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The Underlying Mechanisms by Which Estrogen Regulates Body Composition Including Bone and Muscle Mass

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THE UNDERLYING MECHANISMS BY WHICH ESTROGEN REGULATES BODY COMPOSITION INCLUDING BONE AND MUSCLE MASS

By

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DEDICATION

To my mother who has been there for me during my ups and downs throughout this project, giving me unconditional love and support. And in the memory of my father, whose presence and support is sorely missed.

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ABSTRACT

Ovariectomized (ovx) rats gain excess body weight (BW) even when they are pair-fed to sham animals. This higher BW, contrary to common belief, does not result in higher bone mineral density. The purpose of the present study was to evaluate the potential mechanisms underlying these paradoxical observations. We evaluated the effects of ovariectomy and 17β-estradiol (E_2) using two age groups female Sprague-Dawley (SD) rats: 5- and 10-month old rats.

Thirty-six female SD rats, 5- and 10-months old were acclimatized for 5 days. Animals in each category were divided into three groups: sham, ovx, and ovx+E_2. There were 18 rats per age category with sample size of six rats per treatment group. Five- and ten-month old rats were chosen to model young and middle age women, respectively. Immediately after surgery, rats in all groups received a semi-purified control diet (AIN-93M). Animals in the ovx+E_2 group were injected with E_2 (10μ/kg BW subcutaneously, twice per week). Ovx control rats received solvent vehicle (sesame oil; subcutaneously, twice per week). Rats in ovx groups were pair-fed to the mean food intake of the sham group and their food intake was adjusted every three days. Rats' voluntary running activity was measured by activity wheel and distance counter in individual cages for 24 hours between one to two weeks before sacrifice.

To confirm bone loss due to ovariectomy, whole body bone mineral density (BMD) was measured by dual energy x-ray absorptiometry (iDXA) at baseline, mid-point and at the end of study period. The losses of whole body BMD in five- and ten-month old ovx rats occurred after three and half- and five-months from the time of the removal of ovaries, respectively. In spite of pair feeding the ovx rats, the final body weights of ovx rats in both age categories were significantly ($P<0.05$) higher than those of sham-operated rats. Estrogen completely prevented the ovariectomy-induced weight gains in both age categories. Ovariectomy either significantly decreased (10-month old) or tended ($P<0.1$) to decrease (5-month old) voluntary wheel running activity and in comparison with sham animals. Estrogen treated rats voluntary wheel running activity was able to prevent this ovariectomy-induced decline in physical activity. Mean fat mass of ovx rats in comparison with sham animals was significantly higher in both age groups. Rats that received E_2 did not experience gain in fat mass. The mean tibial and vertebral BMD

values of ovx rats were significantly lower in comparison with sham rats in both age categories. Microstructural properties of tibiae and vertebrae, including bone volume/over total volume, connectivity density, structure model index were all unfavorably altered by ovariectomy in both age categories. In younger rats, E_2 administration was able to prevent ovariectomy-induced alterations in lumbar vertebrae microstructural parameters such as bone volume/total volume (BV/TV), structure model index (SMI) and trabecular thickness (Tb. Th.). However, in this age group E_2 administration was only able to prevent ovx-induced increase in tibial trabecular separation (Tb. Sp.). These findings suggest that E_2 is more effective in preventing microstructural properties of trabecular rich bone such as vertebral bones. In older rats, in addition to the loss of BV/TV, ovx also caused significant decreases in connectivity density (Conn. D.) and trabecular number (Tb. N.) of both tibial and lumbar vertebral bones while increasing SMI and Tb. Sp. in the same bones. E_2 administration was able to prevent changes in Conn. D. and SMI only in lumbar bones. E_2 was also able to prevent the ovx-induced deleterious effects on tibial Tb.N. and Tb.Sp. by maintaining these values to those of sham levels. Overall, these observations suggest that while E_2 is capable of preventing some structural properties as a result of ovx, it is not able to replace ovarian hormones as a whole. Ovariectomy significantly increased serum levels of both biomarker of bone resorption, C-telopeptides of type I collagen, and bone formation, alkaline phosphatase levels. These findings are in agreement with earlier reports that indicate ovariectomy increases the rate of bone turnover with resorption exceeding that of formation. Our findings do not suggest that the bone loss due to ovx is through inflammatory processes as the serum levels of tumor necrosis factor alpha (TNF-α) remained below the detectable level in ovx rats in both age categories. In spite of ovx-induced significant increases in systemic levels of insulin-like growth factor-I (IGF-I) in both age categories, its localized mRNA expressions were either significantly or tended ($P < 0.1$) to be reduced in gastrocnemius (GAS) and soleus (SOL) muscles of 10-month old rats. These local reductions in IGF-I mRNA expressions were in parallel with reductions in normalized to total body weight muscle mass, signifying the importance of IGF-I at the tissue level. Similar trends ($P < 0.1$) were also observed in 5-month old rats, albeit not significantly. Our findings for the first time, to our knowledge, suggest that elevated systemic IGF-I may down-regulate its synthesis in GAS and SOL of ovx rats. Overall, the findings of this study indicate that ovariectomy causes significant losses of BMD, microstructural properties and muscle mass. However, body fat increases as such

that the weight of ovx animals exceeds those of sham and E_2 treated animals, which indicates that ovarian hormone deficiency causes body weight gain mainly due to a gain in fat mass.

Additionally, our data suggest that the detrimental effects of ovarian hormone deficiency are exacerbated as a result of aging. Our observations, especially in older rats, suggest that estrogen deficiency alters body composition which resembles that of osteosarcopenic obesity.

CHAPTER ONE

INTRODUCTION

Obesity is among the most burdensome public health concerns in the United States (1, 2). The prevalence of obesity is higher among females compared to males and yet higher in postmenopausal women than premenopausal women (2, 3). Obesity is a significant risk factor for development of chronic conditions in humans. In particular, women after the onset of menopause experience a drastic increase in weight gain. Obesity associated with ovarian hormone deficiency has been linked to a number of chronic diseases such as cardiovascular (4-6) and osteoporosis (7-9). The detrimental effects of estrogen deficiency are due in large part to associated increased visceral adiposity (10-12) with simultaneous loss of bone mass (9). Estrogen combined with progesterone administration to postmenopausal women appears to reduce the increases in abdominal fat characteristic of the postmenopausal period (13-16). Haarbo and colleagues (13) investigated postmenopausal women who were followed for up to 2 years in a prospective, randomized, placebo-controlled trial. Assessment of body composition by dual-energy x-ray absorptiometry (DXA) revealed that estrogen-progesterone treatment prevented abdominal fat gains in postmenopausal women.

A number of epidemiological (16-18), clinical (19, 20), and animal studies (21, 22) have demonstrated that increased levels of body fat during menopause are directly or indirectly associated with ovarian hormone deficiency. Poehlman et al. (19) demonstrated that no significant changes in body composition were present in healthy menstruating women up to the age of 50 years, the average age at which menopause occurs. After the age of 50 years, dramatic declines in fat-free mass concomitant with significant increases in fat mass were observed. It may be argued that age is the major contributing factor to increased body fat levels; however, younger women with premature ovarian failure have also been found to have body compositional changes similar to postmenopausal women (18).

Although this excess weight gain has been linked to estrogen deficiency, the exact mechanism of action is unclear. Animal studies also indicate that the ovariectomized (ovx) animal continues to gain weight beyond that of intact animals in spite of equal food consumption (21- 23). Ovx rats

have been shown to gain fat mass up to 200 percent in comparison to the fat levels gained by sham operated animals (24). Furthermore, ovx rats receiving estrogen supplementation did not demonstrate the hyperphagia nor the suppression of running wheel activity observed in the untreated ovx group (24). Rosenblatt and colleagues (25) observed reductions of appetite during the midcycle estrogen and gonadotropin surges. It has been suggested (26, 27) that the suppression of weight gain as well as appetite observed with estrogen treatment occurs through decreased neuropeptide Y levels in the hypothalamic sites. Neuropeptide Y levels have been shown to increase food intake as well as decrease metabolic rate, leading to increased body fat accumulation (26-28). At the cellular level, the exact mechanisms by which estrogen exerts its effects on bone and muscle are not fully understood, whereas estrogen appears to protect against loss of lean mass through estrogen receptors (ERs). The presence of ERs in both bone and skeletal muscle suggests that these tissues are direct targets of estrogen. Estrogen acts on osteoclastic cells through ERα to suppress its activity resulting in reduced bone break down. Although the anabolic effect of estrogen on osteoblastic cells is somewhat known (29), its mechanisms of action on muscle are just emerging. For instance, a recent study by Velders and colleagues (30) suggest that estrogen promotes skeletal muscle regeneration after injury via ERβ.

In the absence of ovarian hormone, animals including rats (31) in a compensatory attempt to establish homeostasis increase the production of anabolic factors including insulin like growth factor I (IGF-I) and growth hormone (GH) on a transitory basis. The aforementioned study by Kalue et al. (31), ovariectomy increased the serum levels of IGF-I and its binding proteins and concluded that IGF-I plays a role in the pathogenesis of the increased lean mass turnover that occurs early in ovarian hormone deficiency.

Taken together, estrogen deficiency, while causing the loss of BMD and lean muscle mass, increases body weight by mainly increasing fat mass. However, the mechanisms by which these unfavorable changes occur are not clear. Hence, the intent of the present study was to explore the underlying mechanism by which this imbalance occurs. The *long-term* goal of this proposed research was to improve the health of postmenopausal women by understanding the etiology of this weight gain which in turn would enable us to offer them some practical solutions. The

overall objective of this study was to demonstrate the negative effects of estrogen deficiency in altering body composition using ovx rats of two ages, namely five- and ten-month old rats.

1.1. Research Hypothesis

Our earlier findings (21-23) suggest that estrogen deficiency in ovx rats results in increased food intake accompanied by a sustained elevation of body weight. We have also observed that ovariectomy alters the distribution of the adipose tissue, e.g. increased abdominal fat, perhaps due to increased production of pro-inflammatory and oxidative molecules (32-35). The *rationale* for conducting the proposed study was to illustrate the underlying mechanisms by which estrogen regulates body composition using a rat model. Therefore, we ***hypothesized*** that estrogen deficiency slows down resting metabolic rate and energy expenditure while exerts catabolic effects on lean mass, including bone mass. Estrogen deficiency also may increase adiposity which results in higher body weight gain in both young and old rats. Furthermore, we postulated that this hypothesis was independent of age.

1.2. Specific Aims

Our hypothesis was tested through the following **Specific Aims** based on our preliminary findings:

Aim 1: To examine the extent to which ovarian hormone deficiency unfavorably alters body composition by increasing fat mass while decreasing lean mass. This aim was to test the *working hypothesis that ovariectomy causes unfavorable changes in body composition*. To test this working hypothesis we measured total body weight (BW), bone mineral density (BMD), bone mineral content (BMC), lean mass (LM) and fat mass (FM). Site specific alterations in bone mass and muscle mass were similarly examined. Microstructural properties, e.g. cortical thickness, trabecular number, bone volume/total volume and bone area of newly-formed bone utilizing μ-CT (micro-computed tomography), as well as gastrocnemius (GAS) and soleus (SOL) mass were measured.

Aim 2: To explore the effects of ovarian hormone deficiency on anabolic and catabolic factors playing a role in regulating body composition as well as its effects on the production of select anabolic and catabolic markers involved in maintaining bone and muscle mass. This aim was to test the *working hypothesis that ovariectomy upregulates the biomarkers of bone and muscle turnover.* To test this working hypothesis we measured bone specific alkaline phosphatase (B-ALP), a serum marker of bone formation; C-telopeptides of type I collagen (CTX), a serum marker of bone formation; IGF-I, an anabolic growth factor affecting both bone and muscle growth and maintenance; and tumor necrosis factor alpha (TNF-α), a proinflammatory that negatively affects both bone and muscle.

Aim 3: To measure the degree by which estrogen and/or estrogen deficiency affect the select gene expressions of protein turnover relevant to body composition. This aim was to test the *working hypothesis that ovariectomy down-regulates the gene expressions of anabolic factors and upregulates the gene expression of catabolic molecules.* To test this working hypothesis we measured the gene expressions of IGF-I, TNFα and atrogin-I in GAS and SOL.

In order to conduct this study, we used five- and ten-month old female SD rats. Five- and ten-month old rats were chosen to model pre- and postmenopausal women, respectively. Rats were either sham operated (sham) or ovx. Rats in one of the ovx group was received 17β-estradiol (E_2) and served as the positive control group.

1.3. Limitations

The main limitation of this study is the small sample size which may have compromised the statistical power to detect significant differences for each variable aside from BMD (the primary variable). Another limitation of this animal study is that the findings of this study cannot be directly applicable to women. However, if the results suggest that estrogen exerts beneficial effects on body composition, this would provide justification for investigating the positive effects of low-dose estrogen on body composition in postmenopausal women. In this study, we used five- and ten-month old SD virgin female rats. It would have been more interesting to have

younger and older groups which would have enabled us to examine the effects of ovariectomy and E_2 on body composition across a greater age-span

CHAPTER TWO

REVIEW OF LITERATURE

2.1. Introduction

Women aged 45 or older have almost 50 percent greater median annual per capita expenditures in health care than men in the same age adding significantly to the cost of medical care in the United States (36). This disparity in health care spending may be explained in part by onset of menopause. Although the detrimental effects of estrogen deficiency are due in large part to increases in susceptibility to osteoporosis (9), a growing body of knowledge suggests that estrogen deficiency may also increase cardiovascular disease (CVD) risk through a predisposition towards visceral adipose tissue accumulation (4-6) and obesity-related dyslipidemia. Before menopause, women tend to gain weight within the hip area; following menopause, women gain weight in the abdominal region (37, 38). Such increases in the waist-to-hip ratio have been associated with mortality risk in older women (39), and have been specifically correlated with CVD, even after controlling for hypertension, glucose intolerance, blood lipids, smoking, and body mass index (4, 5). Furthermore, increases in visceral adiposity directly correlate with glucose intolerance (4, 5), and can contribute to increased triglyceride, total cholesterol, and low-density lipoprotein cholesterol levels (4, 5).

Although the positive relationship between increased adiposity and estrogen deficiency has been reported directly and indirectly in both human (16- 20) and animal studies (21, 22), there might be an argument regarding the detrimental effect of aging in increased fat mass in postmenopausal women. However, consistent with our hypothesis; younger women with premature ovarian failure have also been found to have altered body compositional similar to postmenopausal women (18).

Unfortunately, the body compositional changes due to ovarian hormone deficiency are to be considered unfavorable because increased body weight and fat mass occur in the face of decreased muscle and bone mass.

2.2. Obesity

There has been a dramatic increase in the incidence of obesity during the past 20 years with higher prevalence in women than men (1, 2). This gender difference in obesity may partly be due to the fluctuations in female sex hormones (12, 17). The decline in estrogen production during menopause increases body weight which is associated with numerous chronic diseases such as cardiovascular disease and diabetes (4, 5). Epidemiological studies (41, 42) have shown that the weight gain in postmenopausal women on hormone replacement therapy (HRT) is far less than those not on HRT. Menopausal obesity has been linked to multiple CVD risk factors such as abdominal obesity, high blood pressure, dyslipidemia and hyperglycemia (4- 6). Yet, in spite of these known phenomena, there is little, if any, understanding of this tilt in body composition including increased body fat mass, and decreased lean mass and bone mineral density (BMD). Hence, there is an urgent need to elucidate the etiology of this weight gain due to estrogen deficiency which is necessary for development of economical and practical strategies to curb the growing obesity epidemic and/or ameliorate associated chronic disease risk factors in postmenopausal women.

2.2.1. Estrogen Deficiency Alters Patterns of Adiposity

Estrogen has been considered a major regulator of adipose tissue development and deposition in the female body (10, 40). Women have significantly higher levels of body fat compared to men (10, 40). During puberty, women develop more extensive subcutaneous fat, however, few years prior to the onset of menopause, women experience an increase in visceral adiposity (11, 12). Cross-sectional data also supports the observation that postmenopausal women have a higher total and visceral fat mass (40) but lower lean mass (10). Furthermore, lower levels of visceral adiposity (41) and lower waist-to-hip ratio (42) have been reported in postmenopausal women on HRT, confirming a role for estrogen in controlling adiposity.

Increased visceral adiposity that occurs with estrogen deficiency in menopause may be due to altered metabolism of adipose tissue (43-45). Several studies (43, 44) suggest a direct role for estrogen on adipose tissue lipoprotein lipase (LPL) activity and lipolysis. Higher levels of

estrogen are known to increase lipolytic response in subcutaneous abdominal adipocytes (46). However, this effect has not been seen in intra-abdominal adipocytes, indicating that estrogen preferentially promotes subcutaneous adipose deposition (46). As a result, lower lipolytic rate in intra-abdominal adipocytes caused by ovarian hormone deficiency in postmenopausal women leads to increase fat deposition in this area. Animal studies (21, 22) also indicate that when ovaries are removed, the visceral fat mass increases in the ovx animal and administration of estrogen reverses this effect. Furthermore, it has been seen that estrogen affects adipose tissue by increasing hormone-sensitive lipase activity (43), lipolytic effects of epinephrine (43) and beta oxidation of fatty acids (45). Abdominal adiposity is known to be associated with metabolic abnormalities and increased risk of cardiovascular disease (4, 5).

2.2.2. Estrogen Deficiency, Food Intake and Physical Activity

In addition to the direct role of estrogen on adipose tissue, estrogen may indirectly affect adiposity via regulation of food intake and energy expenditure through hypothalamus (47-48). Hypothalamus, the key regulator of food intake and energy homeostasis in the brain (48), widely expresses estrogen receptors, making it a target tissue of estrogen action (47, 49). Accordingly, there are reports (49-51) suggesting that food intake varies due to fluctuations in estrogen levels in women of any age with the lowest intake when estrogen is at the highest concentration. Additionally, postmenopausal women have been reported (51) to have a 30 percent decrease in energy expenditure compared to premenopausal women. Similar results have been reported (21, 22, 52) from ovx animals; displaying an increase in food consumption and a decrease in voluntary physical activity.

A number of clinical trials (41, 42) as well as animal studies (21, 22) have demonstrated that estrogen administration attenuates the body compositional changes associated with ovarian hormone deficiency. Ovariectomized (ovx) rats have been shown to have gains in body fat of up to 200 percent in comparison to sham-operated animals (53) and such fat gain is observed to be attenuated with estrogen treatment. Furthermore, ovx rats receiving estrogen supplementation do not demonstrate the hyperphagia nor the suppression of running wheel activity observed in the untreated ovx group (53).

Although the observed increased in body weight and altered body composition due to menopause maybe explained in part by increased food intake and decreased energy expenditure, ovx animals weight gain cannot totally be explained by increased food intake and decreased energy expenditure which requires further investigation.

2.3. Osteoporosis

Osteoporosis is a disease characterized by low BMD and deterioration of bone structure which leads to increased bone fragility and increased risk of fracture (7). Clinically, a BMD measurement of 2.5 standard deviations below mean values of a young reference population is considered osteoporosis (7). Three types of osteoporosis have been defined as follows: (1) primary osteoporosis in which the underlying causes are not fully understood; (2) secondary osteoporosis with known underlying causes (e.g. steroid use) and (3) rare forms of osteoporosis, such as juvenile, pregnancy-related, and postpartum osteoporosis (7).

Primary osteoporosis is typically seen in the elderly population with a higher prevalence in women compared to men (8). Although the underlying cause of this disease is not completely understood, a decreased level of estrogen has been considered as one of the main factors.

2.3.1. Mechanism of Bone Modeling and Remodeling

Bone is a dynamic organ that undergoes a continuous process of modeling and remodeling which is bone resorption by osteoclasts and bone formation by osteoblasts (54). This integrated process involves four main steps which occur at specific multi-cellular units: 1) activation; 2) resorption; 3) reversal; and 4) formation (55).

It has been speculated that bone resorption is signaled by systemic and local factors existing on osteoblastic cells rather than directly activating osteoclastic cells or their precursors (56). Cells which may possibly be involved in initiating resorption are stromal, pre-osteoblasts, osteoblasts, pre-osteoclasts, osteoclasts, and osteocytes (55, 56). After the initiation, the osteoclasts begin the

resorption process at various sites. This process involves adherence to the bone and removal of mineralized matrix by acidification and protein matrix degradation by enzymatic digestion (46). The resorption phase continues until osteoclast apoptosis (57). If an extensive amount of bone is resorbed at a given time, it may remove the template and make it impossible to replace the entire bone mass at the remodeling site (55-57). The reversal phase involves the disappearance of the osteoclasts followed immediately by the appearance of monocytes, osteocytes and preosteoblasts at the surface of the bone (56). The last stage of remodeling is formation which consists of osteoblastic cells producing osteoids which is a mixture of collagen and other proteins in the area of resorption. The osteoids are mineralized into new bone (57).

Critical to these events are receptor activator of nuclear factor -kB (NF-kB) ligand (RANKL), its receptor RANK, and its decoy receptor osteoprotegerin (OPG) (58, 59). At the cellular level, osteoblasts regulate the recruitment and activity of osteoclasts through the expression of the receptor activator of nuclear factor -kB (NF-kB) ligand also known as RANKL and osteoprotegerin (OPG) (58- 61). RANKL expresses on the surface of osteoblasts or bone marrow stromal cells. Binding of RANKL with its receptor, RANK which presents on preosteoclast, stimulates differentiation and maturation of osteoclast in the presence of the permissive factor macrophage colony-stimulating factor (M-CSF) (62). OPG is a decoy receptor and a member of the tumor necrosis factor (TNF) receptor superfamily that is produced by osteoblast, binds to RANKL and inhibits its interaction with RANK (63). Therefore, a fine balance between the ratio of RANKL to OPG determines the bone mass and bone homeostasis.

There are numerous growth factors, interleukins and proinflammatory molecules that also greatly influence bone mass and integrity. Examples include insulin like growth factor I (IGF-I), known to correlate with bone density (64- 66), interleukin 1-β (IL-1β) and TNF-α known to stimulate bone resorption. All of these factors are altered as a result of ovarian hormone deficiency in postmenopausal period (67, 68). This is why postmenopausal women experience bone loss, particularly five to seven years after the onset of menopause (8).

2.3.2. Postmenopausal Osteoporosis

Both human and animal studies (34, 64-67) suggest that the accelerated rate of bone loss at menopause is associated with the elevated levels of pro-inflammatory cytokines such as IL-1β and TNF-α. Bone loss in premenopausal women who have gone under ovary removal surgeries has been associated with significant increases in IL-1β and TNF-α (69). HRT in surgically-induced menopause women for four weeks has been reported to decrease the elevated levels of pro-inflammatory cytokines to the pre-operation levels (69). Similarly, decreased BMD in ovariectomized rats has been associated with increased production of IL-1β and TNF-α (70). Confirming the aforementioned role of IL-1β and TNF-α, when their actions are blocked the rate of bone loss is considerably slowed down in ovx rats (71). Similar observation (72) has been made in ovx mice in which osteoclastogenesis were halted by administration of either antibodies to IL-6 or the implantation of estrogen pellets. Another line of support (73) for the role of IL-6 in bone is that IL-6 knockout mice, unlike the wild-type have, much higher bone mass as a result of ovariectomy. These data suggest that pro-inflammatory cytokines are key mediators in the process of ovariectomy-induced osteoclast differentiation and bone resorption (64-73). It is established that upregulated proinflammatory cytokines such as IL-1, IL-6 and TNF-α are capable of stimulating osteoclast activity through the expression of RANKL that increases osteoclastogenesis, and OPG, that promotes osteobalstogensis (67). Overall, estrogen is necessary for the maintenance of homeostasis critical in bone environment and in its absence, drastic loss of bone occurs (64). Unfortunately, when bone mass is lost muscle is also lost. Hence, the remainder of this review concentrates on the role that estrogen plays in muscle homeostasis.

2.4. Sarcopenia

Progressive decline in muscle mass, or sarcopenia, is a syndrome characterized by the loss of mass, function or both (74). Clinically, skeletal muscle mass index within one to two standard deviations below mean values of a younger reference population is considered class I sarcopenia and class II sarcopenia is defined as skeletal muscle index lower than two standard deviation below the mean values of the same reference population (74). Stages for sarcopenia that reflect

the severity of this condition were proposed recently by European Working Group on Sarcopenia in Older People (74). These stages include: “presarcopenia”, defined as low muscle mass, “sarcopenia”, which is low muscle mass and strength and “severe sarcopenia”, characterized by low muscle mass, strength and physical performance (74). The underlying cellular changes that cause sarcopenia include down regulation of anabolic molecules responsible for promoting muscle protein formation and up regulation of catabolic molecules contributing to muscle protein degradation. However, to date the causes and mechanisms responsible for loss of muscle mass are, at best, to be considered preliminary and future research is needed to further our understanding in this area.

2.4.1. Mechanisms of Muscle Protein Formation and Degradation

Skeletal muscle is a dynamic tissue and its maintenance requires a fine balance between synthesis and degradation of muscle protein (75). The initiation of muscle protein synthesis is regulated by serine/threonine protein kinase/mammalian target of rapamycin (Akt/mTOR)-mediated pathway. Activated Akt forms a signaling complex of mammalian target of rapamycin (mTOR). mTOR phosphorylates translational regulators, p70S6-K and 4E-BP1 (76). Activation of p70S6-K results in the phosphorylation of ribosomal subunit protein S6 which is required for translation. Phosphorylated 4E-BP1 releases eukaryotic initiation factor-4e (eIF4E), which is required for initiation of translation by binding of mRNA to the ribosome (77). This pathway is pivotal for protein synthesis in skeletal muscle tissue, and has been indicated to regulate hypertrophy and atrophy (78).

Muscle degradation occurs mainly through the ubiquitin–proteasome pathway (79). Activation of ubiquitin ligases such as muscle ring finger 1 (MuRF1) and muscle atrophy box (MAFbx) (68), also known as atrogin-1 (80), accelerate muscle protein breakdown. Both of these ubiquitin ligases can target muscle proteins for proteosome degradation that can be inhibited through the activation of Akt (79, 80).

On the other hand, anabolic molecules such as IGF-I stimulate muscle protein synthesis (81) and inhibit muscle protein break down (82) through upregulation and down regulation of the

Akt/mTOR pathway. Furthermore, catabolic molecules such as TNF-α and IL-1 not only decrease muscle response to IGF-I (75, 82, 83) but also up regulate expression of MuRF1 (75, 78) and MAFbx (75, 79) through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and P38 mitogen-activated protein kinases (p38 MAPK) pathways, respectively.

2.4.2. The Association between Ovarian Hormone Deficiency and Sarcopenia

It has been reported (11, 83, 84) that the prevalence of sarcopenia in women increases following hormonal changes (from the third to sixth decades) and thereafter remains relatively constant. However, the incidence of sarcopenia in the older women compared with the older men is higher (17, 86). After the third decade of life, muscle mass in women tends to decrease and this decline is accelerated after the fifth decade (11, 84). A 0.6 percent per year decline in muscle mass in women has been reported in postmenopausal period (86). Although a positive relationship between muscle mass and plasma levels of estrogen (87), estrone and estradiol (88) in women has been reported, the underlying mechanisms by which decreased levels of estrogen negatively affect muscle mass is poorly understood. It has been suggested (89-94) that estrogen may have direct and indirect effects on muscle mass. There is evidence that estrogen exerts direct effects on skeletal muscle through ERs located on the cell and the nuclear membrane and in the cytoplasm of the muscle fibers (89, 90). Interestingly, it has been reported (89) that expression of ERs in postmenopausal women is lower compared to men, women and children, suggesting a direct role for estrogen and muscle protein synthesis or maintaining lean tissues which is reversed by estrogen administration. Additionally, in ovx rat model, estrogen administration attenuates the muscle atrophy due to disuse (91). Estrogen has been shown to act through estrogen receptor-alpha to up-regulates the Akt/mTOR pathway in mice (92). Furthermore, estrogen deficiency has been associated with elevated levels of pro-inflammatory molecules (93). Serum levels of pro-inflammatory cytokines including TNF-α, IL-6 and IL-1, have been shown to increase as a result of estrogen deficiency after menopause (18). Elevated levels of these cytokines promote the ubiquitin–proteasome pathway which is the main mechanism for protein degradation in skeletal muscle (79, 80). A limited number of studies (94-96) have reported that ovarian hormone deficiency delays recovery of the skeletal muscle mass from disused-induced

muscle atrophy in rats. While the effect of estrogen on bone is better understood, its effect on other tissues including skeletal muscle is poorly understood and needs further investigation.

2.5. Age-Related Changes on Bone and Muscle

With advancing age in humans and other vertebrate animals, it is believed that the rate of bone formation gradually diminishes while the rate of bone resorption either is unaltered or accelerated, resulting in net bone loss (97–99). These events, in part, have been linked to the gradual age-associated modulation in immune cell mediators (cytokines and prostaglandins) and formation of reactive oxygen species (ROS) either in the bone microenvironment or in the cells that serve as osteoclastic precursors such as monocyte-macrophage lineage (100, 101). Bone contains a plethora of local cytokines and lipid mediators such as IL-1, IL-6 and TNF-α (102). The age-associated increase in certain immune cell mediators, such as IL-1, IL-6 (103) may be partially responsible for the stimulated-osteoclastic bone resorption in senescence with unclear mechanisms of action.

Aging is also associated with an increase in fat mass and a decrease in muscle mass (104-108). While visceral fat and intramuscular fat tend to increase with age, the subcutaneous fat decreases (108). As it was discussed earlier, the decline in muscle mass may be associated with an increase in pro-inflammatory cytokines leading to an imbalance between muscle protein synthesis and protein breakdown (111-112).

ROS, such as superoxide and hydrogen peroxide, are produced during normal cellular metabolism (e.g., mitochondrial electron transport) or from environmental stimuli (e.g., cytokines, UV radiation) (100). ROS production also accelerates with advancing age in humans and other vertebrate animals, impairing the integrity of DNA, proteins, and lipids. Age-associated increases in oxidative stress have a detrimental effect on bone and muscle. ROS is considered to be the major contributor to the aging process (100) and various degenerative diseases including osteoporosis (112-114) and sarcopenia. Furthermore, ROS increases osteoclastogenesis (115,116) and contributes to pathogenesis of sarcopenia (117).

2.6. Effects of IGF-I on Bone and Skeletal Muscle Metabolism

Insulin-like growth factor-I has an essential role in muscle and bone cellular growth, development, survival, and metabolism (33, 116, 117). IGF-I is the major circulating growth factor produced by various tissues; however, the liver is the major site for contributing to circulating levels of IGF-I (117-119). The production of IGF-I is mainly in response to growth hormone (GH) and normally its serum level is used as a surrogate measure of GH due to the fluctuations in GH levels (117-119). IGF-I has the ability to act on various cells via autocrine and paracrine signaling (117-119). Both human (120-124) and animal (125-128) studies have shown a positive correlation between serum IGF-I concentrations and bone mass. It has been reported that serum IGF-I levels are positively correlated with the rate of skeletal acquisition at puberty (120). Similarly, positive associations have been demonstrated between serum IGF-I levels and bone mass at various sites including hip, radius and lumbar spine in women (122, 123). Furthermore, lower rates of serum IGF-I levels in older men and women were related to higher risk of hip fracture (123, 124).

The mechanisms underlying the role of IGF-I in bone remodeling have been investigated using genetically modified mouse models (125). Mice lacking liver-specific IGF-I exhibited a marked reduction in cortical bone volume, bone mineral density and periosteal circumference (126, 127). It also has been shown that IGF-I receptor knockout mice have significantly decreased cortical bone size and shortened femoral length compared to the control mice (125). Overall, published data suggest that serum IGF-I is an important anabolic factor that influences bone formation and bone development.

Studies (129, 130) have shown that local IGF-I signaling is essential for proper bone development. It was reported (129) that bone mineral density and trabecular bone volume increased in osteoblast-specific transgenic mice despite normal levels of circulating IGF-I. Additionally, trabecular bone volume was decreased following deletion of osteoblast-specific IGF-I receptor (130). A more recent study (131) has shown that overexpression of IGF-I gene in the livers of transgenic mice has been shown to cause three-fold increases in serum IGF-I levels resulting in a significantly increased skeletal size, improved microarchitectural properties of

trabecular bone as well as mechanical properties of femurs. This study (131) also has reported that when local production of IGF-I is compromised, circulating IGF-I levels can compensate for this deficiency, albeit with a delayed lag phase. For instance, when the genes encoding insulin like growth factor I (igf1) was overexpressed in livers of igf1 null mice (KO-HIT mice), elevated levels of serum IGF-I were shown to overcome growth and skeletal deficiencies after 4 weeks, which included an improvement in microstructural properties of femur bone (131).

IGF-I has also been shown to exert a pronounced effect on skeletal muscle growth and repair both in human (132-134) and animal studies (135-143, 81, 82). IGF-I stimulates skeletal muscle protein synthesis (81, 135), inhibits proteolysis (82, 139, 142), promotes the delivery of amino acids and glucose to myocytes (134), and stimulates proliferation and differentiation of the myoblast (140). Administration of IGF-I increases functional recovery of the skeletal muscle after injury (141), decreases exercise induced muscle damage (141), and improves both contractile (143) and endurance (144) function.

While the growth promoting effects of serum IGF-I in humans and animals are well documented (132-143), IGF-I's role in increasing muscle mass has been linked to the locally produced IGF-I rather than its circulating levels. In humans, an increase in muscle IGF-I has been reported both at the mRNA (132) and protein (133) levels following resistant exercise. Additional evidence comes from animal studies (136, 137, 145, 146), for instance, overexpression of IGF-I in the muscle of the transgenic mice caused a significant hypertrophy of skeletal muscles without any effects on serum levels of IGF-I (136, 137). In further support of this notion, infusion of IGF-I directly into rat skeletal muscle has been shown to increase muscle mass (145), enhance resistance training response (145) and prevent muscle atrophy following a detraining period (146). Additionally, in IGF-I and IGF-I receptor knockout mice, significant postnatal retardation in skeletal muscle growth and development (142) have been demonstrated. Interestingly, it has been reported that when gene encoding IGF-I was overexpressed in transgenic mice, circulating IGF-I was increased by 1.5-fold. However, this elevation in circulating IGF-I had no significant effect on muscle mass (147). Not only systemic elevation of IGF-I has been shown to be ineffective in increasing muscle mass, in contrast, its higher circulating levels may interfere with the action of IGF-I produced locally. Overall, to date literature suggests that while circulating

IGF-I plays little or no effect on muscle mass, its local production by muscle cells enhance muscle mass and hypertrophy.

2. 7. Effects of TNF-α on Bone and Skeletal Muscle Metabolism

Tumor necrosis factor alpha is a cytokine that induces systemic inflammation (148). It is produced mainly by activated macrophages while other cells have the ability to produce this cytokine as well (149). Both in vitro and in vivo studies (150,151) suggest that TNF-α has a central role in the pathophysiology of bone loss following menopause. TNF-α increases bone resorption while simultaneously inhibiting the expected homeostatic response of new bone formation. TNF-α exerts direct and indirect effects on osteoclastogenesis. Directly, it affects osteoclast precursors and indirectly, it up regulates the production of M-CSF and RANKL in mesenchymal stem cells (152-154). Numerous studies (152- 156) have reported that TNF-α stimulates osteoclast-mediated bone resorption via RANKL upregulation. TNF-α has also been shown to increase osteoclastogenesis by upregulating the expression of osteoclast-associated receptors (OSCAR) (155, 156). In addition to promoting osteoclastogenesis via RANKL, TNF-α stimulates osteoblasts to produce other cytokines that facilitate maturation of osteoclasts (148, 149).

TNF-α impairs the function of osteoblasts by suppressing production of a matrix (157) that is an essential component for mineralization and blocking the differentiation of new osteoblasts from their progenitors (158). TNF-α inhibits the production of type I collagen and decreases the expression of osteocalcin mRNA (157), two skeletal matrix proteins necessary for bone formation. TNF-α inhibits activity of alkaline phosphatase which is required for normal bone mineralization (159). TNF-α also suppresses the production of IGF-I at mRNA level (160). Overall, higher levels of TNF-α are associated with chronic inflammatory conditions such as osteoporosis.

TNF-α has a dual role in skeletal muscle. Its autocrine action is essential for the initiation of myogenesis as a result of either injury or mechanical stimulation (161, 162). TNF-α production in injured myofibres is increased due to the release of infiltrating inflammatory cells (163, 164).

However, acute increases in TNF-α levels are required for the stimulation of satellite cells which are essential to repair the damaged muscle (165, 166). TNF-α is necessary in order for satellite cells to differentiate (167) and proliferate (165, 166) as well as muscle protein synthesis (168). These processes are essential in repairing muscle due to injury (169). However, the acute effect of TNF-α disappears at high levels (167). For instance, the differentiation of cultured myoblasts only could take place when TNF-α concentration was 0.05 ng/mL of medium. Indeed, TNF-α at higher concentrations (0.5 to 5 ng/mL) caused apoptosis of satellite cells although initially these concentrations triggered proliferation of the satellite cells (170). Additionally, direct injection of TNF-α into muscles has been shown to cause muscle atrophy and impaired muscle regenerative capacity (171-174). Furthermore, TNF-α injection into rat muscle has led to significant losses in body and muscle mass (175, 176). Some investigators believe that muscle wasting as a result of TNF-α injection is through activation of the ubiquitin–proteasome pathway (177, 178) with decreased rate of protein synthesis (179). Interestingly, there are reports suggesting that TNF-α interrupt IGF-I signaling resulting in compromised protein syntheses which are under the control of IGF-I (180). In summary, literature suggests that TNF-α affects target tissues differently depending on its concentration and the exposure time.

In conclusion, obesity continues to be a major health issue among all segments of population particularly among postmenopausal women. Although obesity in postmenopausal women has often been linked to excess caloric intake and physical inactivity, there is a need for better understanding of the role of estrogen and or its absence that may exert a profound effect on energy metabolism and body composition. Thus, understanding the role of estrogen throughout menopausal period on body composition has important implications for discovering new therapies and needs further investigation.

CHAPTER THREE

METHODS

3.1. Introduction

The ovariectomized (ovx) rat is the most commonly used model demonstrating bone loss (33). Other models include immobilized rat model (or disuse model) created by limb unweighing or resection of nerve or tendon (21-23, 33). However, the ovx rat model appears to be a suitable model for studying postmenopausal bone loss (33). Similar to menopause, the ovx rats experience increased rates of bone resorption and new bone formation with the rate of resporption exceeding that of formation (21-23, 33). This would result in a net loss of bone mass. Estrogen deficiency in the ovx rat model appears to be the most appropriate method available for modeling excess resorption not accompanied by formation deficit (21-23, 33). Although it is well established that bone loss can be prevented in rats by estrogen replacement, the investigation of its effect on muscle mass is sparse. To examine the potential role of estrogen in modulating body composition including bone mineral density (BMD) and muscle mass, two mature age groups of female rats have been utilized in this study. These age rats are prone to marked muscloskeletal sensitivity to ovariectomy-induced alterations in body composition.

3.2. Animals and Diets

Guidelines for the ethical care and treatment of animals from the Animal Care and Use Committee at The Florida State University were strictly followed. Thirty-six female SD rats, 5- and 10-month old (18 rats per age group; for each age category: n=6 per treatment group), after 5 days of acclimation, rats in each age group were divided into sham, ovx, and ovx+E_2. Five- and ten-month old rats were chosen to model young and middle age women, respectively. The average life expectancy of rats is about two years. Treatments were initiated immediately after surgery and continue for 3.5 and 5 months due to the fact that the younger the rats are the faster they lose bone mass as a result of ovariectomy (33). Rats in all groups received a semi-purified control diet (AIN-93M). Animals in the ovx+E_2 group were injected with17β-estradiol (E_2; 10μ/kg body weight (BW) subcutaneously, twice per week). Ovx cortol rats received solvent

vehicle (sesame oil; subcutaneously, twice per week) (Table 1). Ovariectomy is known to influence food intake which affects body weight gain and bone and muscle mass. Therefore, it was important to control dietary intake of all animals to similar amounts. Hence, rats in ovx groups were pair-fed to the mean food intake of the sham group and their food intake was adjusted every three days. Rats were weighed weekly during the course of the study and food intake was monitored every three days. Rats' voluntary running activity was measured by activity wheel and distance counter in individual cages for 24 hours one week before sacrifice.

Table 1. Experimental design

Surgery		**Intervention**
5-month old	sham	Solvent vehicle (Sesame oil)
	ovx	Solvent vehicle (Sesame oil)
	ovx + E_2	17β-estradiol (10μ/kg BW)
10-month old	sham	Solvent vehicle (Sesame oil)
	ovx	Solvent vehicle (Sesame oil)
	ovx + E_2	17β-estradiol (10μ/kg BW)

3.3. Animal Necropsy and Tissue Collection

At the termination of the study, animals were anesthetized with ketamine/xylazine (100 mg/5 mg/kg BW) and bled from their abdominal aortas. Tissues, including bone, muscle, liver, heart and uterus, were harvested. An incision through the skin from the medial side of the thigh to the abdomen was made. The skin was then reflected to expose the muscles of the lower leg. With scissors, an incision along the white facial line demarcating lateral aspects of the lower leg were made from the ankle to 3-5 mm proximal from the ankle. Muscles from hind-limb (gastrocnemius and soleus muscles) were isolated from the distal end using forceps and scissors, weighted, frozen in liquid nitrogen, and stored at -80°C for future analyses. Bone specimens

including vertebrae, tibiae were collected, cleaned of adhering tissues, and stored for further analysis. Additionally, in order to confirm the success of ovx, ovaries were removed, nicked and weighed.

3.4. Assessment of Body Composition and Bone Density

The whole body bone mineral density (BMD), bone mineral content (BMC), lean mass and fat mass of all rats were assessed prior to surgery and at the end of the study using dual energy x-ray absorptiometry (iDXA; GE Healthcare). Following the termination of the study, BMD and BMC of bone specimens including right tibiae and 4^{th} lumbar vertebrae were measured using DXA (Hologic Inc., Bedford, MA. US). Gastrocnemius and soleus muscles wet weights were used to determine any changes in muscle mass in association with lean body mass from DXA (Hologic Inc., Bedford, MA. US).

3.5. Microcomputed Tomography (μCT_{35}) for Analysis Microstructural Properties of Trabeculae in Tibia and 4^{th} Lumbar Vertebra

Microarchitectural properties of the right tibia and 4th lumbar vertebra were measured using using μCT 35 scanner (Scanco Medical, Brüttisellen, Switzerland). All specimens obtained at sacrifice were frozen at −20°C until the time of scanning. The tibia was scanned from the proximal growth plate in the distal direction (16 μm/slice). This region included 350 images obtained from each tibia using 1024 × 1024 matrix resulting in an isotropic voxel resolution of 22 μm3 (). An integration time of 70 ms per projection was used, with a rotational step of 0.36° resulting in a total acquisition time of 150 min/sample. The volume of interest (VOI) was selected as a region twenty five slices away from the growth plate at the proximal end of the tibia to 125 slices. The 3D images were also obtained for visualization and display. Lumbar vertebra were scanned from the caudal to the dorsal end (530 slices; 16 μm/slice). This region was included 530 images obtained from each vertebra using the same isotropic voxel resolution and integration time as described with the tibia. The VOI was selected 25 slices away from the appearance of the growth plate at each end of the vertebral body resulted in approximately 300 slices. Bone morphometric parameters, including bone volume over total volume (BV/TV),

trabecular number (Tb.N.), separation (Tb.Sp.), and thickness (Tb.Th), structure model index (SMI) and connectivity density (Conn. D.), were obtained by analyzing the volume of interest (VOI). The operator conducting the scan analysis was blinded to the treatments associated with the specimen.

3.6. Blood Parameters

Following collection of blood samples, they were centrifuged (4°C) at 1500 g for 15 minutes. Serum samples were immediately separated, liquated and stored in -20°C for analysis of 17β-Estradiol and two of the most widely accepted markers of bone turnover (22, 34) for rats namely C-telopeptides of type I collagen (CTX) as a marker of bone resorption and bone specific alkaline phosphatase (B-ALP) as a marker of bone formation. These measurements were done via enzyme linked immunoassay (ELISA) kits from TSZ ELISA (Framingham, MA, USA) and Immunodiagnostic Systems Inc (Fountain Hills, AZ, USA), respectively following the manufacture's protocols. Additionally, insulin like growth factor I (IGF-I), the anabolic factor associated with increased bone and muscle formation, was measured using ELISA kits (R&D Systems, Minneapolis, MN).

In order to determine the effect of estrogen on inflammatory markers, tumor necrosis factor alpha TNF-α was measured in serum using an enzyme-linked immunosorbent assay (ELISA) kit and following the instructions that came with the kit (Life Technology, Grand Island, NY).

3.7. Analysis of Select Skeletal Muscle Gene Expressions

3.7.1. RNA Isolation

Total RNA was extracted from 30-40 mg of gastrocnemius (GAS) and soleus (SOL) muscle tissues using TRI Reagent (Molecular Research Center, Cincinnati, OH) based on the acid-phenol extraction method as described in detail previously (181, 182). In brief, after precipitating the extracted RNA from the aqueous phase with isopropanol, it was washed twice with ethanol, dried and suspended in nuclease-free water at a ratio of 0.8 µl per mg of muscle. RNA was

quantified spectrophotometrically and RNA samples were stored at −80°C for later analyses of specific mRNAs using relative reverse transcription polymerase reaction (RT-PCR) procedures (181, 182).

3.7.2. Semi Quantitative Reverse Transcription Polymerase Reaction (RT-PCR)

From each tissue sample, one microgram of total RNA was denatured by heating at 70°C and reverse transcribed in presence of a total volume of 20 µl using SuperScript II Reverse Transcriptase with a mixture of oligo (dT) (100ng/reaction) and random primers (200ng/reaction) as described in detail previously (181- 183). After 50 minutes incubation at 44°C, the reaction was stopped by heating at 88°C for 5 minutes and samples were stored at -80°C for later analyses of PCR. A relative reverse transcription polymerase chain reaction method (181- 183) using 18S ribosomal RNA (Invitrogen, Life Technology, GIBCO-BRL, Carlsbad, CA) as an internal standard was used to determine relative expression levels of mRNAs for IGF-I, TNF- α , and atrogin-I. The primer sequences for the specific target mRNAs are indicated in Table 2.

Table 2. Primer sequences of gene products for RT-PCR

Target mRNA	PCR Primer Sequence 5'→3'	Product Size	Gene Bank Association No.
IGF-I	Sense 5'-GCATTGTGGATGAGTGTTGC-3' Anti-sense 5'-GGCTCCTCCTACATTCTGTA-3'	202	X06043
TNF-α	Sense 5'-TGGCGTGTTCATCCGTTCTCTACC-3' Anti-sense 5'CCCGCAATCCAGGCCACTACTT-3'	215	NM-012675
atrogin-I	Sense 5'-CAGAACAGCAAAACCAAAACTC-3' Anti-sense 5'-GCGATGCCACTCAGGGATGT-3'	323	NM-133521

Each set of forward and reverse primers was designed using DNA Star Lasergene 7 software. For optimal conditions all primer sets were tested first. To express each PCR reaction as a ratio of target mRNA/18S, 18S (with a 324-bp product) was co-amplified with each target cDNA (mRNA). After PCR, 23 μl of PCR product were separated by electrophoresis (100V) in a 2 percent agarose gel for 1.5 h. The gels were stained with ethidium bromide (0.1μg/ml) for visualization using a UV light source and the gene products were quantified using a Bio-Rad ChemiDoc™ EQ densitometer and a Bio-Rad QuantityOne® software (Bio-Rad Laboratories, Hercules, CA, USA).

3.8. Statistical Analysis

3.8.1. Power Calculation

Powers for detecting differences were calculated based on a range of possible decreases in BMD of ovx rats when compared to sham operated rats. The range of possible increases is set beginning at 1 percent and increases by 1.0 percent increments to 8 percent. The power was calculated as the:

Probability (rejecting mean (i) = mean (j) given difference = x).

A two-tailed significance level of 0.05 was used. The difference stated in the above probability statement was expressed as a percentage of a reported standard deviation. Thus, 5 percent increase was one standard deviation, and the square of this value was what was assumed to estimate the pooled variance estimate calculated in the analysis of variance. Six rats per treatment group provided us with a power of more than 0.80 at an $\alpha = 0.05$ to detect a difference in BMD of one standard deviation.

3.8.2. Data Analysis

For each age category, a completely randomized design was utilized. SigmaStat 3.5 (Systat Software Inc., San Jose, CA) was used to analyze the data and when a one way ANOVA indicated any significant differences among the means, Tukey's post-hoc test was performed.

Statistical significance was set at the $P < 0.05$ level for all analyses. Descriptive statistics were calculated on all variables and included means, standard deviations, minima and maxima.

CHAPTER FOUR

RESULTS

For the purpose of clarity and distinction, results are presented in 5-month and 10-month old rats separately.

4.1. Findings of Five-month Old Rats

4.1.1. Food intake, 24 Hour Voluntary Wheel Running Distance, Body and Organ Weights

In spite of pair feeding the ovx rats to the mean food intake of sham rats, the final body weights of ovariectomized (ovx) rats were significantly (P=0.04) higher than those of sham-operated rats (Table 3).

Table 3. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on food intake, body and organ weight in 5-month old rats

Parameter	**5-month old**		
	Sham	***Ovx***	***Ovx+E_2***
Food intake (g/day)	16.38±9.1	16.39±9.8	16.05±9.4
Body Weight (g)			
Initial	294±6.96	293±6.65	293±4.99
Final	366±9.8 ^{a}	400±9.8 ^{b}	350±13.38 ^{a}
Organ weight (mg)			
Uterus	0.618±0.05 ^{a}	0.098±0.01 ^{b}	0.287±0.03 ^{c}
Liver	10.640±0.653	9.362±0.236	8.750±0.617
Heart	1.050±0.057	0.965±0.0293	0.950±0.029

Values are means ± SEM. Values that do not share the same superscript letters are significantly (P<0.05) different from each other.

Estrogen prevented the ovariectomy-induced weight gain as the mean weight of rats in the ovx+E_2 group was not different from that of the sham group. As expected, ovariectomy caused atrophy of uterine tissue, indicating the success of the surgical procedure, and E_2 administration prevented this atrophy. No significant differences were found between mean heart and liver weights. These results are present in Table 3.

Rats in ovx group in terms of activity tended (P=0.096) to have lower activity as measured by 24-hour voluntary wheel running compared with sham-operated animals (Figure 1).

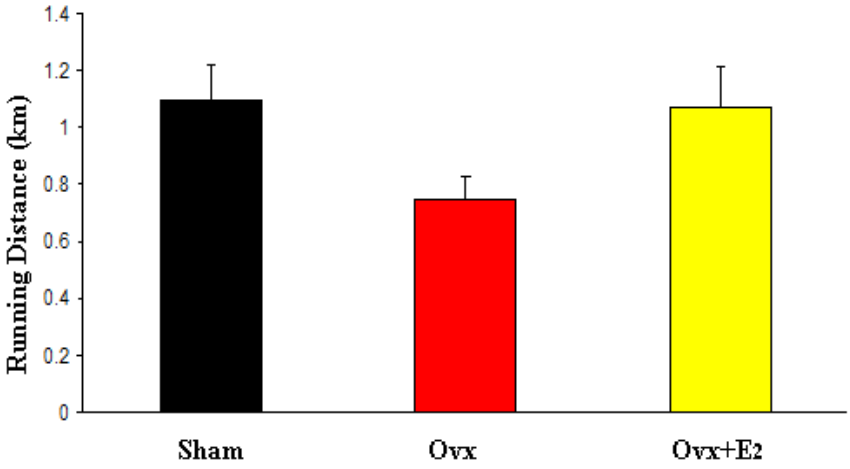


Figure 1. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on 24 hour voluntary running distance in 5- month old rats. Values are means ± SEM. There were no significant differences among the treatment groups.

4.1.2. Body Composition

Baseline values in terms of fat mass, lean mass, bone mineral density (BMD) and bone mineral content (BMC) were not significantly different among the groups (Table 4). However, at the end of the study period, mean fat mass of ovx rats in comparison with sham animals was significantly (P=0.006) higher. Rats that received E_2 did not experience an increase in fat mass. In contrast to fat mass, animals in all groups numerically lost lean mass compared to their

corresponding baseline values. The losses in lean mass were 22 percent, 27 percent, and 25 percent, respectively for sham, ovx and E_2 group.

Table 4. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on body composition in 5-month old rats

Parameter	**5-month old**		
	Sham	***Ovx***	***Ovx+E_2***
Total Fat Mass (g/cm²)			
Baseline	69.400±6.478	70.500±5.948	73.200±5.517
Final	172.00±10.5 ^{a}	226.00±8.68 ^{b}	170.20±13.24 ^{a}
Total Lean Mass (g)			
Baseline	204.600±10.567	203.833±7.485	203.000±7.120
Final	167.00±10.96	146.16±9.08	155.20±9.61
Total BMD (g/cm²)			
Baseline	0.159 ±0.0008	0.160 ±0.004	0.161 ±0.002
Final	0.173±0.003 ^{a}	0.158±11.96 ^{b}	0.172±0.004 ^{a}
Total BMC (g)			
Baseline	8.940 ±0.108	8.800 ±0.144	9.100 ±0.224
Final	11.54±0.42	11.60±0.004	11.18±0.004
4^{th} Lumbar			
BMD (g/cm²)	0.246±0.005 ^{a}	0.190±0.002 ^{b}	0.240±0.005 ^{a}
BMC (g)	0.130±0.007 ^{a}	0.089±0.005 ^{b}	0.128±0.006 ^{a}
Right Tibia			
BMD (g/cm²)	0.211±0.003 ^{a}	0.193±0.004 ^{b}	0.209±0.002 ^{a}
BMC (g)	0.311±0.003	0.292±0.007	0.309±0.007

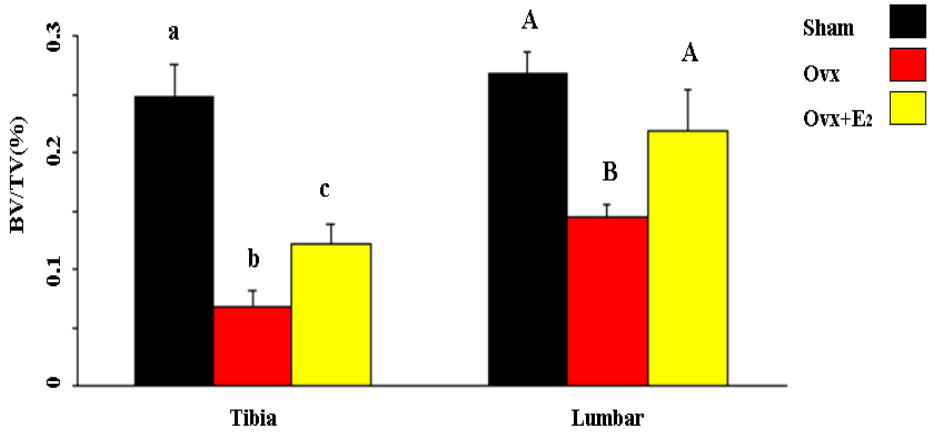
Values are means ± SEM. Values that do not share the same superscript letters are significantly (P<0.05) different from each other.

There were no differences in the whole body BMC among the three groups; however, ovx significantly (P=0.05) lowered the whole body BMD (Table 4) in comparison with sham animals and E_2 prevented the loss of whole BMD. The mean tibial and vertebral BMD and the mean vertebral BMC values of ovx rats were significantly (P=0.05 and P=0.001, respectively) lower in comparison with sham rats. E_2 was able to increase the BMD and BMC in these bones to the level of sham animals. These findings are reported in Table 4.

4.1.3. µCT Analysis

Representative images of the three-dimensional (3-D) trabecular microstructures of proximal tibia of the three groups in 5-month age category are presented in Figures 2 through 4. Similar observations were made in 10-month old rats (figures are not shown). Analysis of data indicated that ovx decreased proximal tibial and lumbar trabecular bone volume (BV)/total volume (TV) significantly by 73 percent and 46 percent, respectively when compared to the sham operated animals (P=0.001 and P=0.005) (Figure 2 A). E_2 administration was not able to prevent the ovx-induced alterations in tibial trabecular architectural properties. Ovx significantly (P=0.001) decreased the proximal tibial trabecular number (Tb.N.) (Figure 2 B) but increased trabecular separation (Tb.Sp.) (P=0.01) (Figure 3 A). Although trabecular thickness (Tb.Th.) decreased in both tibiae and vertebrae in response to ovx, the decrease in vertebral Tb. Th. only reached a significant level (P=0.03). Tibial and vertebral Tb.N. (Figure 2 B) were decreased by 65 percent and 16 percent, respectively. While ovx significantly (P=0.001) reduced connectivity density (Conn. D.) (Figure 4 B) of the tibiae, the mean value of Conn. D. of vertebrae was not significant from that of sham animals. SMI, which quantifies the pattern of trabeculae as either more rod- or plate-like, was 1.69 and 0.572 in tibia and vertebra of sham rats, respectively (Figure 4 A). SMI values for both bones increased in ovx rats, i.e. structure model index (SMI) = 2.753 and 1.18 in tibia and vertebra, respectively indicating a shift to less favorable rod-like trabecular bone. In contrast, E_2 was able to prevent the ovx-induced alterations in microarchitectural properties of lumbar vertebra including the reduction in BV/TV, Conn. D, Tb.Th. and SMI. Interestingly, E_2 had no effect on vertebral microstructural properties (Figure 3 A).

A



B

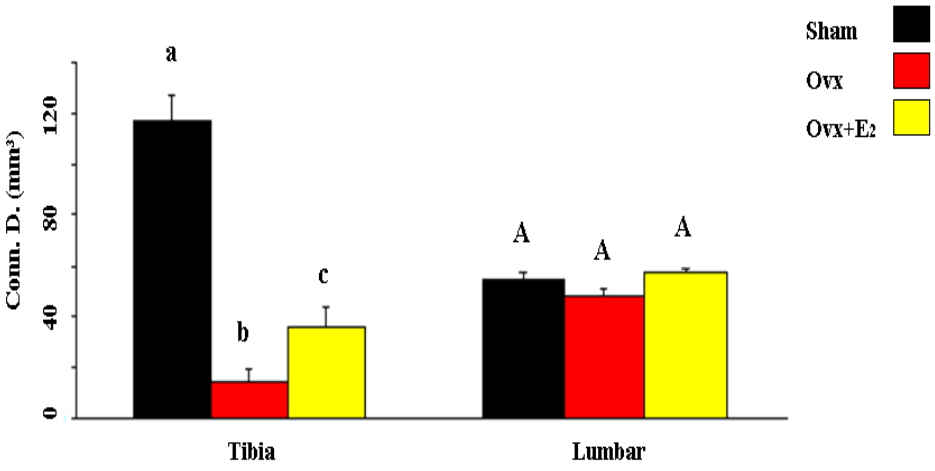
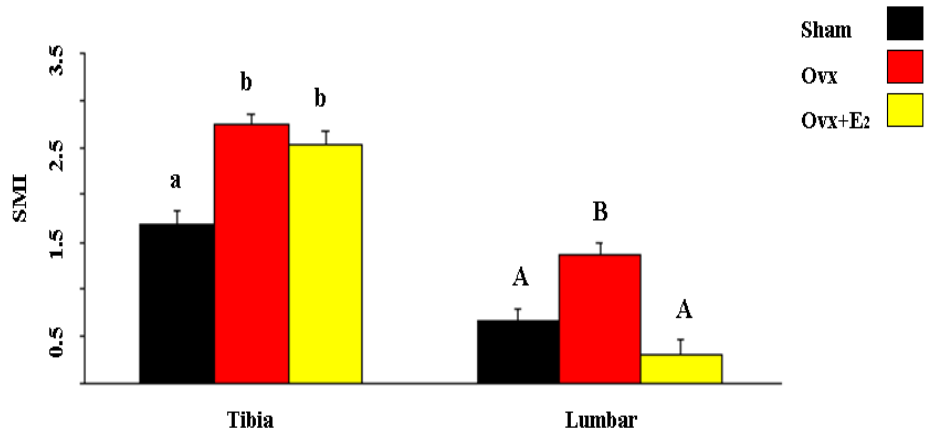


Figure 2. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on bone volume/total volume (BV/TV) (A) and connectivity density (Conn. D.) (B) of right tibia and 4th lumbar vertebrae in 5-month old rats. Values are means ± SEM. Bars that do not share the same superscript letters are significantly ($P<0.05$) different from each other. Small and large letters represent tibia and lumbar, respectively.

A



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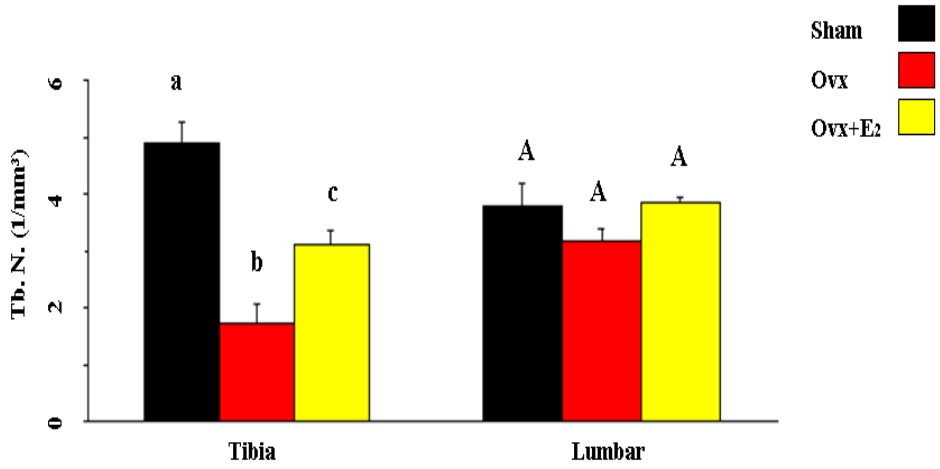
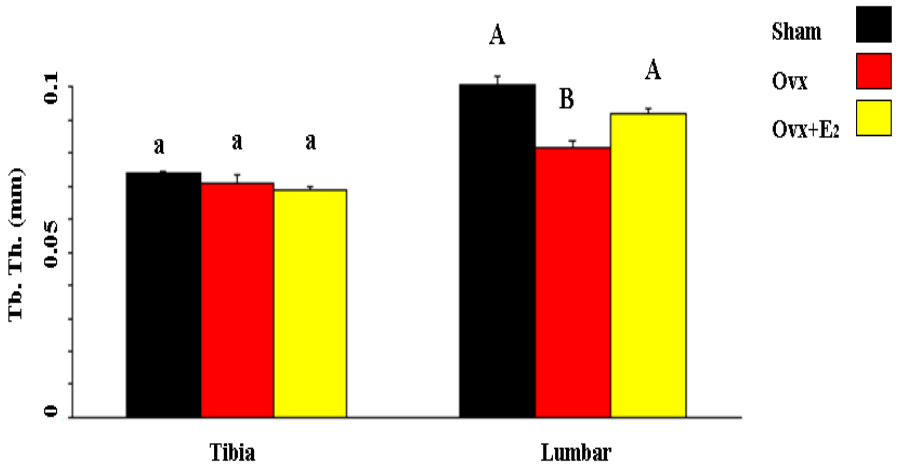


Figure 3. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on structure model index (SMI) (A) and trabecular number (Tb. N.) (B) of right tibia and 4th lumbar vertebrae in 5-month old rats. Values are means ± SEM. Bars that do not share the same superscript letters are significantly ($P<0.05$) different from each other. Small and large letters represent tibia and lumbar, respectively.

A



B

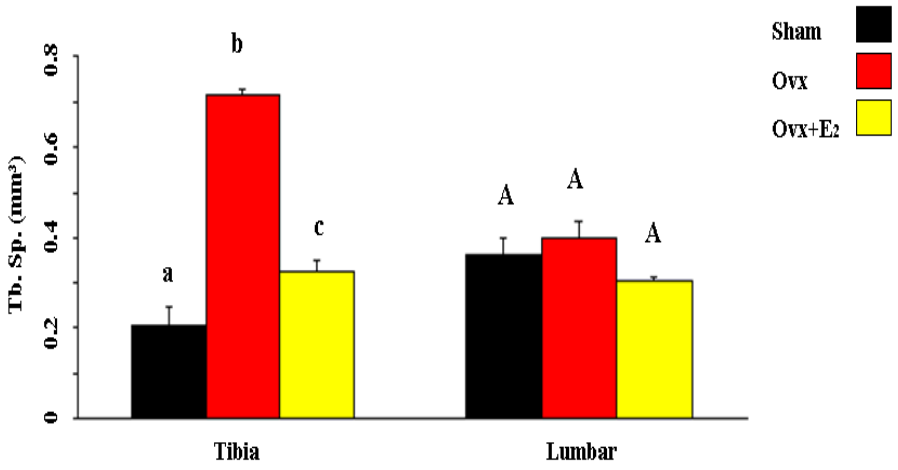


Figure 4. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on trabecular thickness (Tb. Th.) (A) and trabecular separation (Tb. Sp.) (B) of right tibia and 4th lumbar vertebrae in 5-month old rats.Values are means ± SEM. Bars that do not share the same superscript letters are significantly ($P<0.05$) different from each other. Small and large letters represent tibia and lumbar, respectively.

4.1.4. Muscle Mass and Muscle Weight to Body Weight Ratio and Select mRNA Expression in GAS and SOL

Ovarian hormone deficiency increased total body weight (P=0.04) in spite of similar food intake and this excess body weight gain was mainly due to increased fat mass. Although muscle mass was not significantly affected by ovariectomy, when soleus (SOL) and gastrocnemius (GAS) values were normalized to total body weight, mean GAS value in the ovx+E_2 group was tended (P=0.09) to increase compared with that of sham value. Table 5 represents the above findings.

RT-PCR measurements of mRNA expressions of insulin like growth factor I (IGF-I) (Figure 5), tumor necrosis factor alpha (TNF-α) (Figure 6), and atrogin-I (Figure 7) in GAS an SOL muscle specimens were not significantly altered by either ovariectomy or E_2 administration.

Table 5. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on muscle and normalized muscle mass (muscle mass/body mass) in 5-month old rats

Parameter	**5-month old**		
	Sham	***Ovx***	***Ovx+E_2***
Muscle Mass (mg)			
Gastrocnemius	1781.4±58.86 [a]	2076.3±44.42 [b]	1882.8±57.47 [a]
Soleus	135±9.2	163±9.1	139±12
Normalized Muscle Mass (mg/g)			
Gastrocnemius	4.881±0.231	5.191±0.118	5.458±0.197
Soleus	0.371±0.029	0.408±0.024	0.402±0.034

Values are means ± SEM. Mean values that do not share the same superscript letters are significantly (P<0.05) different from each other.

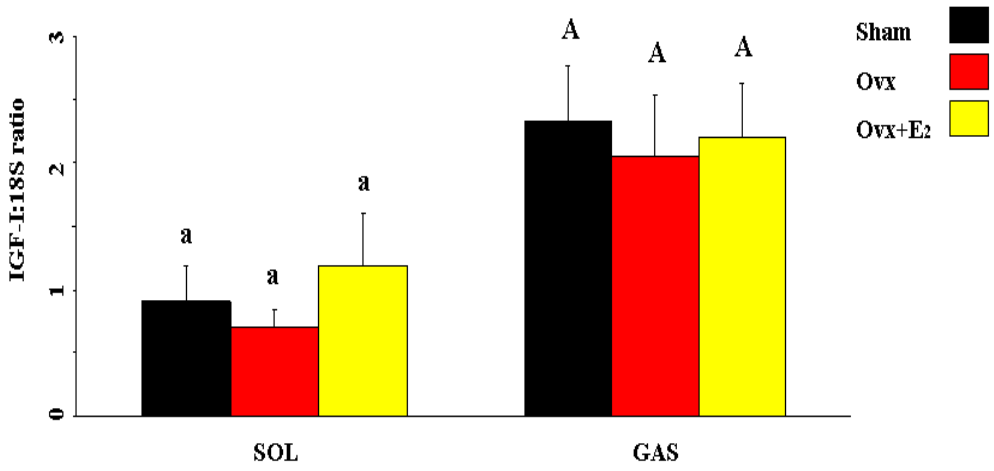


Figure 5. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on IGF-I mRNA expression in 5-month old rats. Values are means ± SEM. There were no significant differences among the treatment groups. Small and large letters represent GAS and SOL, respectively.

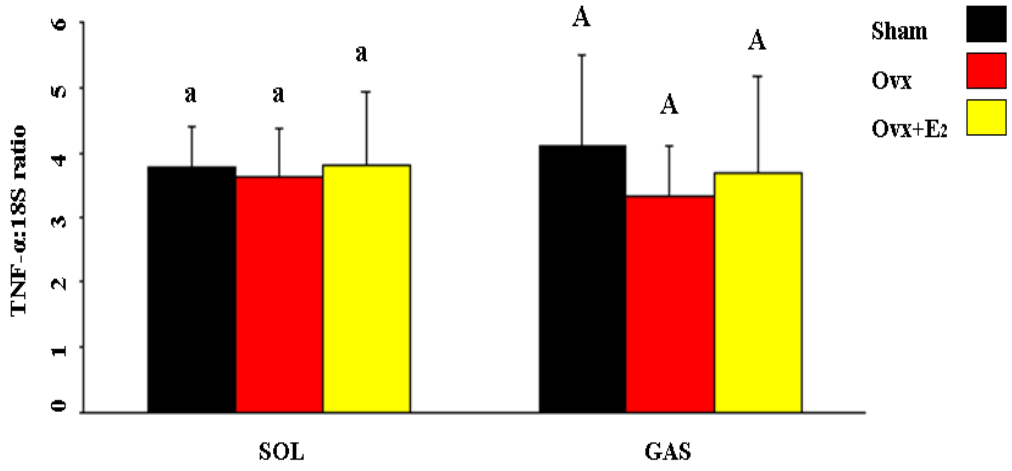


Figure 6. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on TNF-α mRNA expression in 5-month old rats. Values are means ± SEM. There were no significant differences among the treatment groups. Small and large letters represent GAS and SOL, respectively.

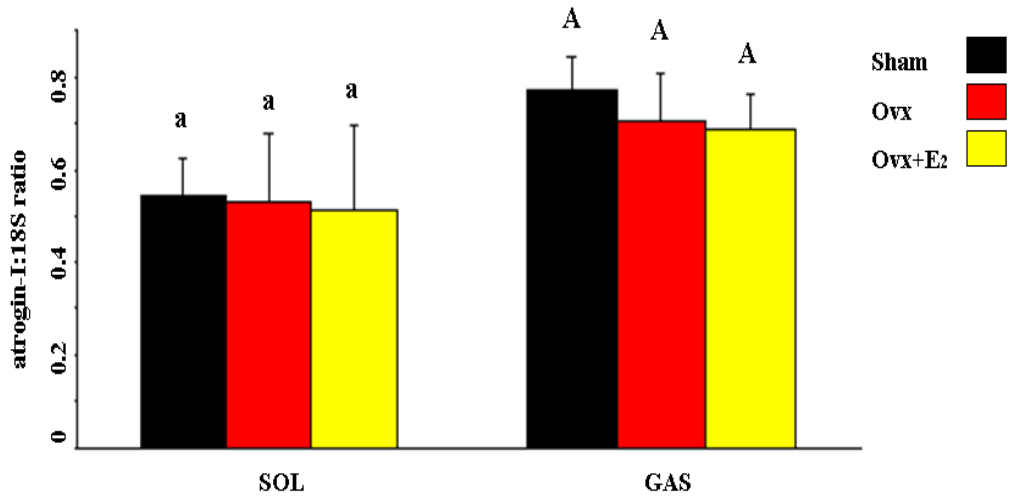


Figure 7. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on atrogin-I mRNA expression in 5-month old rats. Values are means ± SEM. There were no significant differences among the treatment groups. Small and large letters represent GAS and SOL, respectively.

4.1.5. Blood Parameters

Mean serum 17β-estradiol (E_2) level was significantly lower in ovx controls in comparison with sham group. E2 administration resulted in a two fold increase in serum levels of ovx rats. Mean bone specific alkaline phosphatase (B-ALP), level was significantly increased in ovx controls by 40 percent. And though E2 administration lowered B-ALP level, but still its mean was significantly higher than the sham group. Similarly, the mean C-telopeptide of type I collagen (CTX) level was increased by 31 percent in ovx control rats and E2 somewhat reduced its level but did not bring it down to that of sham group (Table 6). Although its mean level was reduced as a result of E2 administration, it remained to be higher than that of sham group. Although, we tried to assess serum TNF-α, its level was below the detection limit of the commercially available kit (Fountain Hills, AZ, USA).

Table 6. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on blood parameters in 5-months old rats

Parameter	5-month old		
	Sham	***Ovx***	***Ovx+E_2***
E_2 (ng/mL)	18.50±4.5 [a]	7.89±3.8 [b]	36.05±9.4 [c]
IGF-I (ng/mL)	631.43±10.8 [a]	667.01±9.7 [b]	651.10±13.38 [a]
B -ALP (U/L)	24.16±13.51 [a]	33.89±10.91[b]	28.10±11.43 [c]
CTX (ng/ml)	84.25±9.05 [a]	110.22±11.01[b]	90.03±10.03 [c]

Values are means ± SEM. Values that do not share the same superscript letters are significantly (P<0.05) different from each other.

4.2. Findings of Ten-month Old Rats

4.2.1. Food Intake, 24 Hour Voluntary Wheel Running Distance, Body and Organ Weights

Similar to 5-month, the mean final body weight of ovx rats were also significantly (P<0.008) higher than the sham-operated rats (Table 7). This happened in spite of pair feeding the ovx rats to the mean food intake of sham animals. In this age group, estrogen also was able to completely prevent the ovariectomy-induced weight gain as the mean weight of rats in the ovx+E2 group was not different from that of the sham group. As expected, ovariectomy caused atrophy of uterine tissue 32 percent, indicating the success of the surgical procedure, and administering E2 significantly (P=0.001) increased the uterine weight compared to ovx animals (Table 7). No significant differences were found between mean heart and liver weights. Ovx significantly (P=0.001) decreased 24 hour voluntary wheel running activity by 57 percent compared with sham operated animals (Figure 8).

Table 7. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on food intake, body and organ weight in 10-month old rats

Parameter	10-month old		
	Sham	***Ovx***	***Ovx+E_2***
Food intake (g/day)	15.65±9.1	15.68±9.8	15.01±9.4
Body Weight (g)			
Initial	369±6.96	370±6.65	361±4.99
Final	421±9.8 ^{a}	474±10.35 ^{b}	404±6.91 ^{a}
Organ weight (mg)			
Uterus	0.733±0.05 ^{a}	0.140±0.02 ^{b}	0.232±0.01 ^{c}
Liver	12.98±1.33	13.22±2.03	10.66±0.06
Heart	1.186±0.029	1.167±0.035	1.125±0.027

Values are means ± SEM. Values that do not share the same superscript letters are significantly (P<0.05) different from each other.

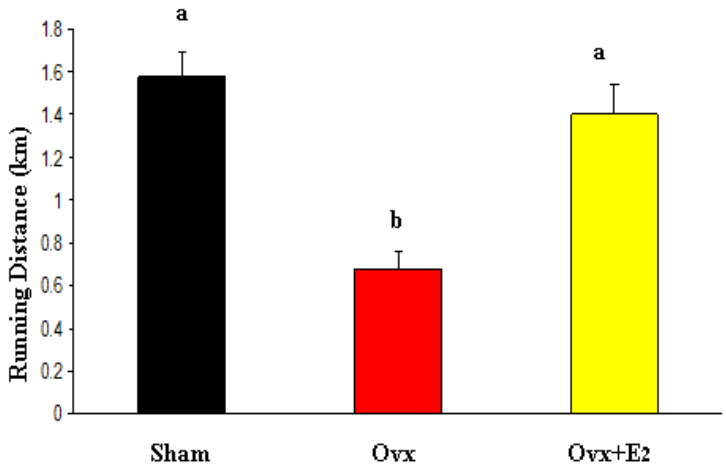


Figure 8. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on 24hour voluntary running distance in 10-month old rats. Values are means ± SEM. Bars that do not share the same superscript letters are significantly (P<0.05) different from each other.

4.2.2. Body Composition

The mean fat mass of ovx rats in comparison with sham animals at the end of the study period was significantly (P=0.02) higher but E_2 was able to prevent this ovx-induced increase in fat mass (Table 8).

Table 8. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on body composition in 10-month old rats

Parameter	**10-month old**		
	Sham	***Ovx***	***Ovx+E_2***
Total Fat Mass (g/cm²)			
Baseline	102.600±14.90	100.333±12.78	101.333±9.97
Final	218.60±11.54 [a]	284.00±7.48 [b]	217.66±7.72 [a]
Total Lean Mass (g)			
Baseline	221.400±9.31	231.167±8.27	219.833±6.63
Final	168.20±10.48	152.167±11.06	160.15±4.61
Total BMD (g/cm²)			
Baseline	0.186±0.007	0.187±0.003	0.181±0.003
Final	0.181±0.008 [a]	0.169±0.002 [b]	0.179±0.006 [a]
Total BMC (g)			
Baseline	11.460±0.93	11.350±0.19	10.517±0.53
Final	13.60±0.80	13.73±0.34	13.17±0.38
4^{th} Lumbar			
BMD (g/cm²)	0.266±0.016 [a]	0.207±0.004 [b]	0.230±0.004 [c]
BMC (g)	0.162±0.017	0.115±0.003	0.127±0.005
Right Tibia			
BMD (g/cm²)	0.240±0.009 [a]	0.204±0.004 [b]	0.218±0.004 [c]
BMC (g)	0.375±0.002 [a]	0.331±0.009 [b]	0.338±0.006 [a]

Values are means ± SEM. Values that do not share the same superscript letters are significantly (P<0.05) different from each other.

In contrast, rats in all three groups lost lean mass compared to their corresponding baseline values by 24 percent, 34 percent, and 30 percent, respectively for sham, ovx and E_2 groups.

Although none of these differences reached a significant level, the loss of lean mass in ovx animals tended to be the highest (P=0.06).

As expected, ovariectomy significantly (P=0.05) reduced whole body BMD without affecting the BMC due to the simultaneous increase in bone area and E_2 prevented these alterations (Table 8). The mean tibial and vertebral BMD and BMC values of ovx rats were significantly (P=0.001 and P=0.04, respectively) lower in comparison with sham rats and E_2 administration prevented these alterations.

4.2.3. µCT Analysis

Similar to 5-month old rats, the 3-D visual images of proximal tibia showed a clear loss of trabecular bone of the ovx rats compared with sham and E_2 groups (Figure 9).

As presented in Figures 10 through 12, the analysis of data indicated that ovariectomy significantly decreased tratrabecular BV/TV in proximal tibial by 86 percent and vertebral by 49 percent (P=0.009 and P=0.006, respectively) (Figure 10 A) in comparison with sham values. E_2 was unable to prevent these ovx-induced reductions in trabecular bone volume in these osteopenic rats. While, ovariectomy significantly decreased tibial and vertebral Tb.N. (P=0.002 and P=0.001, respectively) (Figure 11 B), it caused significant increases in Tb.Sp. in both tibiae and vertebrae (P=0.001 and P=0.001, respectively) (Figure 11 B). Tibial and vertebral Tb.N. (Figure 11 B) were decreased by 73 percent and 45 percent, respectively and Tb.Sp. (Figure 12 B) was increased in tibiae by 74 percent and in vertebrae by 46 percent compared to those of sham values.

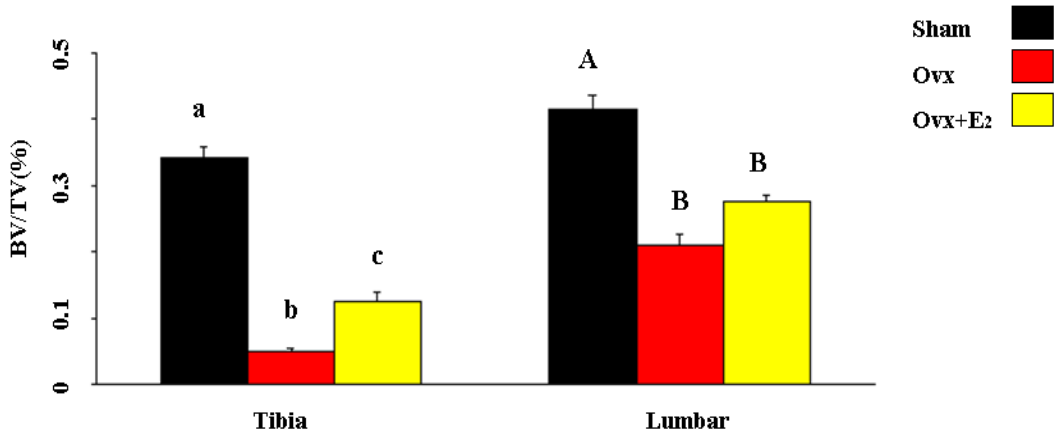
E_2 treatment was able to prevent the ovx-induced reduction in Tb.Sp. (Figure 12 B) in both tibiae and vertebrae. Although it was expected that Tb.Th. increases as a result of the loss of Th.N., the mean values of Tb.Th. were not significantly altered due to ovx (Figure 12 A). E_2 administration prevented the ovariectomy-induced reduction in Tb.N. in tibiae but not in vertebrae (Figure 11 B).

Ovariectomy significantly reduced the mean Conn.D. in both tibiae and vertebrae (P=0.008 and P=0.02, respectively) and E_2 treatment was able to prevent ovariectomy-induced reduction in these values (Figure 10 B).

An increase in SMI value normally indicates a shift in the pattern of trabeculae towards the formation of more rod-like trabecular bone which is inferior bone quality. In the present study, the SMI values for tibiae and vertebrae were both increased compared to those of sham values, i.e. 2.78 vs. 1.49 and 1.07 vs. 0.41 (Figure 11 A) suggesting that ovariectomy not only causes bone loss but also decreases bone quality. Although E_2 administration was able to prevent the increase in SMI value of tibiae due to ovariectomy, it was not able to modulate the vertebral SMI.

Figure 9. 3D trabecular images representative of proximal right tibia of sham, ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2). The images were acquired using μCT. Ovariectomy decreased trabecular bone structure when compared to sham in 10-monht old animals. This effect was prevented by E_2 administration.

A



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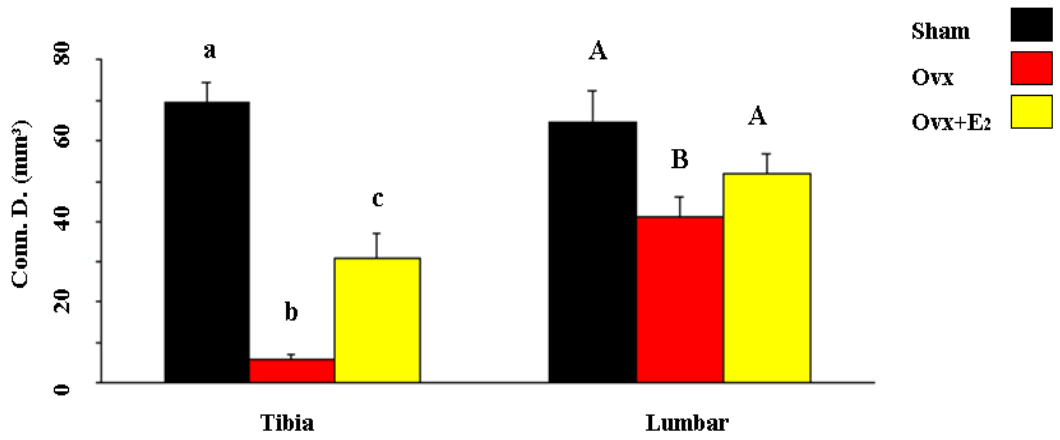
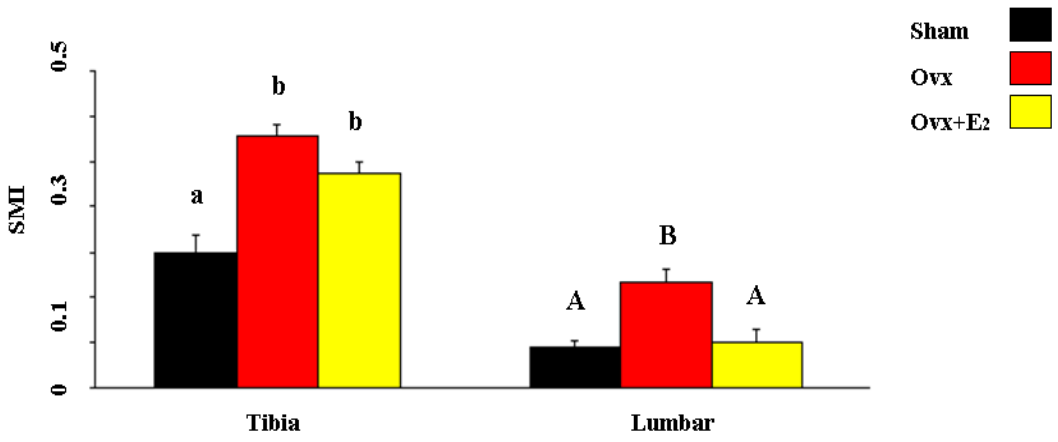


Figure 10. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on bone volume/total volume (BV/TV) (A) and connectivity density (Conn. D.) (B) of right tibia and 4th lumbar vertebrae in 10-month old rats. Values are means ± SEM. Bars that do not share the same superscript letters are significantly ($P<0.05$) different from each other. Small and large letters represent tibia and lumbar, respectively.

A



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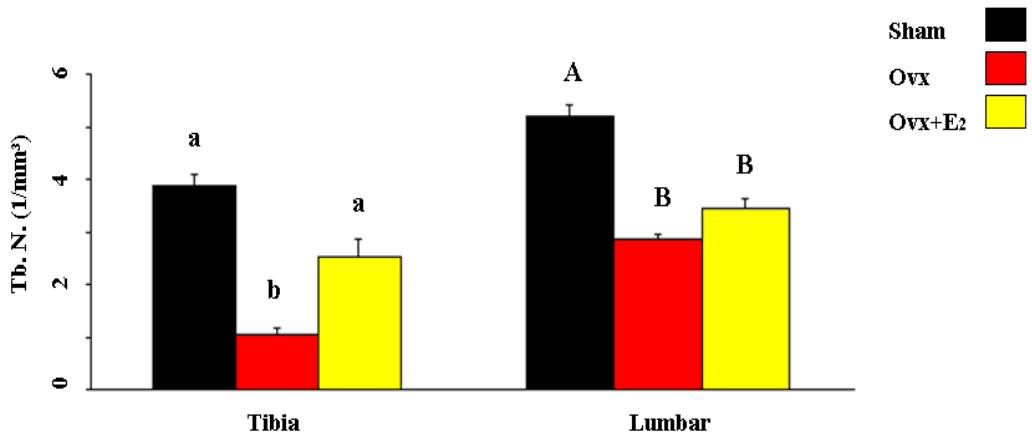
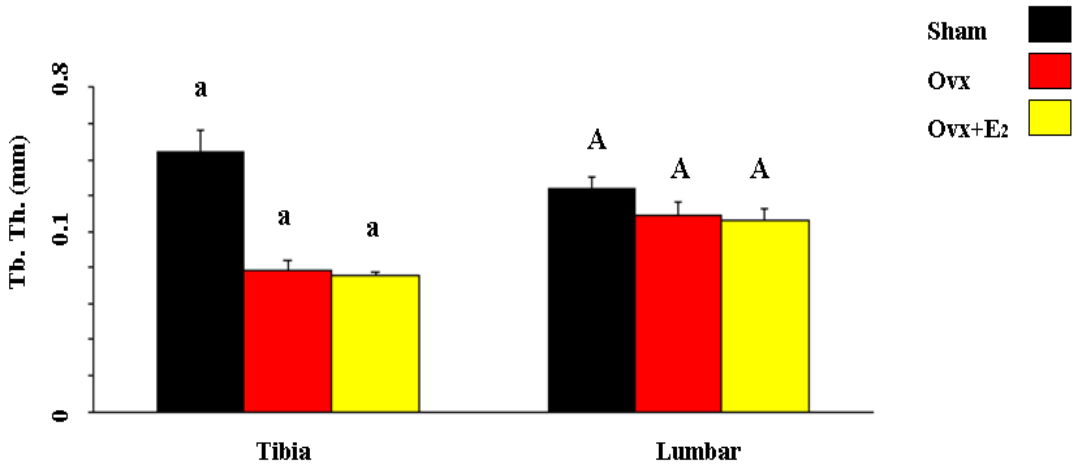


Figure 11. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on structure model index (SMI) (A) and trabecular number (Tb. N.) (B) of right tibia and 4th lumbar vertebrae in 10-month old rats. Values are means ± SEM. Bars that do not share the same superscript letters are significantly ($P<0.05$) different from each other. Small and large letters represent tibia and lumbar, respectively.

A



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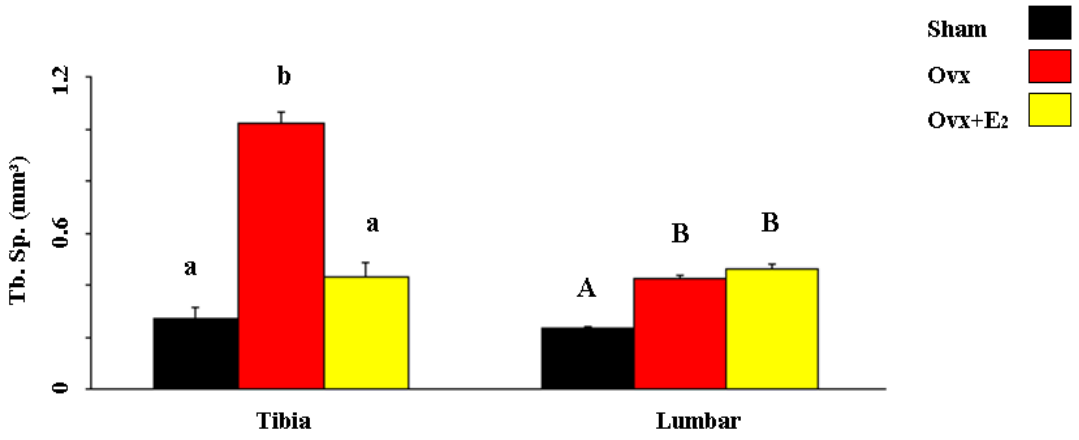

Figure 12. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on trabecular thickness (Tb. Th.) (A) and trabecular separation (Tb. Sp.) (B) of right tibia and 4th lumbar vertebrae in 10-month old rats. Values are means ± SEM. Bars that do not share the same superscript letters are significantly ($P<0.05$) different from each other. Small and large letters represent tibia and lumbar, respectively.

4.2.4. Muscle Mass and Muscle Weight to Body Weight Ratio and Select mRNA Expression in GAS and SOL

There were no differences in the GAS and SOL muscle weights among the groups (Table 9). When these values were normalized to total body weight, the GAS value of ovx group was significantly (P=0.003) lower than that of the sham and/or ovx+E_2 groups (Table 9). Similarly, SOL mass of the ovx group tended (P=0.094) to decrease when normalized to total body weight in comparison with either sham or ovx+E_2 groups (Table 9). E_2 treatment was able to prevent the ovariectomy-induced decreases in SOL, and GAS normalized values by 50 percent and 100 percent, respectively (Table 9). Although mRNA levels of TNF-α and atrogin-I were not affected by either ovx or E_2 administration, IGF-I levels were significantly decreased in both GAS and SOL muscle samples of ovx rats, E_2 admiration prevented these decreases in IGF-I levels (Figures 13 and 15).

Table 9. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on muscle and normalized muscle mass (muscle mass/body mass) in 10-month old rats

Parameter	**10-month old**		
	Sham	***Ovx***	***Ovx+E_2***
Muscle Mass (mg)			
Gastrocnemius	2228.4±65.38	2242.0±67.45	2128.8±54.68
Soleus	155±7.2	150±9.1	140±5.3
Normalized Muscle Mass (mg/g)			
Gastrocnemius	5.285±0.054 ^{a}	4.733±0.011 ^{b}	5.277±0.059 ^{a}
Soleus	0.368±0.014 ^{a}	0.317±0.02 ^{b}	0.347±0.003 ^{a}

Values are means ± SEM, values that do not share the same superscript letters are significantly (P<0.05) different from each other.

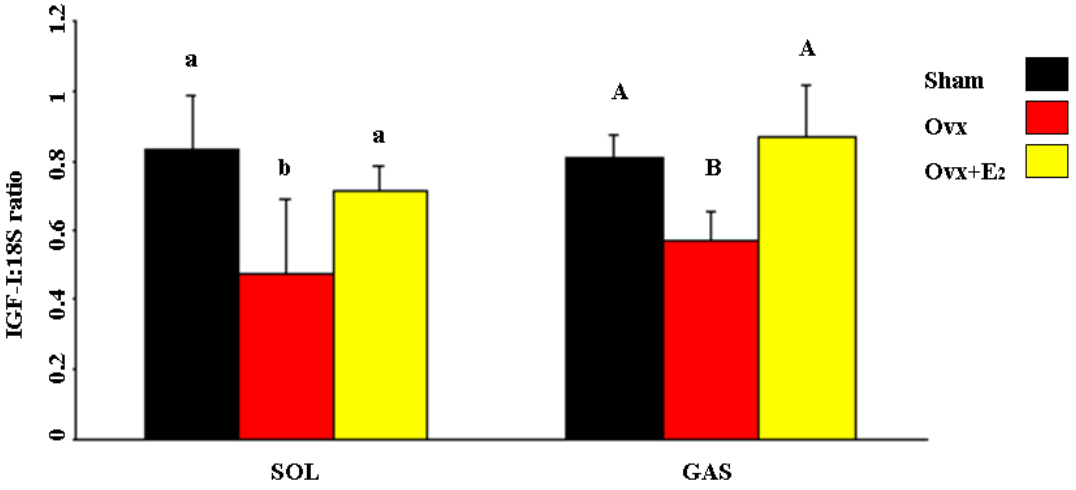

Figure 13. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on IGF-I mRNA expression in 10-month old rats. Values are means ± SEM. Bars that do not share the same superscript letters are significantly ($P<0.05$) different from each other. Small and large letters represent GAS and SOL, respectively.

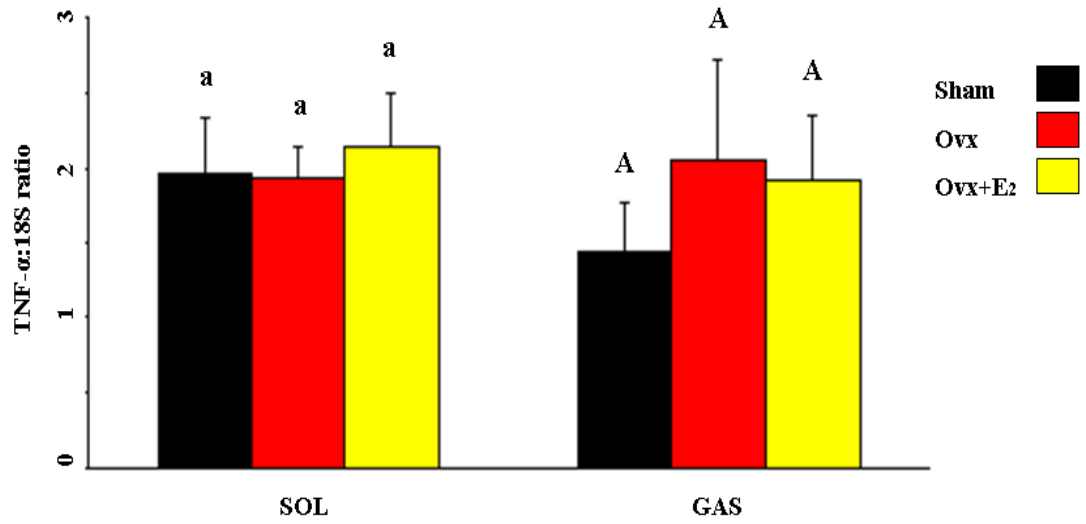

Figure 14. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on TNF-α and mRNA expressions in 10-month old rats. Values are means ± SEM. There were no significant differences among the treatment groups. Small and large letters represent GAS and SOL, respectively.

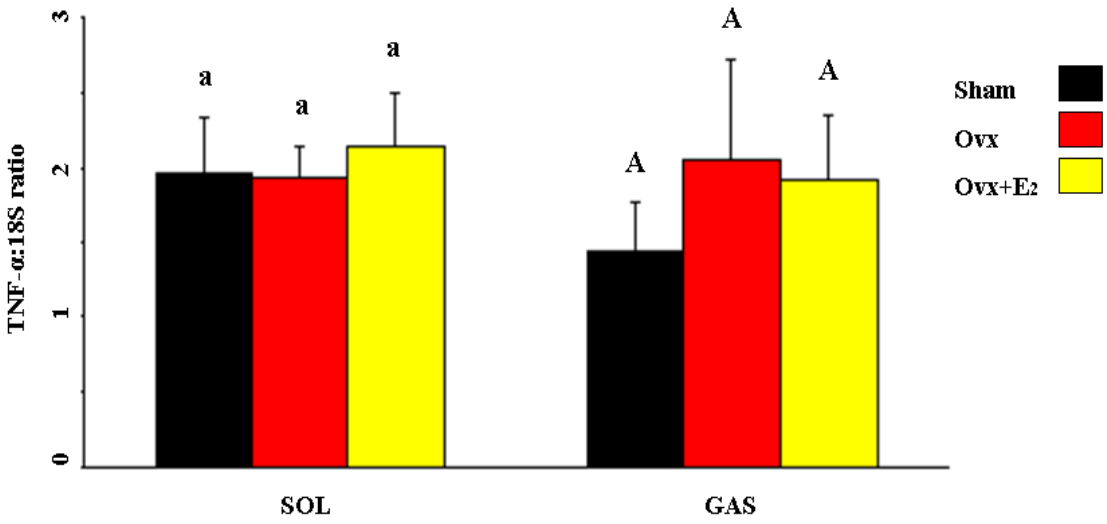

Figure 15. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on atrogin-I (B) mRNA expressions in 10-month old rats. Values are means ± SEM. There were no significant differences among the treatment groups. Small and large letters represent GAS and SOL, respectively.

4.2.5. Blood Parameters

Blood parameters measured in 10-month old rats are present in Table 8 and included serum E_2, B-ALP and CTX. These parameters followed similar patterns in comparison with 5-moth rats. Mean serum E2 level was significantly lower in ovx controls in comparison with sham group by 40 percent. E2 administration increased serum level of E2 in ovx rats significantly by 6 fold which was higher than that of the sham group. The mean B-ALP level of ovx rats was also significantly higher than those of sham and E2 groups. Ovx also significantly increased the mean CTX level by 25 percent compared with that of the sham group. Estrogen administration reduced the CTX level by 20 percent; however, the mean CTX level in E2 group was different from those of both ovx and sham groups. Although TNF-α was measured using three different concentrations, its serum values were undetectable and hence are not being reported here. The mean serum value of IGF-I was significantly higher in ovx rats, however, E2 administration was able to bring its level to those of sham value (Table 10).

Table 10. The effects of ovariectomy (Ovx), and 17β-estradiol (Ovx+E_2) on blood parameters in 10-months old rats

Parameter	**10-month old**		
	Sham	***Ovx***	***Ovx+E_2***
E_2 (ng/mL)	21.07±7.1 ^{a}	8.45±2.1 ^{b}	48.90±9.4 ^{c}
IGF-I (ng/mL)	663.66±9.8 ^{a}	915.75±10.35 ^{b}	689.50±16.91 ^{a}
B -ALP (U/L)	21.56±7.75 ^{a}	35.91±9.82 ^{b}	27.35±10.09 ^{c}
CTX (ng/ml)	77.50±9.05 ^{a}	103.95±11.02 ^{b}	82.32±8.01 ^{c}

Values are means ± SEM. Values that do not share the same superscript letters are significantly (P<0.05) different from each other.

CHAPTER FIVE

DISCUSSION

Ovariectomy caused whole body weight gains in both 5-month and 10-month rats. These increases were not due to excess food intake because they were fed similar amounts of food. We pair-fed Ovariectomized (ovx) rats to the average food intake of sham animals, because earlier studies (185-187) have shown that ovx rats eat more food than intact rats. Our current findings suggest that ovx animals gain higher body weight despite consuming similar amounts of food to those of sham-operated rats in both age categories. These observations are consistent with our previous studies (21-23) and those of other investigators (183, 184) who have demonstrated that ovx rats despite, similar food intake, gain additional body weight compared with sham animals. Although Fisher et al. (183) have suggested that this weight gain was mainly due to increased insulin like growth factor I (IGF-I) and growth hormone production, the exact reason for this excess body weight gain due to ovarian hormone deficiency is yet to be elucidated.

Some investigators (51, 185) have speculated that estrogen deficiency may reduce metabolic rates and some other studies (43, 47) have suggested that estrogen deficiency alter hormones that are involved in energy metabolism. Yet, there are studies (18, 93) that suggest ovariectomy causes up-regulation of pro-inflammatory molecules leading to excess body weight while exerting a deleterious effect on muscle mass (79, 80). In the present study, ovx animals in both age categories had significantly greater amounts of fat mass than the sham-operated and ovx+E_2 animals, suggesting a shift in lower energy metabolism. Similar body compositional changes in postmenopausal women (10, 40-42) and adult ovariectomized rats (23, 182-184) have also been observed. In the case of rat model, in the absence of estrogen, there is an increase in body fat, therefore, an increase in body weight as seen in the ovx control group is mainly due to increased fat mass. Additionally, E_2 may directly regulate energy metabolism by binding to estrogen receptors with fat mass including the abdominal and subcutaneous fat (186- 188). E_2 may also affect body composition by altering serum levels of leptin, a hormone that regulates energy expenditure as suggested by animal (189, 190) and human (191, 192) studies.

In the present study, although ovx rats in both age categories reduced their physical activity as measured by 24-hr voluntary wheel running, this reduction reached a significant level only in older rats. Older ovx rats significantly reduced their voluntary wheel running by 57 percent compared with sham rats, whereas in younger rats ovariectomy had reduced their physical activity by 31.2 percent which tended to be lower than their corresponding sham counterparts. E_2 administration prevented this decline in physical activity due to ovariectomy. These observations agree with those of the others (193, 194) who also reported that ovx rats had lower activity which was reversed by E_2 administration. Hence, these findings suggest that postmenopausal women may become overweight for at least two reasons; namely, increased appetite and decreased physical activity. Yet some other investigators (195) suggest that this energy imbalance lies upon the fact that estrogen deficiency down-regulates sodium/potassium pump. In support of this suggestion, human studies (196, 197) also have demonstrated that postmenopausal women have higher appetites with less desire for physical activity. Similar to our findings that this excess body weight gain is due to a gain in fat mass, other studies (11, 84) have also demonstrated that women, particularly a few years after the onset of menopause gain weigh mostly due to increased fat mass.

Our findings in the present rat study suggest that this weight gain due to ovx occurs in spite of loss of lean mass. Interestingly, rats in all three groups lost lean mass and this was the case for both age categories. Although we cannot offer an explanation for these losses in lean mass, one probable reason for these losses in muscle mass could be the stress of surgery. Nonetheless, this is least likely because Kalu et al. (33) did not observe such losses due to surgery. Additionally, a study by Fisher et al. (183), suggested that ovx rats also gain muscle mass due to increased serum IGF-I level and that there were correlations between serum IGF-I and total body weight. However, when they normalized muscle mass to body weight, ovx rats neither gained nor lost muscle mass.

Another conclusion that can be drawn from our findings is that E_2 does not play an important role in the maintenance of lean mass. Although E_2 prevented the decline in physical activity, it is speculated that this effect of exercise is not adequate enough to override the effects of ovarian hormone deficiency which included the losses of several hormones aside from E2. Indeed, Banu

et al. (34) demonstrated that in this rat model the effect of wheel running exercise is minimal in comparison with growth hormone administration. But they demonstrated that exercise along with growth hormone can produce a synergistic effect in terms of both bone and muscle. Lean mass gain may be more related to growth hormone and anabolic growth factors such as free IGF-I. When IGF-I is bound to its binding protein, e.g. IGF-binding protein-3 (IGFBP-3) it is no longer free to exert its anabolic effect (188). However, this is speculative as we have not measured serum IGFBP-3 and this can be considered a limitation of this study. Another interesting finding of the present study is that E_2 may exert site-specific protective effect on muscle through its receptors (ERα and ERβ) as was the case in the present study where E_2 administered rats had similar soleus (SOL) and gastrocnemius (GAS) muscle weights as those of sham animals, irrespective of age. The site specific action of estrogen on bone and muscle has been known for some time and we are not the first group to report this (34).

There are several studies (196, 197) which have reported that estrogen replacement therapy (ERT) effectively reduces postmenopausal food consumption with elevated aptitudes for increased physical activity. In accordance with these human studies, in the present study, E_2 administration was able to prevent excess body weight gain and hyperphagia as the ovx+E_2 groups in both younger and older groups never ran out of food in comparison with ovx control groups.

In reference to bone, the findings of the present study in both age categories are consistent with those of our earlier studies (47, 48) as well as those of other investigators (68, 73) that ovariectomy causes loss of bone mineral density (BMD); however, the effects of ovariectomy and estrogen on bone microstructural properties are not well studied. The microarchitectural properties of the trabecular bone in both tibia and lumbar vertebra, contrasting the effects of ovariectomy, ovx+E_2 with those of sham values in each categories, clearly demonstrate the importance of ovarian hormones. Besides BMD, bone microarchitectures are deteriorated due to ovarian hormone deficiency in women, and ovx animals (22, 199). The findings of present study also confirmed that ovariectomy caused the loss of tibial bone volume (BV)/total volume (TV), trabecular number (Tb.N), and connectivity density (Conn.D.) and increased trabecular separation (Tb.Sp.) though the degrees of losses were greater in older rats than those of younger

rats. These findings indicate that aging, aside from other factors that deleteriously affect bone microstructures such as estrogen deficiency, can be an independent factor in terms of bone loss. In terms of trabecular thickness (Tb.Th.), our findings showed that ovariectomy causes increase in this parameter. This is unlike the observations made in postmenopausal women (200) and osteoporotic men (201) often experience increases in trabecular thickness due to reduced trabeculae. This, in part, can be explained by the morphological differences between the two species in reference to bone and muscle structures. Rats, due to their structuralphysilogy, do not experience fractures even in the case of sever bone loss (199). On the other hand, the skeletal system in humans tries to compensate for the loss of trabeculae by increasing the thickness of the remaining trabeculae. Although this may postpone fracture up to a certain point, if bone loss continues, humans eventually experience fracture due to stress risers in which the thinning and perforation of trabeculae occur (200-202). In the present study, microcomputed tomography (μCT) analysis indicated that E_2 treatment partially was able to prevent the loss of trabecular structures. Our findings agree with that of Lark et al. (203, 204). However, E_2 administration had a more pronounced modulating effect in older rats than younger rats for which we have no explanation for.

Besides the structural parameters, other morphological parameters also contribute to bone quality and strength, i.e. the structure model index (SMI). The SMI value is based on surface density and thickness of a 3 dimensional structure. Its value is multiplied by a factor to yield more practical numbers, 0 for ideal plates, 3 for ideal rods, and 4 for true spheres (205). Traditionally, plate-like structure has been considered stronger than rod-like structure (206-208). In accordance with these investigators findings, the SMI of ovx rats in both age groups were higher in ovx rats in comparison with sham which indicate that ovariectomy not only causes bone loss, but at the same time makes the remaining bone inferior.

In terms of muscle, IGF-I is known to modulate muscle mass, as it is considered to be amongst the most effective anabolic factors (117). In the present study, paradoxically, ovx rats though had higher levels of IGF-I, lost muscle mass and bone mass. This may be due to the lack of the bioavailability of IGF-I. IGF-I is regulated by its binding proteins, of which at least 6 different classes are known, with the most predominant binding protein being IGFBP-3 (117, 138). Serum

IGFBP-3 is known to interfere with IGF-I/growth hormone axis and render their anabolic actions on bone and muscle (127, 141, 144). This may be the case in the present study; however, we have not assessed serum or tissue levels of IGFBP-3 and we cannot state this affirmatively. IGF-I gene expression is also known to be suppressed by tumor necrosis factor alpha (TNF-α) but again our findings do not show that ovx affects TNF-α levels in the blood or its expression in gastrocnemius (GAS) and soleus (SOL) muscles. In further support of this notion, isolated myoblasts (209) have been utilized to elucidate the mechanisms by which cytokines and growth factors influence muscle metabolism. For instance, myoblast cell line, C2C12, has been shown to contain muscle precursor phenotype similar to satellite cells embodied in muscle. Frost et al. (209) used this cell line to study the effect of TNF-α on IGF-I gene expressions and demonstrated that TNF-α down-regulated the gene expression of IGF-I. As it was stated earlier, in the present study, the TNF-α systemic levels or gene expression was not altered by ovx and hence the modulation in IGF-I levels cannot be attributed to the effect of ovx on TNF-α. In agreement with the findings of Kalu and colleagues (198), ovx in both age categories increased circulating levels of IGF-I, but these increases not only did not elevate the IGF-I gene expressions in SOL and GAS muscles, but rather decreased its expression. This may be due to at least two factors, e.g. it is quite possible that most of the circulating IGF-I concentrations are bound to it binding protein, mainly IGFBP-3, which makes its action rendered or alternatively elevated circulating levels of IGF-I could have down-regulated its mRNA expression in these muscles. Nonetheless, we are being speculative since we did not measure IGFBP-3 levels in blood or tissue and it is to be considered one of the limitations of this study. Further research is needed to examine the effect of ovx on IGF-I synthesis and its binding proteins in an extended period of time. With reference to atrogin-I, the 10-month old rats, ovx numerically elevated its mRNA expression. This increase could have become significant if we had a larger sample size. This is also to be considered a limitation of this study. If future findings show that ovx increases the gene expression of atrogin-I, this would indicate that E_2 deficiency increases the catabolic arm of bone and muscle turnover. Banu et al. (34) reported that from 1 to 6 months of age, bone mass parameter such total BMC, cortical BMC, and cancellous BMC increased faster in females than in males with similar muscle area, and at 3 and 6 months of age, the above vertebral indices of bone mass were significantly higher in female than in male rats. Their observations demonstrated that E_2 during growth plays a crucial anabolic role in terms of both bone and

muscle mass. These observations are similar to what has been reported in humans during puberty when serum estrogen levels are high in females. Hence, in the future studies, this model can be further used for studying the effects of serum estrogen on the skeletal response of voluntary muscle forces as has been reported in humans during growth.

5.2. Conclusions

Both bone and muscle mass are decreased after the onset of menopause or surgical ovariectomy while fat mass is increased. This is reflected by decreased BMD and relative decrease in normalized GAS and SOL muscle mass. These alterations in part can be explained by increases in serum biochemical indices of bone turnover including C-telopeptides of type I collagen (CTX) and bone specific alkaline phosphatase (B-ALP). Although previous studies have shown that serum levels of IGF-I are correlated with BMD, unlike postmenopausal women, ovx rats in the present study in both age categories experienced elevated levels of IGF-I. These observations suggest that IGF-I may also behave like other biochemical markers of bone and muscle turnover.

The results of this study suggest that estrogen protects lean mass against ovariectomy. However, E_2 could not completely prevent the deteriorations in microstructural properties of tibiae and lumbar vertebrae. Similarly, E_2 was unable to completely prevent the rise in serum levels of bone and muscle turnovers. Another interesting finding of this study was an apparent anabolic effect of E_2 in five-month old animals as the mean normalized GAS weight of E_2 treated rats was higher than both sham and ovx groups. The findings in both 5- and 10-month old ovx rats suggest that removal of ovaries causes body commotional changes similar to what is recently described as osteosarcopenic obesity in postmenopausal women. Future studies are needed to examine the effects of ovarian hormone deficiency on changes that occur in energy metabolism, including the regulation of mitochondrial function and bioenergetics and the role that E_2 plays in promoting homeostasis and the mechanistic crossroads that lead to divergent outcomes following estrogen exposure.

APPENDIX A

LIST OF ABBREVIATIONS

B-ALP	bone specific alkaline phosphatase
BMC	bone mineral content
BMD	bone mineral density
BV	bone volume
BW	body weight
CTX	C-telopeptides of type I collagen
CVD	cardiovascular disease
E_2	17β-estradiol
eIF4E	eukaryotic initiation factor-4e
ELISA	enzyme linked immunoassay
ER	estrogen receptor
ERT	estrogen replacement therapy
GAS	gastrocnemius
GH	growth hormone
HRT	hormone replacement therapy
iDXA	dual-energy x-ray absorptiometry
igf1	genes encoding insulin like growth factor I
IGF-I	insulin-like growth factor-I
IGFBP-3	insulin-like growth factor binding protein-3
IL-1β	interleukin 1-β
IL-6	interleukin 6
LF	lean fat
LM	lean mass
LPL	lipoprotein lipase
μCT	microcomputed Tomography

MAFbx	muscle atrophy box
M-CSF	macrophage colony-stimulating factor
mTOR	mammalian target of rapamycin
MuRF1	muscle ring finger 1
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
OPG	osteoprotegerin
ovx	ovariectomized
P38 MAPK	P38 mitogen-activated protein kinases
RANK	nuclear factor -kB (NF-kB) ligand
RANKL	receptor activator of nuclear factor -kB (NF-kB) ligand
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SD	Sprague-Dawley
SMI	structure model index
SOL	soleus
Tb N	trabecular number
Tb Sp	trabecular separation
Tb Th	trabecular sthickness
TNF	tumor necrosis factor alpha
TV	total volume
VOI	volume of interest

APPENDIX B

ANIMAL CARE AND USE COMMITTEE

Animal Care and Use Committee (ACUC)
101 Biomedical Research Facility
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MEMORANDUM

TO: Dr. Bahram Arjmandi
Department of Nutrition Food and Exercise Science

FROM: Dr. Paul Q. Throbley, Chair [#27]
Animal Care and Use Committee

SUBJECT: Approval of Protocol **#1023**

DATE: August 4, 2010

"YOUR NEW PROTOCOL IS APPROVED"

The Animal Care and Use Committee approved new **Protocol #1023, "Ferutinin's ability to reverse ovariectomy induced bone loss and prevent excess weight gain in rats"**, for proposed vertebrate animal use at the **July 29, 2010** ACUC meeting. You are approved for the following species and numbers for the proposed protocol approval period.

Species	*Number Animals Approved*	*Protocol Approval Expiration Date*	*Rewrite Due*
Sprague- Dawley rats (*Rattus norvegicus*)	60	July 29, 2013	June 1, 2013

Enclosed for your records are:

- ✓ A copy of the **Committee Comments**
- ✓ A copy of the **Protocol**

When you order animals on this protocol, please remember to convey the ACUC number to the LAR at 644-4262. In addition, if you do not currently have animal housing or procedural space assigned or should you need additional animal housing or procedural space, please make a request for space in writing to the Biomedical Advisory Committee (BAC) care of Kristin Auter at kauter@fsu.edu. Animals will not be ordered unless adequate animal housing/procedural space is confirmed by the LAR Facility Manager.

We appreciate your contribution to assuring that animal research at Florida State University complies with federal guidelines and regulations. Let us know if we can be of further assistance.

PQT/kj
Enclosures

Animal Care and Use Committee (ACUC)
PROTOCOL REVIEW
COMMITTEE COMMENTS AND ACTION

Reviewed by the Animal Care and Use Committee on July, 29, 2010

Comments:

#1023, Dr. Bahram Arjmandi, New, Approved, non-USDA covered species
A brief summary of new **Protocol #1023, "Ferutinin's ability to reverse ovariectomy induced bone loss and prevent excess weight gain in rats"**, was provided by Dr. Spector. This protocol proposes research to look at a rat model of post-menopausal complications; osteoporosis and weight gain, and examine the effect of a phytoestrogen, ferutinin on both. Ferutinin, from a native plant grown in Syria and Lebanon, is suggested to play an important role in preventing bone loss and excess body weight gain in post-menopausal women based upon anecdotal evidence. The issue of food restriction via pair feeding was discussed and determined to have no adverse effects upon the rats. The issue of proficiency of lab staff for daily gavage of animals for a 2 month period was raised. The PI has extensive experience with the procedure and will teach lab members the technique. Follow up monitoring for proficiency demonstration was raised.

The committee raised the following issues and concerns:

- The scientific notation for Estradiol, E_2, should be clearly identified at its first use for ease of review the document for by non-scientific individuals.
- The Environmental Health & Safety representative indicated that Monica Figueroa had submitted an incomplete Medical Monitoring form. The ACUC Secretary will follow-up on the completion of this form immediately following the meeting.
- The LAR staff will perform post-approval monitoring for proficiency with the gavage technique due to the large number of personnel expected to be trained performing the procedure. Ensuring the laboratory personnel are appropriately trained will decrease the risk of inhalation pneumonia and any trauma associated with gavaging, as well as reduce potential stress to the animals.

There were no other questions or comments.

It was moved and seconded to approve new protocol #1023. Approved unanimously.

ACUC Committee Comments
July 29, 2010
Protocol #1023

OFFICIAL ACTION

APPROVED	REQUIRES MODIFICATIONS	DISAPPROVED

Dr. Paul Q. Trombley, ACUC Chair — July, 29, 2010 Date

Dr. Kathleen Harper, LAR Director and Attending Veterinarian — July 29, 2010 Date

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BIOGRAPHICAL SKETCH

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Education

PhD Candidate, Nutrition and Food Sciences, Faculty of Nutrition, Food and Exercise Science, Florida State University, Tallahassee, FL, USA

MS in Nutrition, Metabolism & Genetics, Faculty of Kinesiology, University of Calgary, Calgary, AB, Canada

BS in Nutrition Sciences GPA 17.9/20, 1st honour student Department of Nutritional Sciences Shaheed Beheshti University, Tehran, Iran

Awards

2012 Dissertation Award Program, College of Human Sciences
2012 Florence Smith McAllister Scholarship, College of Human Sciences
2012 Anna Marie Erdman Scholarship, Dept. of Nutr. Food and Exc. Sci.
2012 Florida Canada Linkage Institute
2011 Florida Canada Linkage Institute
2011 Eva Maria Erdman Scholarship, Dept. of Nutr. Food and Exc. Sci.
2010 Florida Canada Linkage Institute
2010 Betty Watts Memorial Scholarship Dept. of Nutr. Food and Exc. Sci.
2009 Canadian Institutes of Health Research (CIHR)
2008 Queen Elizabeth II Graduate Scholarships, University of Calgary

Membership

2012 American Society of Nutrition

2012 Glen Society

2011 KON Society

2010 Golden Key Society

2008 Canadian Obesity Network

Published Papers

- Juma S.S., **Ezzat Zadeh, Z.**, Khalil D.A., Hooshmand S.H., Arjmandi B.H. Soy Protein with or without Isoflavones was Unable to Preserve Bone Density in Gonadal Hormone Deficient Male Rat Model of Osteoporosis. Nutr Res. 2012;32(9):694-700.

Published Abstracts

- **Ezzat Zadeh, Z.**, Dodge B.G., Elam M., Feresin R., Brown J., Kim J.S., Arjmandi B.H. The Underlying Mechanisms by Which Estrogen Regulates Energy Metabolism and Body Composition. Experimental Biology. April 21-25 2012. San Diego, CA, USA.

- Hughey C.C., **Ezzat Zadeh Z.**, Johnsen V.L., Belke D.D., Hittel D.S., Shearer J.Paradoxical reduction in cardiac O-GlcNAcylation following short-term high fat feeding. April 21-25 2012. San Diego, CA, USA.

- **Ezzat Zadeh, Z**., Soung D.Y., Khalil D.A., Arjmandi B.H. Vitamin E Reduces the Rate of Osteoclstogenesis in Ovarian Hormone Deficiency by Suppressing the Formation of TRAP Positive Cells. Experimental Biology. April 9-13 2011. Washington, DC, USA. 773.2- P 239.

- Elam, M. L. **Ezzat Zadeh, Z.**, Hooshmand S., Arjmandi, B. H. Examining the Bone Forming Ability of Ferutinin, an Extract of Giant Fennel, on the Function of MC3T3-E1 Osteoblast-like Cells. Experimental Biology. April 9-13 2011. Washington, DC, USA. 581.17- P 160.

- **Ezzat Zadeh, Z**., Hittel, D., Severson, D., Hepple, R., Sensen, C., Shearer, J. High Fat Feeding Increases Mitochondrial Content while Compromising Outer Membrane Integrity in the Mouse Heart. Annual Meeting of the Obesity Society. Oct 03-07 2008. Phoenix, USA. 731-P 16 (1), S261.

- Hughey, C. H., **Ezzat Zadeh, Z**., Mazursky, S., MacIntosh, B., Shearer, J. Fiber Type-Specific Alterations in Skeltal Muscle Mitochondrial Oxidative Phosphorilation following Induction of Heart Failure. NIH – Mitochondrial biology in cardiovascular Health diseases Meeting. Oct 06-08 2008.Bethesda, Maryland. 10. 7. 24. P 580.

Presentations as guest speaker (public or invited lectures)

- **Ezzat Zadeh Z.** Estrogen regulation in energy metabolism and tissue composition. The Florida State University. College of Human Sciences. Research and Creativity Day Presentation (first place winner). Feb 23, 2012.

- **Ezzat Zadeh Z.** UP446, A novel compound that may alleviate the symptoms of osteoarthritis as early as one week. The Florida State University. College of Human Sciences. Research and Creativity Day Presentation (second place winner). Feb 25, 2011.

- **Ezzat Zadeh Z**. Essential role for uncoupling protein-3 in mitochondrial adaptation to fasting but not in fatty acid oxidation or fatty acid anion export. Seifert EL, Bézaire V, Estey C, Harper ME. (2008) J Biol Chem. 283(37):25124-31. Journal Club Presentation. Jan 21, 2009.

- **Ezzat Zadeh Z**. Mitochondrial uncoupling protein 3 and its role in cardiac- and skeletal muscle metabolism. Nabben M, Hoeks J. (2007) Physiol Behav. 94(2):259-69. Journal Club Presentation. Journal Club Presentation. Jan 14, 2009.

- **Ezzat Zadeh Z**. Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes. Mogensen M, Sahlin K, Fernström M, Glintborg D, Vind BF, Beck-Nielsen H, Højlund K. (2007) Diabetes. 56(6):1592-9. Journal Club Presentation. April 11, 2008.

Teaching experience

Fall 2012	Florida State University, HUN 3226 Metab II
Summer 2012	Florida State University, HUN 3224 Metab I
Spring 2012	Florida State University, HUN 3224 Metab I
Fall 2011	Florida State University, HUN 3224 Metab I
Summer 2011	Florida State University, HUN 3224 Metab I
Summer 2010	Florida State University, HUN 3224 Metab I

Other contributions (non-referred)

- **Ezzat Zadeh, Z**. Nutrition is an Important Factor in Prevention Diseases with a Genetic Link. Food Specialized Magazine. No.2, 2002

- **Ezzat Zadeh,Z**. Steam Pressure Cooker Protect Food Nutrients During Cooking Time. Standard, No.100,2003

- **Ezzat Zadeh,Z**. Special Food Technology, a Solution to Snack Food Problem. Standard, No.92,2004

Volunteer Experience

- FSU Persian Student Society, Tallahassee, FL. USA 2010– present
- Calgary Health Region, Diversity Services. Calgary, AB. Canada 2006 – 2008
- Iranian Nutrition Society Newsletter, Tehran, Iran 2003 – 2006