treatment effect on adjusted bone mineral content (BMC)

Right femur BMC corrected for final body weight (n=10). Graphs show means ± SEM. a,b,c: columns without a common letter are different, $p<0.05$

Figure 3.5 Microcomputed tomography images

Representative 3-D images of diatal femur trabecular bone in rats treated with control, vitamin D and high dose combination by scanning microCT (n=4). Graphs show means ± SEM. a,b: columns without a common letter are different, $p<0.05$

Figure 3.6 Treatment effect on number of adipocytes in the bone marrow

Eight-week treatment effect on bone marrow adipocytes. (a) Paraffin sections from the right tibia stained with H&E showing the images of bone marrow adipocytes from the ovariectomized female rats treated with the control and the high dose combination.

(b) The adipocyte number was expressed as the number of adipocytes in a 0.1mm^2 area of bone marrow. Graphs show means ± SEM. a,b: columns without a common letter are different, $p<0.05$

Figure 3.7 Treatment effect on number of osteoclasts in bone marrow

Eight-week treatment effects on bone marrow osteoclasts. (a) Paraffin sections from the right tibia stained with TRAP activity showing the images of osteoclasts along the endosteal surface from the ovariectomized female rats treated with the control and the high dose combination. (b) Osteoclasts were expressed as the number of osteoclasts per bone surface (N.Oc/B.Pm). Graphs show means ± SEM. a,b: columns without a ommon letter are different, $p<0.05$

Table 3.1 Formulations and nutrient composition of experiment diets.[2]

Treatment group	Control	Vit D	Low	Medium	High
Compounds					
Vitamin D3 (IU/kg diet)[3]	1000	2400	2400	2400	2400
Genistein (mg/kg diet)[4]	0	0	64	256	1040
Quercetin (mg/kg diet)[3]	0	0	80	400	2000
Resveratrol (mg/kg diet)[3]	0	0	16	80	400
Ingredient (%)					
Corn starch	46.5	46.5	46.5	46.5	46.5
Dextrin	15.5	15.5	15.5	15.5	15.5
Casein-vitamin free	14	14	14	14	14
Sucrose	10	10	10	10	10
Powdered cellulose	5	5	5	5	5
Safflower oil	4	4	4	4	4
AIN-93M mineral mix	3.5	3.5	3.5	3.5	3.5
AIN-93M vit Px/2.4 IU/g Vit D_3	0	1	1	1	1
Choline bitartrate	0.25	0.25	0.25	0.25	0.25
L-Cystine	0.18	0.18	0.18	0.18	0.18
Energy (%)					
Protein	13.6	13.6	13.6	13.6	13.6
Fat	9.7	9.7	9.7	9.7	9.7
Carbohydrates	76.7	76.7	76.7	76.7	76.7

[2] Treatments were given by average initial body weight of rats (240g).
[3] Prepared by TestDiet, Richmond, IN
[4] Compounds were received from Spectrum, Gardena, CA

Table 3.2 Final body weight and tissue weights in ovariectomized female rats treated with the combination compounds for 8 weeks.[5]

	Control	Vit D	Low	Medium	High
Body weight (g)	302.21±8	301.09±5.4	299.2±5	298.5±5.2	284.16±5.9
Adipose tissue					
BAT (mg)	439±22	445±13	462±23	415±24	449±35
Parametrial (g)	14.0±1.4	16.4±1.2	15.9±0.9	16.9±0.9	16.5±1.2
Retroperitoneal, (g)	9.8±0.5	10.5±0.6	10.1±1.2	9.5±0.8	9.0±0.4
Inguinal (g)	$11.9±0.5^{ab}$	$12.5±0.7^{b}$	$12.1±0.6^{ab}$	$12.1±0.6^{ab}$	$10.8±0.5^{a}$
Muscle					
Gastrocnemius (g)	2.5±0.05	2.6±0.03	2.5±0.05	2.6±0.04	2.5±0.03
Soleus (g)	163±4.4	167±3.8	166±6.1	169±3.5	152±9.2
Uteri (mg)	$160.09±3.7^{a}$	$175.69±4.6^{ab}$	$178.38±8.5^{b}$	$182.25±6.81^{b}$	$194.02±14.1^{b}$
Liver (g)	9.0±0.3	10.2±0.3	9.5±0.3	10.6±0.9	9.5±0.3

[5] Data are presented as means ± SEM. a,b: columns without a common letter are different, $p<0.05$

Table 3.3 Eight-week treatment effects on glucose level and the serum biomarkers.[6]

	Control	Vit D	Low	Medium	High
Glucose	113.5±4.12	113.6±4.01	121.56±6.01	124±8.59	113.79±3.81
GLP (pM)	51.2±4.1	51.9±5.3	35.6±4.8	35.6±6.7	42.0±5.8
Glucagon (pM)	37.1±8.4	42.9±7.1	35.2±6.9	40.0±7.6	31.3±5.9
Insulin (pM)	153.3±23.5	166.2±19.7	154.0±22.3	177.2±22.8	136.1±11.7
Leptin (pM)	93.7±19.1	152.9±29.1	136.0±20.9	142.1±34.5	148.7±16.5
OPG (pg/mL)	382.1±24.7	439.0±30.5	409.6±30.8	396.4±29.9	374.1±38.4
RANKL (pg/mL)	7.8±0.6	6.7±0.5	7.6±1.1	8.2±0.9	7.8±0.6
Osteocalcin (ng/mL)	77.31±3.8	98.78±8.7	90.05±4.8	86.81±5.1	89.8±9.2
IGF-I (ng/mL)	200.37±12[a]	260.22±19[ab]	249.54±20[ab]	256.05±22[ab]	264.54±30[b]

[6] Data are presented as means ± SEM. a,b: columns without a common letter are different, $p<0.05$

Table 3.4 Trabecular bone parameters of distal femur in rats fed the Control, Vitamin D, and High diets for 8 weeks.[7]

Parameters	Control	Vit D	High	p-value
TV (mm^3)	29.43±0.7	31.38±0.6	30.8±1.6	Ns
BV (mm^3)	5.7±1.3[a]	6.3±0.3[a]	12.6±2.6[b]	0.03
BV/TV (%)	19.3±4.1[a]	20.0±0.7[a]	40.2±6.0[b]	0.01
TS (mm^2)	63.34±0.5	65.2±0.8	64.4±1.8	Ns
BS (mm^2)	225.68±36[a]	241.01±13[a]	344.31±25[b]	0.02
BS/BV (mm^{-1})	41±2.1[a]	38.3±0.7[a]	29.1±3.1[b]	0.009
BS/TV (mm^{-1})	7.7±1.2[a]	7.7±0.3[a]	11.2±0.4[b]	0.01
Tb.Th (mm)	0.096±0.001[a]	0.097±0.001[a]	0.12±0.011[b]	0.05
Tb.N (mm^{-1})	2±0.4[a]	2.07±0.07[a]	3.3±0.18[b]	0.008
Tb.Sp (mm)	0.42±0.07[a]	0.38±0.03[ab]	0.25±0.02[b]	0.05
DA	1.79±0.03[a]	1.76±0.04[a]	1.51±0.03[b]	0.002
SMI	1.59±0.3[a]	1.41±0.04[a]	016±0.42[b]	0.001

[7] N=4, Data are presented as means ± SEM. a,b: columns without a common letter are different, $p<0.05$

Figure 3.1

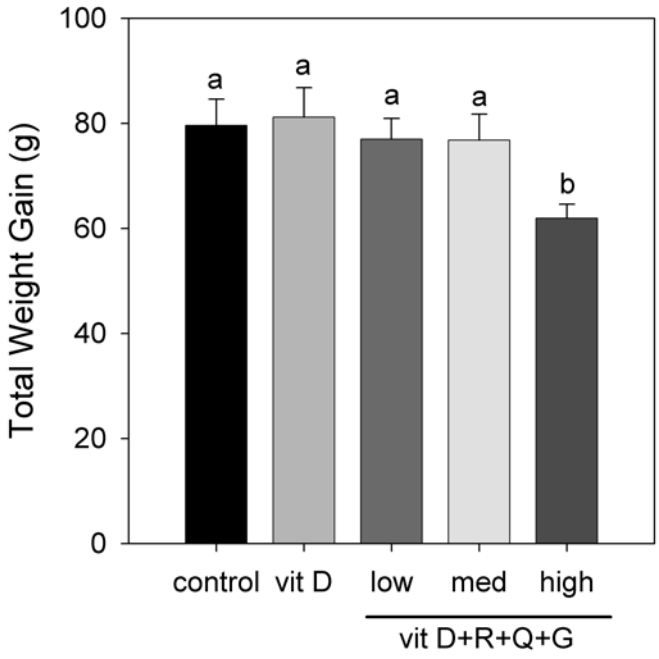

Total Weight Gain (g)
100
80
60
40
20
0
a
a
a
a
b
control
vit D
low
med
high
vit D+R+Q+G

Figure 3.2

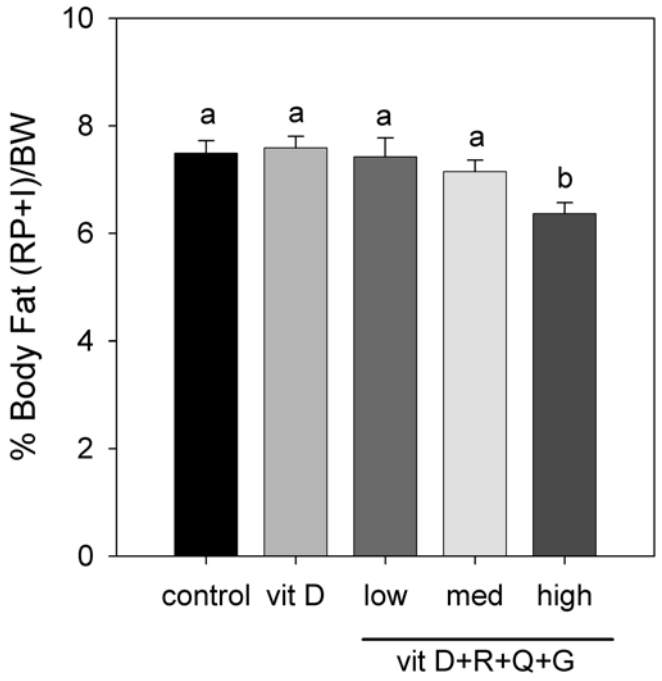

10
8
6
4
2
0
% Body Fat (RP+I)/BW
a
a
a
a
b
control
vit D
low
med
high
vit D+R+Q+G

Figure 3.3

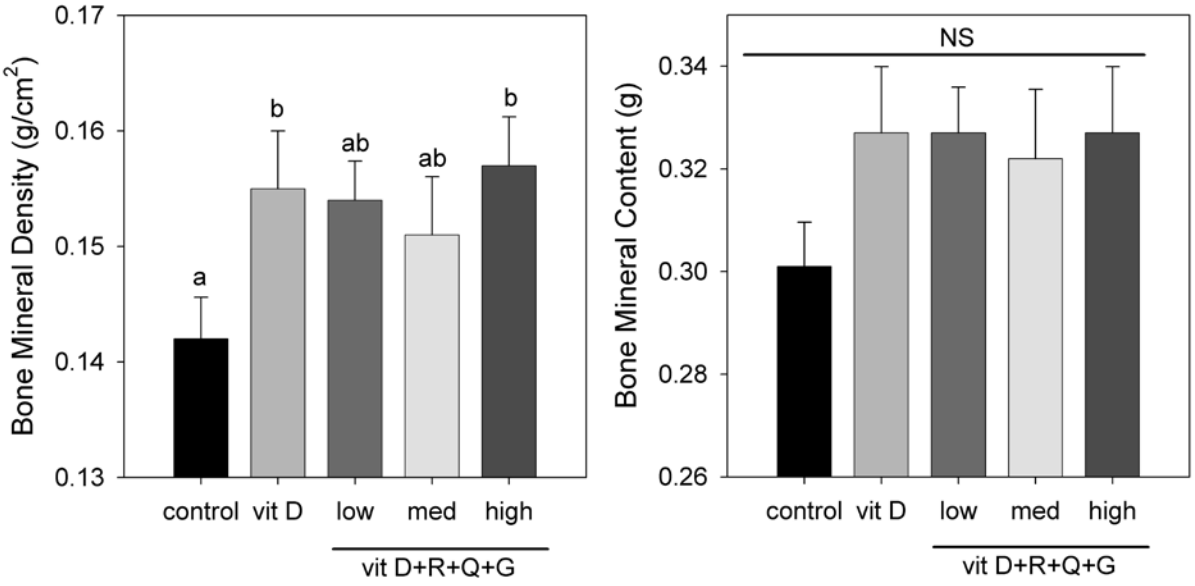

Bone Mineral Density (g/cm^2)
0.17
0.16
0.15
0.14
0.13
a
b
ab
ab
b
control
vit D
low
med
high
vit D+R+Q+G
Bone Mineral Content (g)
0.34
0.32
0.30
0.28
0.26
NS
control
vit D
low
med
high
vit D+R+Q+G

Figure 3.4

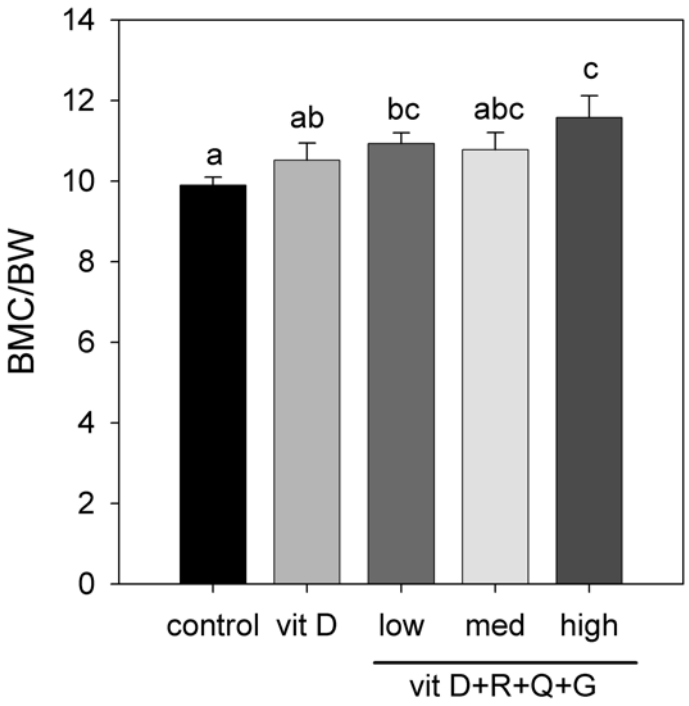

14
12
10
8
6
4
2
0
BMC/BW
a
ab
bc
abc
c
control
vit D
low
med
high
vit D+R+Q+G

Figure 3.5

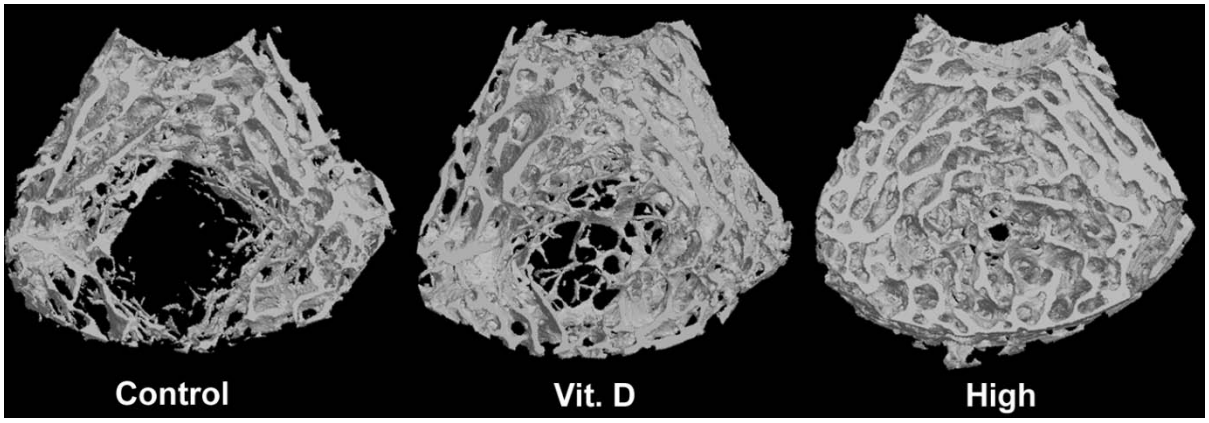


Figure 3.6

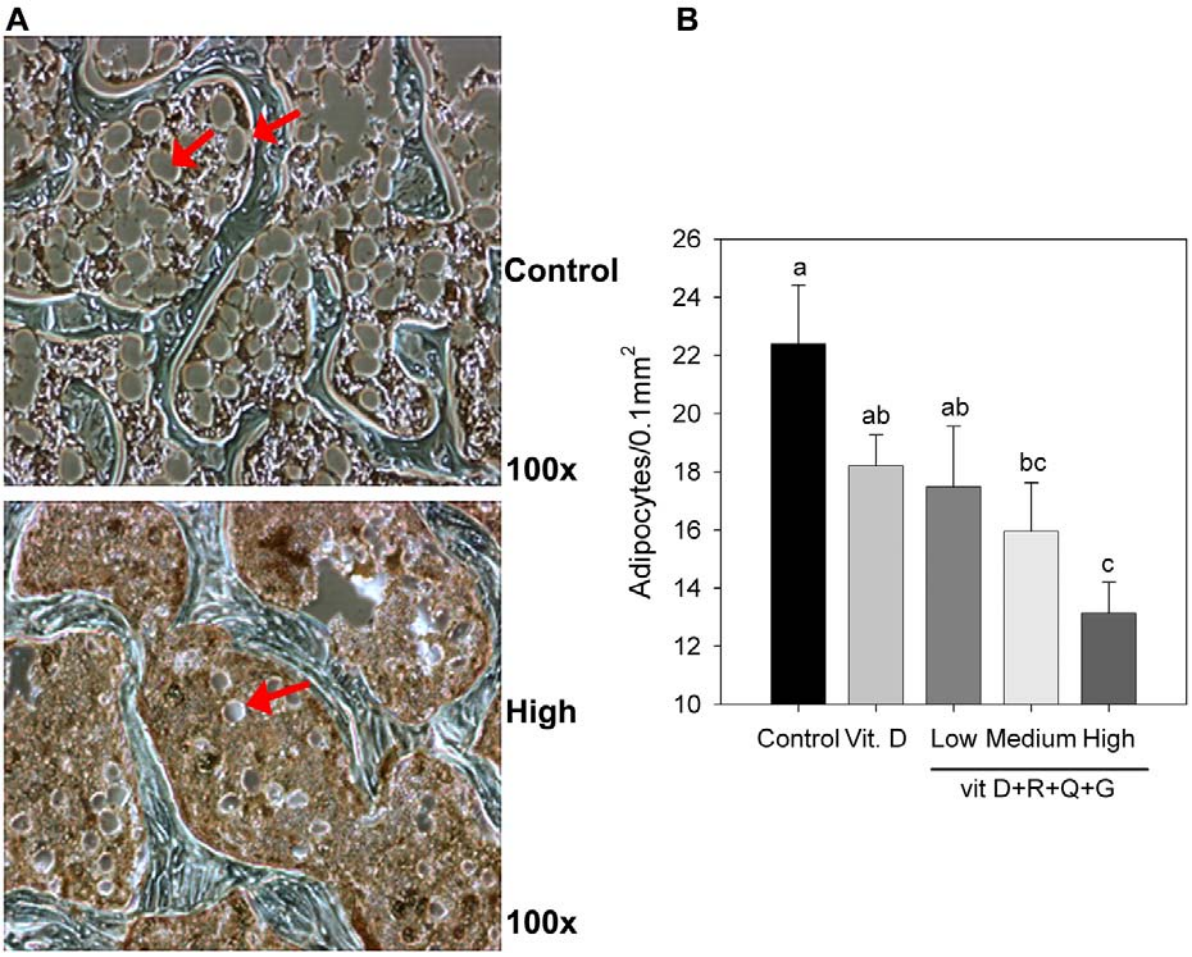
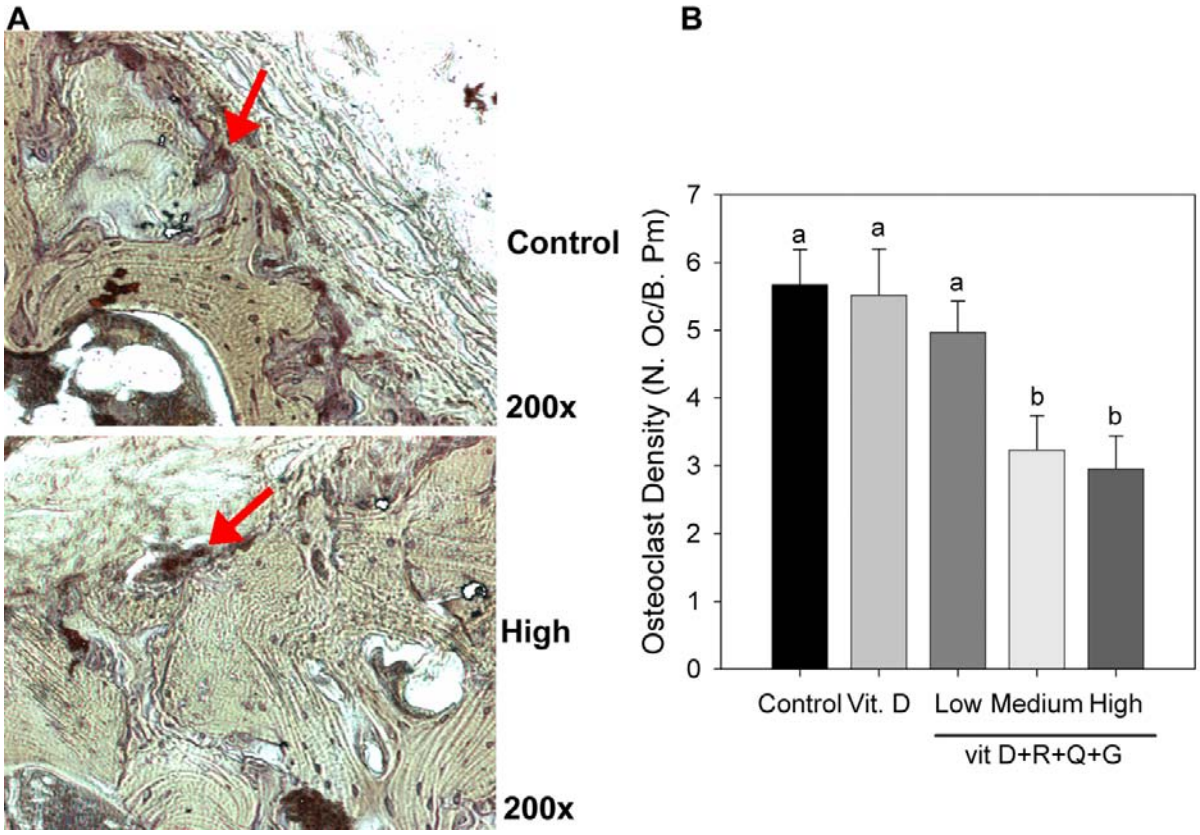

A
Control
100x
High
100x
B
Adipocytes/0.1mm2
26
24
22
20
18
16
14
12
10
a
ab
ab
bc
c
Control
Vit. D
Low
Medium
High
vit D+R+Q+G

Figure 3.7


A
Control
200x
High
200x
B
Osteoclast Density (N. Oc/B. Pm)
0
1
2
3
4
5
6
7
a
a
a
b
b
Control
Vit. D
Low
Medium
High
vit D+R+Q+G

CHAPTER 4

CONCLUSION

The high dose combination treatment significantly reduced weight gain, and fat pad weights as a percent of body weight. In addition, BMD and BMC were significantly increased by the high dose combination, although they were not different from vitamin D alone. However, BMC and BMD corrected for body weight were significantly greater in the high dose combination treatment compared to both control and vitamin D alone. The weight adjusted improvement in bone density, along with a reduction in weight gain and adiposity is an important finding, because decreased adiposity has been associated with decreased bone density. We conclude that in ovariectomized female rats supplemented with vitamin D when combined with genistein, quercetin, and resveratrol improved bone density and reduced body weight gain. Most likely, multiple phytochemicals acting on numerous targets on adipocytes and osteoblasts simultaneously can achieve synergistic beneficial effects at a dose known to be well within safe ranges in humans. Thus, we propose that the synergistic effects of a combination of flavonoids with vitamin D would be effective in preventing osteoporosis after menopause. These synergistic effects of the combination tested in this rat study remains to be tested for efficacy for treatment of obesity and in preventing osteoporosis in menopausal women.

APPENDIX 1

STANDARD OPERATION PROCEDURE FOR RAT OVARIECTOMY

(Abbreviated from the original document found in the lab's SOP handbook)

1. Autoclave surgical instruments, drapes, swabs, etc. either the day before or the day of the surgery.
2. Fill the anesthesia machine with Isoflurane prior to surgery.
3. Turn on Harvard Apparatus® Homeothermic Blanket Control Unit (HBCU) prior to doing surgery to permit the blanket to warm to ~12ºC.
4. Gown up for surgery with gloves, mask and a lab coat.
5. Weigh and record the rat's body weight on the standard surgical form.
6. Anesthetize the rat by securing the nose cone over the rat's nose and mouth. This is achieved by looping the nose cone's dental floss around the front teeth and pulling on the floss to pull the cone snuggly against the nose.
7. Apply Artificial Tears to the eyes.
8. Once the rat is anesthetized, use an electric hair clipper to shave the area from its lower ribs to the pelvis. Use the vacuum to dispose of cut hair.
9. Inject Meloxicam (1mg/kg body weight) subcutaneously.
10. Spray and wipe the shaved area with Betadine® and then wipe clean with 70% ethanol. Repeat.
11. Discard gloves and put on a sterile pair of surgical gloves.
12. Locate the rat's pelvis/flank with the thumb and use the index finger to locate the last rib. Pull the skin tight between these two bones and make a blunt lateral incision with

a scalpel approximately two-thirds away from the last rib. Press hard to cut through the skin and continue to pull the scalpel toward the rat's spine until a 1.5 cm lateral incision is made.

13. Use scissors to open the area beneath the incision using the following technique: Close the tips and insert them into the center of the incision, then open the tips to separate the muscle. Repeat this several times until the muscle is penetrated and the peritoneal lining is visible. Use the scissors to puncture this lining and then open the tips to expose the white adipose tissue surrounding the ovary. Be careful not to cut the tissue with the scissors.
14. Insert forceps into the opening and pull on the fat tissue attached to the uterine horn. Pull the uterine horn and its surrounding adipose tissue out of the incision.
15. Cut two 6-inch pieces of catgut suture (30", 75 cm).
16. Use the small hemostat to locate the main artery that extends parallel to the uterine horn, through the fat tissue, and connects to the ovary. Slip the hemostat beneath the artery and grab the tip of one piece of catgut. Pull the suture through and ligate the artery by tying three knots.
17. Use the small hemostat again to locate the uterine horn where it connects to the ovary. Ligate the tube approximately 3 mm below its point of attachment to the ovary by tying three surgical knots around the tube.
18. Remove the ovary with scissors at its attachment to the uterine horn and the main artery.
19. Cut off excess catgut to leave approximately 2 mm at the end of the knot.
20. Use a cotton swab soaked with sterile saline to place the uterine horn back into the

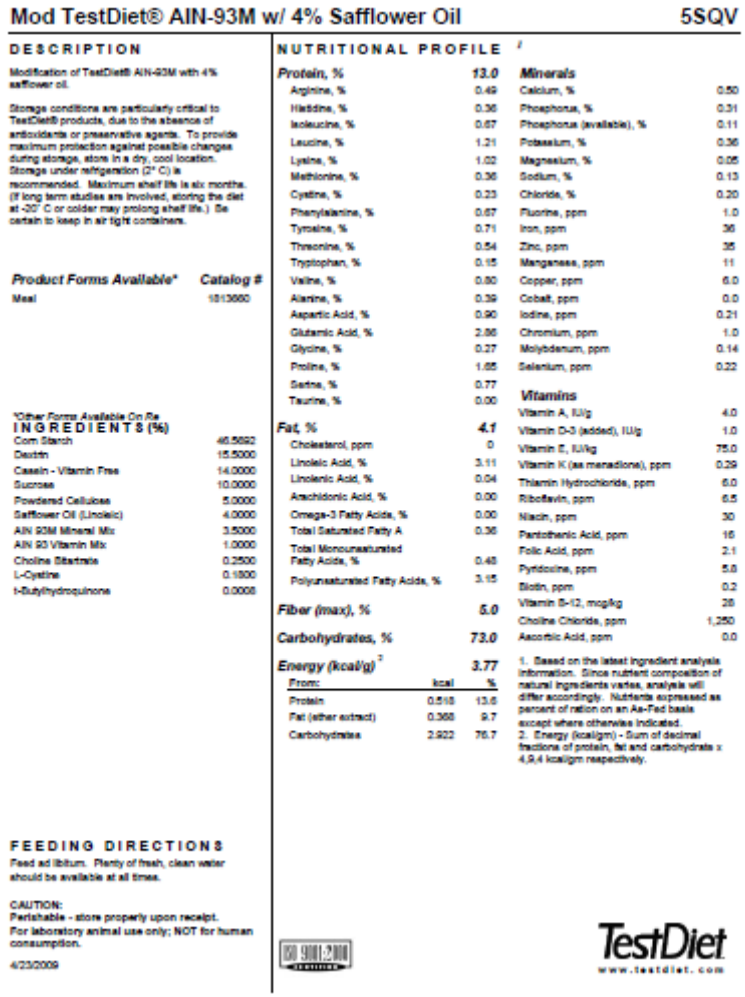
body cavity. Use fingers to ensure the tissue is back in its appropriate place.

21. Pull the incision tight to close it. Insert a wound clip at the tip of the wound clip applicator and clamp the clip at the middle of the incision to hold it closed.
22. Repeat steps 12 through 21 to remove the other ovary from the rat.
23. Wipe away excess blood from the rat's back and apply a small amount of antibiotic ointment to the incisions.
24. Transfer the rat to a pre-heated recovery chamber. Monitor the temperature, respiratory rate, and responsiveness of the rat.
25. Transfer the rat to a clean shoebox cage after it has the ability of sternal recumbancy and capable of purposeful movement which is about 30 minutes.

APPENDIX 2

NUTRITIONAL PROFILE OF THE DIETS

I. Basal diet: Treatment 1



Mod TestDiet® AIN-93M w/ 4% Safflower Oil — **5SQV**

DESCRIPTION

Modification of TestDiet® AIN-93M with 4% safflower oil.

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

*Product Forms Available**	*Catalog #*
Meal	1813660

**Other Forms Available On Re*

INGREDIENTS (%)

Corn Starch	46.5692
Dextrin	15.5000
Casein - Vitamin Free	14.0000
Sucrose	10.0000
Powdered Cellulose	5.0000
Safflower Oil (Linoleic)	4.0000
AIN 93M Mineral Mix	3.5000
AIN 93 Vitamin Mix	1.0000
Choline Bitartrate	0.2500
L-Cystine	0.1800
t-Butylhydroquinone	0.0008

NUTRITIONAL PROFILE [1]

Protein, %	***13.0***	***Minerals***	
Arginine, %	0.49	Calcium, %	0.50
Histidine, %	0.36	Phosphorus, %	0.31
Isoleucine, %	0.67	Phosphorus (available), %	0.11
Leucine, %	1.21	Potassium, %	0.36
Lysine, %	1.02	Magnesium, %	0.05
Methionine, %	0.36	Sodium, %	0.13
Cystine, %	0.23	Chloride, %	0.20
Phenylalanine, %	0.67	Fluorine, ppm	1.0
Tyrosine, %	0.71	Iron, ppm	36
Threonine, %	0.54	Zinc, ppm	35
Tryptophan, %	0.15	Manganese, ppm	11
Valine, %	0.80	Copper, ppm	6.0
Alanine, %	0.39	Cobalt, ppm	0.0
Aspartic Acid, %	0.90	Iodine, ppm	0.21
Glutamic Acid, %	2.86	Chromium, ppm	1.0
Glycine, %	0.27	Molybdenum, ppm	0.14
Proline, %	1.65	Selenium, ppm	0.22
Serine, %	0.77		
Taurine, %	0.00	**Vitamins**	
Fat, %	***4.1***	Vitamin A, IU/g	4.0
Cholesterol, ppm	0	Vitamin D-3 (added), IU/g	1.0
Linoleic Acid, %	3.11	Vitamin E, IU/kg	75.0
Linolenic Acid, %	0.04	Vitamin K (as menadione), ppm	0.29
Arachidonic Acid, %	0.00	Thiamin Hydrochloride, ppm	6.0
Omega-3 Fatty Acids, %	0.00	Riboflavin, ppm	6.5
Total Saturated Fatty A	0.36	Niacin, ppm	30
Total Monounsaturated Fatty Acids, %	0.48	Pantothenic Acid, ppm	16
Polyunsaturated Fatty Acids, %	3.15	Folic Acid, ppm	2.1
Fiber (max), %	***5.0***	Pyridoxine, ppm	5.0
Carbohydrates, %	***73.0***	Biotin, ppm	0.2
Energy (kcal/g) [2]	***3.77***	Vitamin B-12, mcg/kg	28
From:	kcal %	Choline Chloride, ppm	1,250
Protein	0.518 13.6	Ascorbic Acid, ppm	0.0
Fat (ether extract)	0.368 9.7		
Carbohydrates	2.922 76.7		

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly. Nutrients expressed as percent of ration on an As-Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

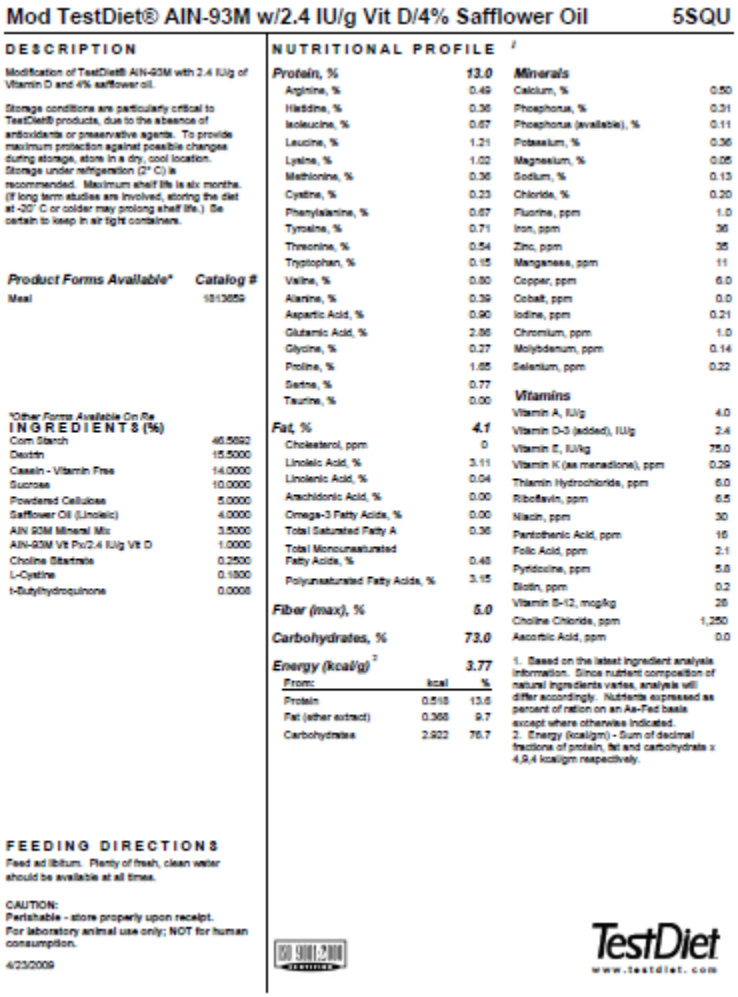
CAUTION:
Perishable - store properly upon receipt.
For laboratory animal use only; NOT for human consumption.

4/23/2009

ISO 9001:2000

TestDiet
www.testdiet.com

II. Enhanced supplementation of vitamin D basal feed for treatments 2-5



Mod TestDiet® AIN-93M w/2.4 IU/g Vit D/4% Safflower Oil **5SQU**

DESCRIPTION

Modification of TestDiet® AIN-93M with 2.4 IU/g of Vitamin D and 4% safflower oil.

Storage conditions are particularly critical to TestDiet® products, due to the absence of antioxidants or preservative agents. To provide maximum protection against possible changes during storage, store in a dry, cool location. Storage under refrigeration (2° C) is recommended. Maximum shelf life is six months. (If long term studies are involved, storing the diet at -20° C or colder may prolong shelf life.) Be certain to keep in air tight containers.

*Product Forms Available**	*Catalog #*
Meal	1813659

*Other Forms Available On Re

INGREDIENTS (%)

Corn Starch	46.5692
Dextrin	15.5000
Casein - Vitamin Free	14.0000
Sucrose	10.0000
Powdered Cellulose	5.0000
Safflower Oil (Linoleic)	4.0000
AIN 93M Mineral Mix	3.5000
AIN-93M Vit Pw/2.4 IU/g Vit D	1.0000
Choline Startrate	0.2500
L-Cystine	0.1800
t-Butylhydroquinone	0.0008

FEEDING DIRECTIONS

Feed ad libitum. Plenty of fresh, clean water should be available at all times.

CAUTION:
Perishable - store properly upon receipt.
For laboratory animal use only; NOT for human consumption.

4/23/2009

NUTRITIONAL PROFILE [1]

Protein, %	***13.0***
Arginine, %	0.49
Histidine, %	0.36
Isoleucine, %	0.67
Leucine, %	1.21
Lysine, %	1.02
Methionine, %	0.36
Cystine, %	0.23
Phenylalanine, %	0.67
Tyrosine, %	0.71
Threonine, %	0.54
Tryptophan, %	0.15
Valine, %	0.80
Alanine, %	0.39
Aspartic Acid, %	0.90
Glutamic Acid, %	2.86
Glycine, %	0.27
Proline, %	1.65
Serine, %	0.77
Taurine, %	0.00
Fat, %	***4.1***
Cholesterol, ppm	0
Linoleic Acid, %	3.11
Linolenic Acid, %	0.04
Arachidonic Acid, %	0.00
Omega-3 Fatty Acids, %	0.00
Total Saturated Fatty A	0.36
Total Monounsaturated Fatty Acids, %	0.48
Polyunsaturated Fatty Acids, %	3.65
Fiber (max), %	***5.0***
Carbohydrates, %	***73.0***
Energy (kcal/g) [2]	***3.77***

From:	kcal	%
Protein	0.518	13.6
Fat (ether extract)	0.368	9.7
Carbohydrates	2.922	76.7

Minerals	
Calcium, %	0.50
Phosphorus, %	0.31
Phosphorus (available), %	0.11
Potassium, %	0.36
Magnesium, %	0.05
Sodium, %	0.13
Chloride, %	0.20
Fluorine, ppm	1.0
Iron, ppm	36
Zinc, ppm	35
Manganese, ppm	11
Copper, ppm	6.0
Cobalt, ppm	0.0
Iodine, ppm	0.21
Chromium, ppm	1.0
Molybdenum, ppm	0.14
Selenium, ppm	0.22
Vitamins	
Vitamin A, IU/g	4.0
Vitamin D-3 (added), IU/g	2.4
Vitamin E, IU/kg	75.0
Vitamin K (as menadione), ppm	0.29
Thiamin Hydrochloride, ppm	6.0
Riboflavin, ppm	6.5
Niacin, ppm	30
Pantothenic Acid, ppm	16
Folic Acid, ppm	2.1
Pyridoxine, ppm	5.0
Biotin, ppm	0.2
Vitamin B-12, mcg/kg	28
Choline Chloride, ppm	1,250
Ascorbic Acid, ppm	0.0

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly. Nutrients expressed as percent of ration on an As-Fed basis except where otherwise indicated.
2. Energy (kcal/gm) - Sum of decimal fractions of protein, fat and carbohydrate x 4,9,4 kcal/gm respectively.

TestDiet
www.testdiet.com

Test Diets were ordered through Stewart Feeds & Garden Center, P.O. Box 1630, 658 Grayson Hwy., Lawrenceville, GA 30046, ph. (770) 963-8335

APPENDIX 3

PROTOCOL FOR THE PARAFFIN EMBEDDING AND ASSAYS

Purpose

Sections for histomorphometry of trabecular architecture and osteoclast numbers are prepared using decalcified specimens. Bone samples are decalcified in EDTA for approximately 3 weeks and dehydrated, cleared in xylene by tissue processor (Sakura Tissue-Tek®) and then embedded in paraffin. In order to be able to produce section consistently, it is necessary to select a wax of suitable hardness at room temperature. The melting point of paraffin is in the range 40-70ºC. Paraffin sections can be used for a variety of different detection techniques including TRAP and immune-histochemistry.

Materials

1. Ethanol Anhydrous (#48218-736, VWR)
2. Fixative 10% Neutral buffer formalin (#84000-024, VWR)
3. EDTA, Disodium salt, dehydrate, crystal (#8993-01, JT-Baker)
4. Xylene reagent (#48218-746, VWR)
5. CLEAR-RITE 3 (#84000-052, VWR)
6. Paraffin histo (#8330, MISC-CLINCAL, VWR)
7. Microtome blades HI PROFI (#84000-164, VWR)
8. Cover glass #1 24*60mm (#16002-534, VWR)
9. Slide UP-RITE WH (#16002-082, VWR)
10. Tissue-Tek mold (31mm*23mm*13.5mm) (#4166, IMEB)

A. Preparation of decalcified rat bones for paraffin embedding

1. Bone tissue collection and fixation

   Remove skin, major tendons and muscles from the bone before fixing in 10% buffered formalin for 24 - 48 hr.

2. Specimen preparation

   2.1. Trim bone of any remaining tendons or muscles from the fixed tissue

   2.2. Store in 70% ethanol for 24 hr at 4ºC

   2.3. Remove samples and thoroughly rinse the specimens with distilled water

   2.4. 1/3 of the bone length (proximal part of the tibia from knee to midshaft) is cut off and place into the cassette for decalcification

3. Decalcification

   3.1. Prepare the EDTA decalcification buffer, mix well using a stirring bar until dissolved and then stored in the 4ºC

   500 ml Distilled water

   20.6g EDTA

   2.2g NaOH

   3.2. Place specimens into the decalcification buffer. Generally, the accepted volume of EDTA decalcification buffer (weak acid) is 20 times the volume of tissue

   3.3. Change the buffer daily during the first week of decalcification

   3.4. Rat bones will take about 3 weeks; change buffer every other day starting at the second week until the end point is achieved. Also randomly check several bones and location within the bone with a needle (26G) that they

have been fully decalcified or scan with PIXImus to verify if the bone is entirely decalcified. The end point of decalcification is when the bone has become discolored and the needle penetrates the bone with ease.

3.5. Once a specimen has been satisfactorily decalcified, it should be thoroughly washed with water for 24 hr.

4. Embedding in paraffin

4.1. The processor requires 24 hr warm up in order to melt paraffin. Meanwhile, turn on dispensing and thermal consoles of paraffin embedding system and check the processor and paraffin embedding system for enough paraffin inside for infiltration and blocking (Figures 1 and 2).

Figure 1. Tissue Tek® tissue processor

Figure 2. Tissue Tek® paraffin embedding system.

Program the tissue processor for dehydration, cleaning and infiltration using the following settings

Stations	**Solutions**	**Time (min)**
1	Formalin	30
2	Alcohol 70%	45
3	Alcohol 80%	45
4	Alcohol 90%	45
5	Alcohol 90%	45
6	Alcohol 100%	45
7	Alcohol 100%	45
8	Alcohol 100%	45
9	Xylene	80
10	Xylene	60
11	Paraffin	45
12	Paraffin	45
13	Paraffin	45

4.2. Block Preparation

Process each bone separately. Fill liquid paraffin into the mold on the embedding station (Figure 2) and then adjust the desired orientation of the bone for sectioning. The section of bone to be cut should face the bottom of mold. Place the block onto the freezer (-4 to 8ºC) for at least 6 hours so that it can harden.

5. Section cutting

5.1. Turn on the water bath and set the temperature to 40ºC about 20 minutes before cutting (Figure 3).

5.2. For cutting paraffin sections use a sliding microtome equipped with a microtome blade holder (Figure 4)

Figure 3. Water bath

Figure 4 Microtome

5.3. Make sections 5-8µm in thickness.

5.4. Move sections to the water bath so that they can be unfolded and stretched using a curved forcep.

5.5. Transfer each tissue section onto a slide. Depending on the section obtained, one to two sections can be placed on the slide.

(Note: Positive charged slides are required for H&E stain and immunostaining.)

5.6. Load the slides in the slide holder place into the 40ºC oven for 24 hours, remove and store at room temperature (Figures 5 and 6)

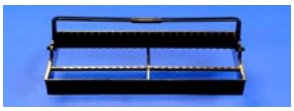


Figure 5. Slide holder

Figure 6. Oven

TRAP assay (Sigma 386)

TRAP staining kit is used for the staining of tartrate-resistant acid phosphatase in osteoclasts. The staining procedure is followed below:

1. Deparaffinization

1.1. Slides are placed into the slide holder (Figure 7)

1.2. Slides are then gently immersed into a staining rack filled with Xylene for 3 min (Figure 8)

1.3. Slide holder is removed from the Xylene and the holder is blotted on a paper towel before gently immersing the slides into a staining rack filled with 100% ethanol for 3 mins. *(Note: Removing the slide holder from the solution and blotting on a paper towel is carried out for the next 4 steps before immersing the holder in the new solution.)*

1.4. Slides are gently immersed into a staining rack filled with 95% ethanol for 3 mins.

1.5. Slides are gently immersed into a staining rack filled with 70% ethanol for 3 mins.

1.6. Slides are gently immersed into a staining rack filled with 50% ethanol for 3 mins.

1.7. Slides are gently immersed into a staining rack filled with deionized water to wash for 10 mins

2. Decalcification

2.1. Slides are immersed in EDTA for 2 mins to remove any residual calcium salts

2.2. Slides are placed in deionized water for a 5 min wash

3. Slides are then incubated for 1.5 hour (37 ºC) in the dark in a solution of 44 ml water, 2 ml acetate solution, 2 ml napthol AS-BI phosphoric solution, 2 ml tartrate solution and contents of 1 capsule Fast Garnet GBC Salt to develop bright red TRAP localization. (Check that temperature is at 37 ºC before adding the slides)

4. Slides are washed for 3 mins in deionized water and counterstained with Hematoxylin for 1 min

5. Slides are washed for 3 mins in deionized water 2 times and then 1 time with 1X PBS, allowed to air dry and mounted with water based mounting medium (item # 84000-112, VWR).

6. Place a drop of the mounting medium on the slide and cover with an appropriate size cover slip

7. Section images are captured at 400X and osteoclasts on each bone surface will be counted. Surface lengths will then be measured using ImagePro image analysis software and N.Oc/B.Pm expressed as number of osteoclasts counted per surface length (mm).

B. **Hematoxylin and eosin staining**[8]

H&E staining is the standard staining method widely used in histology and it gives an overview of the structure of the tissue. The staining procedure is followed below:

1. Slides are placed into the slide holder (Figure 7)
2. Slides are then gently immersed into a staining rack filled with Xylene[9] for 3 mins (Figure 8)
3. Slides holder is removed from the Xylene and the holder is blotted on a paper towel before gently immersing the slides in a staining rack filled with 100% ethanol for 3 mins. *(Note: Removing the slide holder from the solution and blotting on a paper towel is carried out for the next 14 steps before immersing the holder in the new solution.)*
4. Slides are gently immersed in a staining rack filled with 95% ethanol for 3 mins
5. Slides are gently immersed into a staining rack filled with 70% ethanol for 3 mins
6. Slides are gently immersed into a staining rack filled with 50% ethanol for 3 mins
7. Slides are gently immersed into a staining rack filled with water to wash for 10 mins
8. Slides are gently immersed into a staining rack filled with Harris Hematoxylin for 3 mins (use a dry slide holder a staining rack for this step)
9. Slides are gently immersed into a staining rack filled with deionized water to wash for 10 mins
10. Dip slides 10X'ss in 0.003% ammonium water (Ammonium hydroxide, 28.0-30.0%; #9721-00 J.T. Baker)**;** 3 ml of ammonium to 1000 ml distilled water)

[8] Positive charged slides are required for this staining technique

[9] Xylene is used for cleaning which can be substituted by the Clear-Rite 3 (VWR, item #6910)

11. Slides are gently immersed into a staining rack filled with Eosin Y for 1 min and then dipped 5X's in deioinized water

12. Slides are gently immersed into a staining rack filled with 70% ethanol for 30 secs

13. Slides are gently immersed into a staining rack filled with 95% ethanol for 30 secs

14. Slides are gently immersed into a staining rack filled with 95% ethanol for 1 min

15. Slides are gently immersed into a staining rack filled with 100% ethanol for 1 min, 2X's

16. Slides are gently immersed into a staining rack filled with Xylene for 3 mins, 2X's

17. Place a drop of oil based mounting medium (item # 84000-112, VWR) on the slide and cover with an appropriate size cover slip

Figure 7. Slide holder

Figure 8. Staining rack