REVERSAL OF INFLAMMATION-INDUCED BONE LOSS AND VASCULAR PATHOLOGY BY DRIED PLUM'S POLYPHENOLS

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Bachelor of Science in Biochemistry

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Stillwater, OK

2007

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 2009

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ACKNOWLEDGMENTS

I would like to start off by thanking my major advisor Dr. Brenda Smith who provided me with many different support systems which all played a significant role my degree. Dr. Smith became more than a mentor to me, she took me under her wing and created an environment which I was able to thrive in. I cannot express enough gratitude to her for this opportunity and for allowing me to find something I am truly passionate about.

I would also like to thank my committee members, Dr. Stephen Clarke and Dr. Edralin Lucas. They played a vital role in the completion of my degree, as well as offering their support and guidance any time I needed it. Along with my committee members, I'd like to thank the other faculty in the Nutritional Science Department, who were always willing to assist me in any way they could and I have always appreciated that.

Lastly I'd like to thank my family who I have always had a unique bond with. My nephew and niece, who always bring a smile to my face and I cannot express enough how much I love and enjoy them. My sister, Andrea, who is always there to support me and is the "big sister" every girl wants. Finally my parents, who are and have always been so supportive of me and all my endeavors. My mom and dad have continued to support my dreams no matter how they have changed throughout the years, and are always willing to help me in whatever possible. My family has played huge part in my successes and I feel every goal I accomplish in life is a direct reflection on them.

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CHAPTER I

INTRODUCTION

Even with advances in modern medicine, osteoporosis and cardiovascular diseases (CVD) are still considered major public health threats. By definition, osteoporosis is a disease characterized by low bone mass, as indicated by bone mineral density (BMD) measures, and structural deterioration of bone microarchitecture to the point that fracture risk is increased. Recent estimates indicate that over 43 million Americans have been diagnosed with osteoporosis or low bone mass (i.e. osteopenia) (1). By comparison, CVD include a wide range of conditions that involve the myocardium and/or vascular system. One of the most common CVD, coronary artery disease, is a contributing factor in approximately 29% of all American deaths (2). Both osteoporosis and CVD are considered age-related diseases in that they evolve over time and age is considered a major risk factor. This is demonstrated by the fact that osteoporosis is more common in individuals over the age of 50, and CVD increases significantly after the age of 50 (2). The predicted demographic shift toward an older society in the U.S. would suggest that the prevalence of these diseases will only increase unless better prevention and treatment strategies are developed (1).

Clinical observations suggest that chronic activation of the immune system is likely involved in the development of osteoporosis and CVD (3). In recent years, studies

have shown that in postmenopausal women, a population in which bone loss and increased risk for CVD are well documented, a marked increase in the pro-inflammatory cytokines occurs (4). Additionally, patients with chronic inflammatory conditions such as systematic lupus erythematosus (SLE) and rheumatoid arthritis (RA), often experience complications of bone loss and CVD (5-8). Women with SLE have been shown to be at greater risk of having developing osteoporosis (6) and autopsy studies indicate a high incidence of atherosclerotic lesions (9). Likewise, patients with RA experience bone loss throughout the skeleton as well as in the affected joints, and have reduced life-expectancy due in part to increased CVD mortality (7). Elevated levels of circulating pro-inflammatory mediators such as interleukin (IL)-1 and IL-6, tumor necrosis factor (TNF)-α, and macrophage colony stimulating factor (M-CSF) are common in many of these inflammatory conditions as well as in postmenopausal women as estrogen levels decline (10).

At the tissue level, chronic inflammation creates a bone catabolic and vascular pro-atherosclerotic environment. In the bone, increased bone resorption by osteoclasts and decreased bone formation by osteoblasts are likely due to the increase in cytokines and growth factors that up-regulate osteoclast development and activity, and suppress osteogenesis (4). If such alterations in bone metabolism persist, deterioration of bone tissue and bone loss will result. Within the vascular walls, immune cells such as monocytes and macrophages produce cytokines which have been identified in all stages artheromatous lesions. Several of the same inflammatory mediators that play a role in bone loss such as IL-1, IL-6, interferon (IFN)-γ, TNF-α, several growth factors, are also involved in vascular disease (11). Both male and female patients with CVD exhibit much

higher serum IL-6 and TNF-α than those without CVD (12). Continued research is focused on establishing a better understanding the role of these mediators in osteoporosis as well as CVD.

Accumulating evidence over the past six decades has suggested that the pathophysiology of both osteoporosis and CVD may be related (13-18). Individuals with low BMD have been shown to have a higher incidence of CVD (19). Hyder *et al.,* found an inverse relationship between patient's lumbar spine BMD and aortic calcification (18). Postmenopausal women that have been diagnosed with osteoporosis tend to have elevated arterial stiffness, predisposing them to a higher risk of developing CVD (20). Based on the role of inflammation in the pathophysiology of osteoporosis and CVD, it seems reasonable that chronic elevation of pro-inflammatory mediators may provide a link between these two conditions.

Recently, an animal model of chronic low grade inflammation was developed for the purpose of studying the effects of low grade inflammation on bone (21). This model was first developed in 3 month-old Sprague Dawley rats that received lipopolysaccharide (LPS) over 90 days via a slow release pellet implanted on the dorsal region of the neck (21). Chronic inflammation was verified by increased monocyte and neutrophil counts in the peripheral blood over the 90 day study period. This chronic inflammatory state was associated with a decrease in femur BMD and loss of trabecular bone at the proximal tibia (21). Further investigation revealed that chronic exposure to LPS also produced a cardiovascular pathology characterized by vascular fibrosis and disruption of the intimal layer of the smaller intramural arteries and arterioles of the myocardium (22). Subsequent studies in mice revealed a similar bone and vascular pathology in the

C57BL/6 mouse after 30 and 90 days (23, 24). Evidence from these initial studies demonstrated that this model provided a system to study the role of chronic inflammation in concurrent bone loss and vascular disease.

Theoretically, if inflammatory pathways are the primary mediators of bone loss and vascular disease, therapeutic agents which have anti-inflammatory properties would serve as an ideal treatment. Statins are a group of drugs commonly prescribed because of their efficacy in lowering serum cholesterol. This class of drugs primarily acts by inhibiting the enzyme HMG-CoA reductase, which catalyzes the conversion of HMG-CoA to the cholesterol precursor, mevalonate (25). More recent studies have suggested that these lipid lowering drugs also have anti-inflammatory properties that may protect against both osteoporosis and CVD (26, 27). In terms of osteoporosis, statin therapy has been associated with an increase in BMD and has positive effects on fracture healing (26) and bone resorption (28). Statins have also been shown to increase bone formation *in vitro* by stimulating bone morphogenetic protein 2 (BMP2), a growth factor that promotes osteoblast differentiation (29). In terms of CVD, pro-inflammatory cytokines participate in atherothrombotic events and it has been suggested that statins may reduce the risk of CVD not only through their positive effects on the lipid profile, but also by their effects on these cytokines (30). While statins provide a potential therapeutic option that could be beneficial in the treatment of both cardiovascular and skeletal health, they are not without some undesirable side effects (e.g. muscle pain/weakness, liver damage). Therefore, natural treatment options that could be incorporated into the diet may provide a more desirable alternative for many individuals.

Plant-based foods such as fruits and vegetables and their bioactive components, are of particular interest due to their ability to reduce the occurrence of bone loss and cardiovascular disease. Dried plums are of particular interest due to their rich source of several nutrients (i.e. potassium, boron, and magnesium) and their polyphenolic compound content (31). Dietary supplementation with dried plum has been shown to prevent and, to some extent, reverse the loss of bone mass (i.e., BMD) in ovariectomized young adult rats (32, 33). Dried plum's osteoprotective effects were also studied in male orchidectomized rats and were found to down-regulate the gene expression of both osteoprotegerin (OPG) and receptor activator for NF-κB ligand (RANKL), and prevent the gonadal hormone deficiency-induced deterioration of trabecular bone microarchitecture (34). *In vitro* studies using polyphenols extracted from dried plum indicated that these compounds may enhance osteoblastic activity (35), and suppress osteoclastogenesis (36). In addition to its effects on bone, dried plum has also been shown to impact risk factors for CVD. Tinkerand colleagues., (37) administered dried plums to men with mild hypercholesterolemia, and found that they were able to reduce total cholesterol. Dried plums have also been shown to protect against the ovariectomy-induced elevation of total cholesterol in rats fed a high dried plum diet (25% w/w) had lower levels of total liver lipids (38). These findings suggest that dietary supplementation with dried plum is able to exert beneficial effects both on both the skeletal and vascular system.

One particular group of compounds found in fruits and vegetables, polyphenols, have been of particular interest due to their anti-inflammatory and antioxidant properties (39). Polyphenols are a class of phytochemicals found in high concentrations in plant-

based foods which are responsible for a fruit/vegetable's vibrant color and offer a natural defense mechanism for plants (39). Red wine, a rich source of the polyphenolic compound resveratrol has been shown to reduce the incidence of CVD and at the same time stimulate proliferation and differentiation of osteoblasts (40). The polyphenolic compound found in high concentrations in green tea, (-) –epigallocatechin-3-gallate (EGCG), has been shown to dramatically decrease the number of osteoclasts *in vitro* (41) and is inversely related to mortality due to CVD (42). The mechanism by which green tea exerts its positive effects appear to be anti-oxidative, anti-inflammatory, and anti-atherosclerotic (43). Furthermore, soy, another rich source of polyphenols (e.g. genistein and daidzine), has been shown to have protective effects on bone (44), and prevent the formation of atherosclerotic lesions (45). Therefore, it is reasonable to expect that dried plum's polyphenols may offer protection and perhaps could even reverse the detrimental effects on the skeletal and cardiovascular systems.

In this study, we propose to induce bone loss and vascular pathology due to chronic low grade inflammation, and that dried plum's polyphenols will be able to attenuate this response. The following hypothesis will be tested and the objectives carried out to accomplish this purpose.

Hypothesis: Polyphenol extract from dried plums will reverse the bone loss and vascular pathology induced by low grade chronic inflammation by reducing key inflammatory mediators (e.g. TNF-α and Il-6) involved in the pathophysiology of osteoporosis and CVD.

Objective 1: X-ray microcomputed tomography (μCT) and dual-energy x-ray absorptiometry (DXA) will be used to assess the alterations in trabecular and cortical bone microarchitecture and bone mineral content and density in response to inflammation and dried plum's polyphenols. The effects of dried plums' polyphenols will be compared to those of the whole dried fruit and the cholesterol-lowering drug simvastatin (25 mg/kg diet) will be used as a positive control.

Objective 2: To determine the extent to which dried plum's polyphenols reverse the pathological changes occurring in the myocardium and vasculature in response to inflammation, histological evaluation will be performed. The influence of dried plums' polyphenols on cardiovascular pathology will be compared to that of similar doses of dried plum and simvastatin.

Objective 3: To assess the local and systemic effects of dried plum's polyphenols on genes involved in the regulation of bone formation and resorption. The effects of dried plum's polyphenols on these indicators of bone resorption (TRAP, RANKL, OPG) and formation (ALP, IGF-1) will be compared to that of dried plum and simvastatin.

Objective 4: To determine the extent to which polyphenols extracted from dried plums transcriptionally alter local and systemic inflammatory mediators. TNF-α, IL-6 and IL-10 gene expression will be assessed to determine the effect different treatment options have on these two inflammatory-induced conditions, using of real time PCR. The effects

of the polyphenols on these inflammatory mediators will be compared to that of dried plum and simvastatin.

Study Limitations:

Our laboratory has developed the animal model proposed in this study to examine the effects of chronic low grade inflammation (21-23, 46). Previously this model has been shown to closely mimic some of the effects of chronic inflammation on the skeletal and vascular system in humans. Although the use of *in vivo* model has advantages compared to cell or tissue culture systems, results from this study cannot be directly extrapolated to humans. It is well documented that effects observed in rodent animal models may vary to some extent with the responses observed in humans. Animal models are also convenient means for researchers to perform initial studies of various dietary regimens, however in humans, adherence to study protocol may be problematic.

Another potential limitation to this study is the fact that total polyphenols were extracted from dried plums. While the extract was analyzed for its polyphenolic, carbohydrate, fat and proetin content, other components may remain in this sample and potentially influence the results. Polyphenol content of dried plum may vary by season and other harvesting techniques, which may alter potency of the dried plum's polyphenols. This study used a single lot of dried plum powder provided by California Dried Plum Board to account for this possible confounding factor.

CHAPTER II

REVIEW OF LITERATURE

Introduction to Osteoporosis and Cardiovascular Diseases

Mounting clinical evidence and epidemiological studies suggest a link between osteoporosis and CVDs such as atherosclerosis, cerebral vascular incidence, peripheral arterial disease (PAD), and coronary heart disease (17, 18, 47-49). Both conditions are major public health problems for developed countries such as the United States and many European countries (50). Coronary artery disease is the leading cause of death for both men and women in the U.S. (51), and osteoporosis affects an estimated is 10 million Americans, with another 34 million at risk (i.e. osteopenia or low BMD) (1). European countries have the highest prevalence of osteoporosis. For example Denmark has a population of approximately 5.46 million people and 29.3% (1.6 million) are affected by osteoporosis (52). CVD is an even greater public health concern in that an estimated 17.5 million people die from cardiovascular disease per year worldwide, representing 30% of all deaths globally (50).

Osteoporosis and CVD are debilitating conditions that affect a patients "physical" health, as well as having a significantly economic impact on the healthcare system. The annual costs associated with CVDs and osteoporosis in the United States have been

estimated at $403.1 billion (53) and $16.9 billion, respectively (54). Combined, these two age-related chronic conditions contribute approximately $420 billion to the total U.S. healthcare expenditures (53, 54). The total direct costs of osteoporosis in European countries was estimated at $41 billion per year (€31.7 billion/year) (55), and overall CVD is estimated to cost Europeans $248.3 billion (€192 billion/year) per year (56). The debilitating nature of the age-related conditions as well as the economic burden to healthcare systems has motivated researchers and healthcare professionals to improve the prevention and treatment strategies.

To date, patients diagnosed with osteoporosis have the option of anti-resorptive bisphosphonate drugs or anabolic parathyroid hormone (PTH) therapy, which are focused on altering bone metabolism. Treatment for CVD is dependent on the specific disease pathology (e.g., coronary artery disease, hypertension, or stroke), and effective treatments usually include lifestyle behavior modifications. For example, patients diagnosed with atherosclerosis may be prescribed a variety of medications, one of the most commonly prescribed drug classes is the cholesterol loweringc statins. A single patient diagnosed with both a CVD and osteoporosis will most likely be prescribed separate drugs (e.g., an anti-resorptive drug combined with lipid-lowering and anti-hypertensive agents) which ultimately lead to polypharmacy. This scenario is quite common due to the tendency for these conditions occur simultaneously (57).

Populations at Risk for Osteoporosis and CVDs

Increased risk for osteoporosis and CVD occurs in populations such as the elderly, postmenopausal women, and people with chronic inflammatory conditions. For instance, as individuals age, skeletal deterioration and the risk for CVD increase. In fact

55% of the people 50 years of age and older have osteoporosis in America and about 84% of cardiovascular disease deaths occur in people age 65 and older (1, 2). Mussolino *et al.,* (1) reported that in a cohort of Caucasian men, phalangeal BMD was inversely associated with cardiovascular-related deaths. Farhat and colleagues (2) found that the incidence of CVD in older men and women (i.e. aged 68-80 years) was also inversely related to BMD of the hip, femoral neck, trochanter, and spine with CVD (2). Browner and colleagues (3) also studied women aged 65 years and older to determine how stroke risk would coincide with skeletal deterioration. They determined that osteopenia was correlated with increased risk of stroke, but indicated that they did not believe the low BMD was causal (3).

In addition to aging, diminished estrogen, associated with natural or surgical menopause, is also a major risk factor for osteoporosis and CVD. Uyama and colleagues (4) found a negative correlation between carotid atherosclerosis and total BMD in postmenopausal women aged 67-85. Von der Recke *et al.,* (5) identified that low bone mineral content (BMC) in postmenopausal women as a risk factor for increased mortality, particularly from cardiovascular disease. Low femoral neck BMD has been specifically associated with the incidence of PAD in postmenopausal women (6). Sumino *et al.,* (7) showed that postmenopausal women with osteoporosis have elevated arterial stiffness, which may increase the risk of developing CVDs. Sennerby and colleagues (8) designed a study to establish a common link between CVDs and osteoporosis. They observed a significant increase in the incidence of hip fractures after the diagnosis of cardiovascular disease in postmenopausal women (8). Hence, these studies indicate that in addition to the elderly, postmenopausal women are also a population at risk for concurrent osteoporosis and CVD.

Interestingly, aging and ovarian hormone deficiency are both associated with immunological changes (9, 10). These changes include increased levels of pro-

inflammatory cytokines, as well as a decrease in levels of anti-inflammatory cytokines (9,10). Thus, as detailed in the following section a potential role of the immune system in the concurrent development of osteoporosis and CVD may be suggested by the fact that bone loss and vascular disease are common complications of chronic inflammatory conditions.

CVD and Osteoporosis: Common Complications of Chronic Inflammatory Conditions

Conditions such as RA, COPD, periodontal disease, HIV and SLE are classified as chronic inflammatory diseases, and have been shown to have detrimental effects on both bone and vascular health. One of the first inflammatory states in which bone deterioration was observed was RA. Bone loss in RA occurs locally in the affected joints and systemically as pro-inflammatory cytokines (e.g., IL-1 and TNF-α) and proteinases are released resulting in cartilage and bone destruction (8). Due to this relationship, RA disease activity is considered an independent risk factor for osteoporosis (58). Attempts aimed at identifying the mechanism of RA associated bone loss have resulted in the TNF-α superfamily molecule RANKL, also known as osteoclast differentiation factor, to be the focus of much research (59). In addition to bone loss, RA patients are also at increased risk of developing CVD (60). Kao *et al.,* (61) found a higher prevalence of asymptomatic coronary artery calcium and CRP in women with RA. A cohort study of 1,010 patients with RA indicated the most frequent cause of death in this patient population was ischemic heart disease and myocardial infarction (7). These observations are likely attributed to the fact that patients with RA have higher circulating pro-

inflammatory cytokines which can have deleterious consequences on the skeletal and cardiovascular systems.

COPD is another chronic inflammatory condition in which osteoporosis and CVD are common complications (62). As many as 72% of patients with COPD have been reported to be osteopenic, and estimates ranging from 36-60% of patients with COPD have osteoporosis (63). Nuti and colleagues (64) identified a strong association between the severity of COPD and fractures in men. Sabit *et al.,* (65) documented that increased arterial stiffness is related to the severity of airflow obstruction and may be a factor in the excess risk for CVD in COPD patients. This same study revealed an increased aortic pulse wave velocity in patients with osteoporosis and the authors concluded that age-related bone and vascular changes occur prematurely in COPD patients (65). Moreover, Anthonisen *et al.,* (66) found that CVD accounted for 42% of the first hospitalizations and 48% of second hospitalization in patients with mild COPD. The results of these studies demonstrate that COPD is a disease in which the pro-inflammatory cytokines originating from the airways dramatically increase the risk of osteoporosis and CVDs.

Another chronic inflammatory condition in which bone loss and CVD complications are often observed are the periodontal diseases. This group of infectious diseases results primarily from gram negative bacteria (such as, *E. coli* and *S. shigella*) Periodontitis is characterized by the destruction of connective tissue and dental bone support after an inflammatory host response secondary to infection by periodontal bacteria (67). Severe periodontitis may result in tooth loss due to the loss of bone and other supporting tissues (67). Kribbs and colleagues (68) were the first to report on the relationship between systemic BMD and mandibular density. In this study, the

osteoporotic group had less mandibular bone mass and density in a thinner cortex then the control group (68). Tezal and *et al,*. (69) showed a strong correlation between the clinical severity of periodontal disease and BMD of the trochanter, Ward's triangle, and femur in postmenopausal women (69). Furthermore, postmenopausal women with osteoporosis and low educational levels have a greater chance of having periodontal disease than do those without osteoporosis (70). The major mechanism of periodontal diseases is the observed destruction of bone and cartilage, which has also been linked to increased risk of for CVD. Inverse relationships have been shown in men between the number of teeth, coronary heart disease (71) and myocardial infarction (72).

Bone demineralization and cardiovascular disease are also common complications among HIV infected patients, which is a relatively recent observation due to the dramatic improvement in life expectancy in this patient population. Women with HIV have been shown to have a lower BMD of the lumbar spine and hip than women without HIV (73). In HIV-infected patients, alterations of both bone resorption and formation markers have been observed. A relationship between $CD4^+$ cell counts, low bone formation and elevated bone resorption have been proposed as the underlying pathophysiology of this bone deterioration (74). With regard to cardiovascular health, the overall rate of incidence of PAH among HIV-infected individuals is 25-fold higher than in the general population (75). Currier *et al.,* (76) showed that the incidence of coronary heart disease among young men and women with HIV infection was significantly higher than in non-HIV-infected individuals. Hence data from HIV patients lend further support to the inflammation, osteoporosis, and CVD connection.

As a final example of a clinical condition in which immune dysfunction is associated with bone loss and vascular disease is evident in patients with SLE. Studies suggest that women with SLE exhibit a lower BMD than healthy female population (74). Many factors contribute to this predisposition, including the systemic inflammatory response associated with the disease. Almehed *et al.,* (6) reported that in SLE patients (*n*=163) more than half had a lower BMD of the radius, lumbar spine or hip than expected and 23% were osteoporotic at one site or more (6). The number of SLE patients experiencing osteopenia has drastically increased within the past decade, supporting that patients with SLE are at greater risk for developing osteoporosis and have a greater fracture risk (6). In addition to bone loss, CVDs such as atherosclerosis, ischemic cerebral vascular event, and coronary heart disease are common in patients with SLE (77). In a case-control study, Svenungsson and colleagues (5) showed a greater occurrence of atherosclerotic plaque among SLE cases compared with controls. Not only was the incidence of plaque higher in this populations, but patients with SLE were more vulnerable for plaque rupture (78). Pre-menopausal women with SLE are 50 times more likely to have a myocardial infarction compared with healthy women (79). The more frequent incidence of CVDs and osteoporosis that occur in SLE patients may be due to on-going elevation of pro-inflammatory cytokines.

Treatment options commonly used to in many of these chronic inflammatory conditions make it more difficult to discern the etiology of the skeletal and vascular pathology. For example many RA, COPD, and SLE patients are treated with corticosteroids, such as glucocorticoid, due to their ability to suppress the immune response. However, glucocorticoids have been shown to induce significant bone loss,

reduce bone strength, and ultimately increase the risk for osteoporotic fractures (80). Other undesirable side-effects of glucocorticoids potentially affecting the cardiovascular system include hyperglycemia and weight gain (80). Another example of a class of drugs that may complicate interpreting the connection between chronic inflammatory conditions, CVD and osteoporosis are the highly active antiretroviral therapy (HAART) used to treat HIV-infected patients. HAART have been associated with accelerated bone loss (81), but recently this concept has been brought into question (82). Antiretroviral drugs have been associated with several metabolic side effects, such as dyslipidemia, impaired glucose metabolism and abnormal body fat distribution that may increase the risk of CVD in this patient population (83). Hence, glucocorticoids and HAART are two examples of drugs used in the treatment of inflammatory conditions that may complicate our understanding of this relationship between inflammation, bone, and vascular health.

Inflammation in Osteoporosis and CVD

Osteoporosis and CVD not only tend to occur simultaneously, but also share some very common features within their initiation and progression. For decades it has been observed that patients with CVD have elevated pro-inflammatory cytokines and that CVDs can be classified as chronic inflammatory conditions. The role of cytokines in the pathogenesis of cardiovascular disease has become increasingly evident as advances in our understanding of the role of the immune system in atherosclerosis and heart failure has evolved (84). It was not until recently that osteoporosis which was traditionally considered a condition resulting from gonadal hormone deficiency was considered an immunological disorder (4). However now many of the pro-inflammatory cytokines are

recognized as playing a critical role in normal bone remodeling and persistent increases in many of these cytokines is involved in the pathogenesis of osteoporosis in peri- and postmenopausal women (85) and the elderly (84).

The skeleton is a very complex system in which coupling of osteoblast activity or bone formation and osteoclast activity or bone resorption) is required to maintain strong bones that are resistant to fractures. Uncoupling of bone resorption and formation can have a significant impact on bone quality and strength, thereby increasing the risk of fracture. Pro-inflammatory cytokines are involved in the regulation of bone metabolism. During the process of normal bone resorption and formation, osteoclasts resorb bone within a bone remodeling unit (BMU) and osteoblasts then migrate to the site. The process of bone remodeling is mediated by signaling that occurs between stromal cell derived osteoblasts and osteoclasts which originate from a mesenchymal lineage. For instance, osteoblasts express RANKL which promotes osteoclast differentiation and bone resorption. The receptor for RANKL, RANK, is expressed on pre-osteoclasts and the RANKL-RANK interaction can promote pre-osteoclasts to differentiate into mature, bone resorbing cells. In addition to RANKL, osteoblasts also secret a protein called OPG which acts as a decoy receptor for RANKL (86) and interferes with RANKL/RANK interaction therefore repressing osteoclastogenesis (86). Hence the ratio of RANKL to OPG is of importance in the regulation of bone resorption during normal remodeling and during states of chronic inflammation (i.e., ovarian hormone deficiency, and periodontitis, RA and ageing) (87, 88). Similarly, OPG-deficient mice exhibit an osteoporotic phenotype due to the inability to suppress RANKL and osteoclastogenesis (89).

The mechanism responsible for the up-regulation of RANKL in response to inflammation may be explained in part by its relation to TNF-α. TNF-α is a pleiotropic cytokine that induces cellular proliferation, production of inflammatory mediators, and cell death (90). TNF-α exerts its biological actions by interacting with two membrane receptors: TNFR1, activated by the soluble TNF-α and TNFR2, activated by membrane-bound TNF-α. TNFR2 exclusively activates pro-inflammatory pathways, but does not induce apoptosis (91). TNF-α has also been observed to stimulate the production of other inflammatory cytokines, such as RANKL and IL-1, which can enhance osteoclast differentiation (92, 93). Furthermore it should also be noted that TNF-α has been shown to impact osteoblasts by inhibiting the maturation of pre-osteoblast cells (94), decreasing osteoblast activity (35) and stimulating osteoblast apoptosis (10). Elevated TNF-α by peripheral blood monocytes has been positively correlated with bone resorption and vertebral bone loss in healthy pre- and post-menopausal women (95). Increasing evidence suggests that the role of pro-inflammatory cytokines in respect to skeletal health is critical and has provided novel insight into their specific action on bone metabolism.

Another family of cytokines that have been observed to have an impact on bone metabolism is the interleukins (IL). Wei *et al.,* (93) established that IL-1 is a potent stimulator of bone resorption and exerts this effect by enhancing stromal cell expression of RANKL. Elevated IL-1 has been linked to the acceleration of bone loss as seen in idiopathic and postmenopausal osteoporosis (96). Likewise, IL-6 is also involved in bone pathophysiology by promoting the differentiation and activation of osteoclasts (97). Recently, a longitudinal study documented a strong negative correlation between

circulating IL-6 and total body BMD in elderly men and women suggesting an important role of IL-6 in age-related bone loss (98).

Inflammation plays a significant role in atherosclerosis, the most common form of CVD. The development of atherosclerosis is believed to be initiated by vascular injury or stress which may elicit an immune response, but in some cases may be initiated by persistent elevation of pro-inflammatory mediators (99). In response to these factors, endothelial cells express adhesion molecules (e.g., intracellular adhesion molecule or ICAM-1 or vascular adhesion molecule or VCAM-1) that attract monocytes and subsequently other immune cell populations to the site (99). Monocytes interact with the endothelium by the chemoattractant proteins (e.g. monocyte chemotactic protein-1 or MCP-1) and are translocated into the intima where they differentiate into macrophages (99). These macrophages may secrete inflammatory cytokines and take-up oxidized low density lipoprotein (LDL) and eventually differentiate into foam cells. Foam cells will continue to secrete pro-inflammatory cytokines (i.e., TNF-α, IL-1β, and IL-8) (100), while simultaneously promoting the proliferation of smooth muscle cells. Thus, these events highlight just a few of the key immunological aspects in the pathophysiology of atherosclerosis.

Recently, RANKL and OPG have been recognized as having important roles on the vascular system, even though their initial function was associated with the bone metabolism (101). McGonigle *et al.*, (102) observed that RANKL regulates endothelial cell proliferation, apoptosis, and signaling. OPG-deficient mice exhibit calcification of the aorta and renal arteries in addition to an osteoporotic phenotype (89). Morony and colleagues (103) found that low density lipoprotein receptor knockout ($ldlr^{-/-}$) mice

developed significant progression of atherosclerosis, but OPG administration reduced aortic osteocalcin, a marker of calcification. Furthermore, stimulation of endothelial cells with TNF-α and IL-1 results in increased OPG is secretion that may represent the cells attempt to counter an increase in RANKL (104). OPG is also beneficial by its ability to promote leukocyte adhesion to endothelial cells (105). The impact that RANKL and OPG have on the vascular system may provide one potential link between the simultaneous bone loss and vascular pathology that are associated with inflammation.

In addition to RANKL and OPG, substantial evidence supports a pro-atherogenic role for TNF-α and some of the interleukins. TNFR1 expression in the arterial wall, greatly contributes to early and late-stage atherosclerosis in mice by enhancing arterial wall chemokine and adhesion molecule expression, as well as by augmenting medial smooth muscle cell proliferation and migration (106). Evidence suggests that TNF-α impairs endothelium-dependent and nitric oxide (NO)-mediated vasodilation in various vascular beds (e.g. mouse coronary arterioles) (107) and rat coronary arterioles (108). Expression of IL-1 and their receptors has been demonstrated in atheromatous tissue, and serum levels of IL-1-cytokines have been correlated with various aspects of cardiovascular disease and their outcome (109). Furthermore, in stroke patients, elevated IL-6 at baseline seemed to be an independent predictor of further deterioration (110). In both men and women serum IL-6 and TNF-α are significantly higher in patients with cardiovascular pathology and have been suggested to play a critical role in the progression of CVDs (111).

Animal Models of Chronic Inflammation, Bone Loss and Vascular Disease

To advance our understanding of the role of many of these inflammatory mediators in the development of osteoporosis and CVDs, it important to have an animal model that closely mimics the pathophysiology of human conditions. Some of the more common models of chronic inflammation used in animals are the LPS-injection model (112), the collagen-induced arthritis model (113), and the colitis model (114, 115). Although all of these models have been shown to activate the immune response by up-regulating inflammatory mediators, they were not ideal to examine the simultaneous development of skeletal and vascular pathology. Confounding factors such as the stress of chronic handling with injection models, lack of weight-bearing on an inflamed foot pad in collagen-induced arthritis models, or alterations in nutrient absorption associated with colitis make these models less than ideal.

Preferably, an animal model used to study chronic inflammation-induced bone loss and vascular pathology would induce a low-grade inflammatory response without significantly altering weight gain and normal animal behavior (i.e., physical activity, food intake, and grooming). Our laboratory developed an animal model to study the effects of a low grade chronic inflammatory state on bone mass and metabolism that involved the use of a slow release pellet containing LPS (21). In the initial study, adult 3-month old male Sprague Dawley rats were surgically implanted with LPS or placebo pellets designed to deliver 0.0, 3.3 or 33.3 μg LPS/day for 90 days (21). The doses were based on the previous work of Jarvelainen *et al.,* (116) in which LPS delivered via an osmotic pump for 4 weeks up-regulated hepatic TNF-α , IL-1β, IL-10, IL-4, and TGF- β, without inducing tolerance or significant weight loss. In this study, both doses of LPS induced a

significant decrease in femoral BMD compared to the controls (21). Histological cross-sections of the myocardium indicated the inflammation not only induced changes in bone mass, but also produced small vessel disease characterized by fibrosis surrounding the arterioles and a roughened intimal border within the intramural vessels (21, 22). At the end of the 90 day study period, inflammatory mediators, cyclooxygenase (COX)-2, TNF-α , and IL-1β, were up-regulated in the bone and myocardium which suggested this novel model may provide an acceptable means of studying inflammation-induced bone and cardiovascular pathology (21, 22). In a subsequent study, eight-week old C57BL/6 female mice were used to establish the dose of LPS that would induce similar bone loss and vascular disease in the mouse, and then to determine if soy-isoflavones could attenuate this response (24). In this study it was determined that 1.33 μg LPS per day (~0.1 mg of LPS/kg/d) produced the greatest decrease in lymphocytes and increase in neutrophils, and decreased trabecular bone volume (BV/TV) and number (TbN), and increased separation (TbSp). Moreover, TNF-α was up-regulated in the endothelium of small myocardial arteries and metaphyseal region of the bone, however soy isoflavones were able to attenuate this response (24). Taken together, these studies suggest that this model provides a system to study how low level inflammation induces simultaneous development of bone loss and vascular disease and to explore potential interventions.

Pharmacological Treatment Options for Osteoporosis and CVD

A number of different drug therapies are available for the treatment of osteoporosis and CVDs that generally offer specific relief for one or the other conditions depending on the patient's gender, lifestyle, and co-morbidities. Most of the available

therapies for osteoporosis target either bone resorption or formation, and thus exert suppression on the loss of bone or stimulate new bone formation.

The most commonly prescribed therapies for osteoporosis for osteoporosis have anti-resorptive properties. Bisphosphonates (i.e. alendronate and risedronate) are the most commonly prescribed pharmacological treatment for osteoporosis (117). Silverman *et al.,* (117) demonstrated that patients receiving risedronate have lower rates of hip and nonvertebral fractures during their first year of therapy than patients receiving alendronate. Oral bisphosphonates are relatively poorly absorbed and are associated with esophagitis, which make them poorly tolerated. Due to these gastrointestinal issues, strict adherence to therapeutic protocols must be followed (e.g., drugs must be taken first thing in the morning, consume no other food or medications and take with a full glass of tap water). Intravenous bisphosphonates are available, however, these potent anti-resorptive agents have been associated with increased incidence of osteonecrosis of the jaw (118). In addition to bisphosphonates, other anti-resorptive treatment options include calcitonin and hormone replacement therapy (HRT). Calcitonin has been shown to decrease the loss of bone in the spine, but is by far the least potent of all of the anti-resorptive treatment options (119). HRT remains a reasonable option for the prevention of osteoporosis, but is not recommended unless there are other indications for use (120).

Another more recently FDA-approved drug available for the treatment of osteoporosis is teriparatide or intermittent PTH therapy. Teriparatide (i.e. recombinant PTH peptide 1-34) is the only anabolic agent that can reverse bone loss in patients with established osteoporosis, and is administered by injection once a day in the thigh or abdomen (121). In most cases, teriparatide is approved for treatment when

bisphosphonates have failed, and should be avoided in the young and in patients with previous radiation therapy and Paget's disease (122). Intermittent PTH has been demonstrated to increase bone mass and reduce vertebral and nonvertebral fractures, and has been approved for use in the US and Europe (122). The most common side-effects of teriparatide are dizziness and leg cramps and elevations of blood calcium and urinary calcium can occur. To date, the safety and benefits of teriparatide have not been evaluated beyond two years, so treatment for longer than two years is not recommended.

Treatment of cardiovascular disease is dependent on the specific form of the disease, but is usually combined with lifestyle behavior modification (e.g. weight loss, altered nutrition, physical activity). Patients at risk for or diagnosed with atherosclerosis may be on medications such as cholesterol lowering statins, blood pressure lowering drugs, or anti-platelet or anticoagulant drugs. Over 11 million Americans are estimated to be on statin therapy which act by inhibiting HMG-CoA reductase, an essential enzyme in mevalonate formation and cholesterol biosynthesis (25). In the late 1990's, evidence emerged that statins may also have anti-inflammatory properties (29). In 1999, Mundy and colleagues (29) reported that of the more than 30,000 compounds screened, lovastatin, stimulated BMP-2, a potent regulator of osteoblast differentiation and activity. This finding was significant due to the fact that most FDA-approved drugs for the treatment of osteoporosis have anti-resorptive properties, but that lovastatin could potentially stimulate bone formation. The findings by Mundy and colleagues led other investigators to evaluate the relationship between statins, BMD, and fracture. Chung and *et al.,* (123) found that patients (n=69) on 3 commonly prescribed statins (i.e. lovastatin, pravastatin and simvastatin) for at least 15 months had increased BMD compared to the

control group after adjustment for age and body mass index. These data suggest that HMG-CoA reductase inhibitors may increase femoral BMD in males. Similar results have also been demonstrated in elderly women with increased hip BMD and reduced fracture risk (124). Given that these initial studies demonstrated a positive association between statin use and BMD, other studies followed in attempt to identify the mechanism by which statins exert these beneficial skeletal effects.

These observational studies suggest a connection between statin therapy and bone metabolism; however, the effects of statins on bone resorption and mineralization remain unknown. Tikiz *et al.,* (125) observed that after three months of simvastatin treatment, postmenopausal women had increased serum markers of bone formation (i.e. ALP and osteocalcin) and a negative correlation was demonstrated between TNF-α and these bone markers. These findings suggested that the anti-inflammatory effects of simvastatin may be involved in the bone remodeling process. While statins seemed to benefit bone forming osteoblast activity, they were also shown to have a substantial effect on osteoclasts activity via the inhibition of isoprenylated protein production, which are needed for osteoclast activity (28). These data suggest that statins may have potent anti-inflammatory effects and are both anti-resorptive and anabolic in terms of bone metabolism.

Although the mechanism by which statins reduce the risk of CVDs is associated with the inhibition of the rate-limiting enzyme, HMG-CoA reductase, in cholesterol synthesis, recent research suggests statin's anti-inflammatory properties are contributing to the lower incidence of CVDs. Studies (110, 126, 127) have shown that serum C-reactive protein (CRP) is highly correlated with CVDs and patients receiving statin

therapy have lower serum CRP compared to controls. Statins have also been shown to suppress the monocyte secretion of pro-inflammatory cytokines, such as IL-1β, IL-6, and IL-8 (128-130). Wolfrum *et al.,* (131) demonstrated that statins confer their beneficial effects by modulating endothelium-derived NO bioactivity, which attenuates endothelial dysfunction and atherosclerotic disease progression. Recent studies recognize simvastatin's role as an anti-inflammatory agent on human peripheral blood monocyte-macrophages, including up-regulation of the atheroprotective factor Kruppel-like factor 2 (KLF-2). This observation may explain in part statins' ability to reduce inflammation in the vessel wall (132).

Natural Treatment Options for Osteoporosis and CVD

Although there are therapeutic agents available for treatment of the individual symptoms associated with CVDs and osteoporosis, many of these treatments are associated with undesirable side-effects. Additionally, some patients may prefer a more natural treatment option. To date there has been no therapeutic agent developed to target both osteoporosis and CVD occurring simultaneously and as a result many patients end up taking multiple medications. A number of dietary supplements are known to have anti-inflammatory effects such as fish oils and phytochemicals found in fruits and vegetables. Specifically in fruits and vegetables, compounds known as polyphenols are one of the components that may be responsible for the anti-inflammatory qualities of the plant-based foods.

Polyphenols are a group of chemical substances found in plants, characterized by the presence of more than one phenol unit or building block per molecule, this unique chemical structure allows for these compounds to have potent anti-inflammatory

capacity. Dietary polyphenols have the ability to scavenge free-radicals, which can have devastating effects on our biological systems. For example, rutin, a glycoside of quercetin found mainly in onions but is also in plums, was able to prevent OVX-induced bone loss in rats and reduced urinary excretion of deoxypyridinoline (Dpd) and calciuria (133). EGCG, one of the primary polyphenols in green tea, appears to induce osteoclastic cell death (41), and enhance osteoblastic differentiation by up-regulating runt-related transcription factor 2 (Runx2) and osterix gene expression (134). Resveratrol, commonly found in red wines, has been shown to enhance the proliferation and differentiation of human osteoblastic cells (135). Both the consumption of tea (136) and red wine (137) are associated with a lower the risk of myocardial infarction in both case-control and cohort studies. Chlorogenic acid is the most abundant polyphenol in dried plum, and *in vitro* has been shown to have positive effects on oxidative stress, which is responsible for deterioration of cardiovascular health (138). Polyphenols have also been shown to improve endothelial dysfunction, which is an early event in atherosclerosis (139, 140). Endothelial-dependant vasorelaxing activity has been demonstrated with wine anthocyanins (141) soy isoflavones (142) and quercetin (143) in animal studies. Droke *et al.,* (24) found that dietary supplementation with soy isoflavones in mice protected against the inflammation-induced effects on skeletal microarchitecture and reduced TNF-α protein in endothelium of small myocardial arteries and arterioles (24). Therefore dietary polyphenolic compounds appear to protect against inflammation-induced osteoporosis and CVDs and may offer consumers an easily obtainable treatment with fewer side-effects.

Dried plums are known to have a very high content of polyphenolic compounds. These characteristics make this fruit an ideal candidate for the possible reversal of the pathology observed in osteoporosis and CVDs. Arjmandi *et al.,* (33) were the first to report that 25% dried plum (w/w) was able to prevent the ovariectomy-induced bone loss, and enhance serum IGF-1 in rats. Subsequently, a clinical study with postmenopausal women designed to assess the effect of dried plum supplementation (compared to dried apples) on bone biochemical markers revealed that women consuming the dried plums (i.e. ~100 g/d) experienced an increase in serum IGF-1 and BAP (144). Dried plum, in doses as low as 5% of total diet, has been shown to reverse the loss of tibial and femoral bone density in ovariectomized rats (32). Further studies have led to the conclusion that some component(s) of dried plum are able to suppress bone resorption (145) and also increase bone formation (32). In a male model of osteoporosis, orchidectomized rats were fed dried plum (5%, 15%, or 25%), the 15% dried plum diet had an increased femoral and lumbar spine BMD, and the 25% dried plum diet has completely prevented the decrease in BMD, this trend was also observed in microarchitectural parameters of the spine (34). Through the use of orchidectomized rats, conclusions were drawn that dried plum was able to restore bone similar to that of the pharmacological treatment PTH, however it seems likely that a different mechanism is involved (146). Bu *et al.,* (35) showed that dried plum's polypehnols were able to increase phosphatase ALP activity under normal and inflammatory conditions, and increased the gene expression of Runx2, Osterix, IGF-1 and nodule formation, while simultaneously down-regulating RANKL expression. Bu and colleagues (36) also determined dried plum's polyphenols significantly reduced nitrite production in LPS treated pre-osteoclast and osteoclast cells,

and decreased TRAP positive cells (36). These *in vitro* studies suggest that the whole fruit dried plum is able to exert anabolic and anti-resorptive effects on bone by the action of its polyphenols. In terms of cardiovascular system, dried plum has also been shown to suppress OVX-induced hypercholesteremia in rats by decreasing serum total cholesterol, triacylgycerides, and non-HDL cholesterol (38). Since it is known that dried plums contain such a high amount of polyphenols and based on *in vitro* studies, it is reasonable to attribute the skeletal and vascular protective properties of dried plum in part to their phenolic compounds.

In short, osteoporosis and CVDs are a major health concern in the U.S as well as many other developed countries. With the demographic shift towards an older society in such countries, these trends will only increase. Accumulating evidence from clinical and pre-clinical studies identified the pathophysiology of osteoporosis and CVD, and established a working premise that inflammation may play a central role in the development and progression of each condition (21). Our previous work suggests that dried plum's polyphenols may be an ideal candidate to explore as a single strategy for the treatment of concurrent CVDs and osteoporosis due to their potent anti-inflammatory abilities. Therefore the purpose of this study is to assess the effects of dried plum and its polyphenols on low grade chronic inflammation-induced changes in bone microarchitecture and vascular histology, and to determine the underlying mechanisms involved.

CHAPTER III

METHODOLOGY

Animal Care

Three-month old female C57BL/6 mice (*n*=192) were obtained (Jackson Labs, Bar Harbor, ME) and housed in environmentally controlled conditions. Following a seven day acclimation period, animals were randomly assigned to treatment groups (**Table 1**) and were surgically implanted with either a placebo (*n*=36) or LPS pellet (Innovative Research of America, Sarasota, FL) designed to deliver 0.1 mg LPS/kg bw /day (*n*=156). Treatments included AIN-93M control diet (147), control diet with low (LDP) or high (HDP) dose dried plum added (low dose = 5% or high dose = 25%, w/w), control diet with comparable dose of polyphenolic compounds as low (LPP) and high (HPP) dose dried plum, or simvastatin (25 mg/kg diet) as a positive control. All animals were maintained on the control diet for an initial 4 week period to allow the bone and vascular pathology to develop in the animals receiving LPS. At the end of 4 weeks, two groups of mice (i.e. a placebo and an LPS group) were sacrificed and data were collected for baseline characterization of bone mass and microarchitecture, gene expression from femoral RNA, and histological evaluation of the myocardium and aorta. The remaining animals were immediately started on their respective treatments fortreatment period food intake was monitored and animals were match fed to the group consuming the least

amount of diet (i.e. adjusted once a week). All animals had free access to deionized water and were weighed weekly.

At necropsy (i.e., baseline, 2 weeks, or 6 weeks), mice were anesthetized with an intramuscular injection of ketamine/xylazine cocktail 100.0/10.0 mg/kg bw and exsanguinated by the carotid artery. Bone (i.e. femurs and tibias), heart, aorta and liver specimens were harvested for future analyses. The femurs and tibias were cleaned of all adhering soft tissue and either snap frozen for RNA extraction or fixed in 10% neutral buffered formalin for μCT analyses. The heart and aorta were removed, fixed in10% neutral buffered formalin for histological examination. Portions of the liver were snap frozen for protein analyses, while the remainder of the liver was fixed in 10% neutral buffered formalin. All procedures were strictly adhered to the guidelines set for by the University of Oklahoma Health Sciences Center IACUC.

Implantation of Pellets

Time release pellets (Innovative Research of America, Sarasota, FL) were designed to deliver 0 or 0.1 mg LPS/kg bw /d for 90 days. The doses were selected based on the results from our previous study in which bone loss and vascular pathology developed (21, 22, 46). Surgical placement of the pellet required animals to be anesthetized with an intramuscular injection of ketamine/xylazine cocktail 10.0 mg/kg and the dorsal back of the mice was shaved. A small incision was made (~2 cm from the site of placement) and forceps were used to tunnel to the interscapular region where the pellet was placed. The incision site was closed with a single suture.

Extraction of Polyphenols from Dried Plum Powder

Polyphenols were extracted from 10 g batches of dried plum powder. This was accomplished by adding 100 mL of 80% ethanol to dried plum powder in an Erlenmeyer flask and sonicating the mixture in an ice cold (~4°C) water bath for 20 min, while being exposed to a continuous flow of N_2 gas to ensure polyphenol integrity. Once sonication was completed, the liquid was vacuum filtered using a chilled Buchner funnel lined with filter paper (8μm particle size). The flask was rinsed twice with 50 mL of 100% ethanol. The remaining dried plum powder was scraped from the filter paper and the extraction procedure repeated. Upon the completion of the extraction, approximately 400 mL of ethanol-extracted polyphenols were transferred to a round-bottom flask and the volume reduced to about 40% (~10 mL remaining) by roto-evaporation at 35°C and 60 rpm. The extract was then collected and stored in -80°C before being freeze dried (VirTis, Gardiner, NY).

Total Polyphenolic Analysis and Diet Formulation

To analyze the total phenolic content of the extract was determined by the Folin-Ciocalteu assay. First, 15 mL of ethanol was added to 150 mg of powder extract, sonicated for 30 min, and then filtered. About 1 mL of filtered extract was added to 100 mL volumetric flask with 60-70% HPLC grade water and 5 mL of Folin-Ciocalteu's phenol reagent was added and mixed. After two hours the absorption was measured at 760 nm. The same solution without extract was used as a blank. Calculation of the percent of total phenolics was determined based on a standard curve. The polyphenolic content of the diets (i.e. low and high) were based on the amount of polyphenolic

compounds provided in the 5% dried plum and 25% (w/w) dried plum diet. Diets were formulated to be isocaloric and have similar carbohydrate, fat, protein, calcium and phosphorous content (**Table 2)**.

BMD Assessment Using DXA

Excised tibias were used for DXA scans (GE Medical Systems,LunarPIXI, Madison, WI) performed on specimens harvested at baseline, 2 and 6 weeks post dietary treatments to determine the bone mineral area (BMA), bone mineral content (BMC), and bone mineral density (BMD). All DXA scans were performed using PIXImus Series Software version 1.4x.

Microcomputed Tomography Analyses

The tibia was scanned using μCT (MicroCT40, SCANCO Medical, Switzerland) to assess trabecular and cortical bone microarchitecture. The proximal tibia was the site of trabecular bone analyses while the tibial mid-diaphysis was analyzed for cortical bone parameters. Tibia scans were performed at high resolution (2048 x 2048 pixels). The proximal tibia metaphysis was analyzed by acquiring 175 slices and evaluating 100 slices (600 μm) in the volume of interest (VOI). Semi-automated contours were placed starting 5 slices (30 μm) distal to the growth plate to assess secondary spongiosa within the VOI. Trabecular bone volume expressed per unit of total volume (BV/TV), trabecular number (TbN), separation (TbSp), and thickness (TbTh), were determined and connectivity density (ConnDens) and structural model index (SMI) were calculated. The midshaft of the tibia was evaluated by acquiring 27 slices at the midpoint, and analyzing 20 slices (120 μm). Cortical porosity, thickness, area and medullary area were assessed to

determine the alterations in cortical bone associated with inflammation and the dietary treatments.

Finite Element Analysis

Images acquired with μCT allowed for further evaluation of the biomechanical parameters on trabecular bone structures using finite element analysis (FE) software (SCANCO Medical). A micromechanical FE model was constructed by converting bone voxels from the VOI into 8-node brick elements (148). The elements in this FE model have been shown to have a linear, elastic and isotropic material properties described by a Poisson's ratio of 0.3 and a Young's modulus of 10 GPa (149). Compression testing was simulated on the reconstructed 3-D images of the proximal tibia, total force and size-independent stiffness was determined (150).

RNA Extraction and Quantitative Real-Time PCR

Whole femurs were pulverized (Spex 6770 freezer mill) and homogenized-bone powder was immediately placed in 1 mL of Trizol Reagent (Life Technology, Rockville, MD, USA). Trizol and bone were centrifuged at 4°C for ~5 min at 12,000 x *g*. The top phase was then removed and transferred to a clean, RNase-free microfuge tube and allowed to incubate at ~25°C for 5 min. Next, 200 μL of chloroform was added and vigorously shaken for 15 sec. The mixture was allowed to incubate for 3 min followed by centrifugation at 12,000 x *g* for 10 min at 4°C. The clear aqueous phase was then transferred to a clean tube and 250 μL isopropanol and 250 μL high salt solution (1.2 M NaCl and 0.8 M $Na_3C_3H_5O(COO)_3$) were added and allowed to precipitate on ice for 30 min. To pellet the RNA, the solution was centrifuged at 4°C for 15 min at 12,000 x *g* and

the RNA pellet was observed. The supernatant was removed, and the pellet was washed with 75% ethanol and centrifuged again at 7,500 x g for 5 min at 4°C. The RNA pellet was resuspended in 20 μL of diethylpyrocarbonate (DEPC) H_2O. To determine quantity and quality of RNA, the concentration and A_{260}/A_{280} ratio was obtained using a Nanodrop Spetrophotometer (Rockland, DE).

Quantitative real time PCR was performed using 2 μg of total RNA pre-treated with DNase I and subjected to reverse-transcription (Superscript II, Invitrogen, Carlsbad, CA). Fifty ng of cDNA were used for each qRT-PCR reaction and all reactions were assayed in duplicate using SYBR green chemistry (Roche, Penzberg, Germany) on the Applied Biosystems 7300 Real Time PCR (Foster City, CA). The primer sequences for genes of interest were designed based on Genebank database or published species-specific sequences (**Table 3**). The criteria used for primer design/validation is the amplicon must span an intron, template titration must have an efficiency slope of -3.3, and demonstrate the formation of a single dissociation curve. All qRT PCR results were evaluated by the comparative cycle number at threshold (C_T) method using hypoxanthine guanine phosphoribosyltransferase 1 (HPRT1) as the invariant control.

Histology

Cross-sections of the aorta and heart were paraffin-embedded and cut into 5-μm sections. Sections were then stained with hemotoxylin and eosin (H&E) for analysis of cellular and tissue pathology. All slides were graded using a scoring system by the study pathologist. The arteries and arterioles were scored based on the degree of narrowing: 0, no observed narrowing of vessel through 3, severe narrowing of vessel. Within the

myocardium, inflammatory infiltrate such as lymphocytes and polymorphonuclear cells (PMN) along with mast cells were graded as such: 0, no visible infiltration through 3, severe infiltration. The aorta scores were totaled from three parameters of thickening of the wall (0-3), thinning of the wall (0-3) and artheromatous plaque (0-3), so the highest possible score is 9. Infarction was graded as either 0, no observed event or 1, in the event of a myocardial infarction.

Statistical Analyses

Descriptive statistics were calculated for all variables and included means and standard error. Data were analyzed using ANOVA (SAS Version 9.1; SAS Institute, Cary NC). Bone and vascular structure and calcification were investigated through linear correlation coefficients. Categorical data derived from the pathology scoring were compared using Chi-squared test followed by Fisher's Exact Test to confirm significance. Values were expressed as means ± standard error (SE), and $p<0.05$ was considered to be statistically significant for all analyses.

Table 1. Treatment Groups

Baseline	2 weeks	6 weeks
Placebo + Control Diet	Placebo + Control Diet	Placebo + Control Diet
LPS + Control Diet	LPS + Control Diet	LPS + Control Diet
	LPS + High Dose Dried Plum (HDP)	LPS + High Dose Dried Plum (HDP)
	LPS + Low Dose Dried Plum (LDP)	LPS + Low Dose Dried Plum (LDP)
	LPS + High Dose Polyphenols (HPP)	LPS + High Dose Polyphenols (HPP)
	LPS + Low Dose Polyphenols (LPP)	LPS + Low Dose Polyphenols (LPP)
	LPS + Statin	LPS + Statin

Slow release pellets delivered 0 (Placebo) or 0.1 mg LPS/kg bw/d (LPS). Baseline animals received only AIN-93M (control) diet. Dietary treatments were: control diet, control diet with low (LDP) or high (HDP) dose dried plum added (low = 5% or high = 25%, w/w), control diet with comparable dose of polyphenolic compounds as low (LPP) and high (HPP) dose dried plum, or simvastatin (Statin) as a positive control (25 mg/kg diet). Dietary treatments started after the baseline sacrifice (4 weeks post pellet implantation) and were maintained for 2 or 6 weeks.

Table 2. Formulation of Diet

Ingredients	Control	LDP	HDP	LPP	HPP
			g/ kg Diet		
Dried Plum	_	50	250	_	_
Polyphenol Extract	_	_	_	32.1	160.7
Carbohydrate					
Cornstarch	466	425.7	265.7	439.4	334
Sucrose	100	100	100	100	100
Dextrinized Cornstarch	155	155	155	155	155
Protein					
Casein	140	138.5	132.5	138.9	134.6
Fat					
Soybean Oil	40	39.75	38.75	39.8	38.9
Fiber					
Cellulose	50	45.5	55	50	50
Vitamin Mix	10	10	10	10	10
Mineral Mix (Ca-P Def)	13.4	13.4	13.4	13.4	13.4
Sucrose	2.2	2.6	4.6	2.4	4.0
Calcium Carbonate	12.5	12.4	12.1	12.4	12
Sodium Phosphate, Monobasic	5.6	5.5	4.8	5.6	5.2
Potassium Phosphate, Monobasic	2.4	2.4	2.4	2.4	2.4
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5
L-cysteine	1.8	1.8	1.8	1.8	1.8
Tert-butylhydroquinone	0.008	0.008	0.008	0.008	0.008

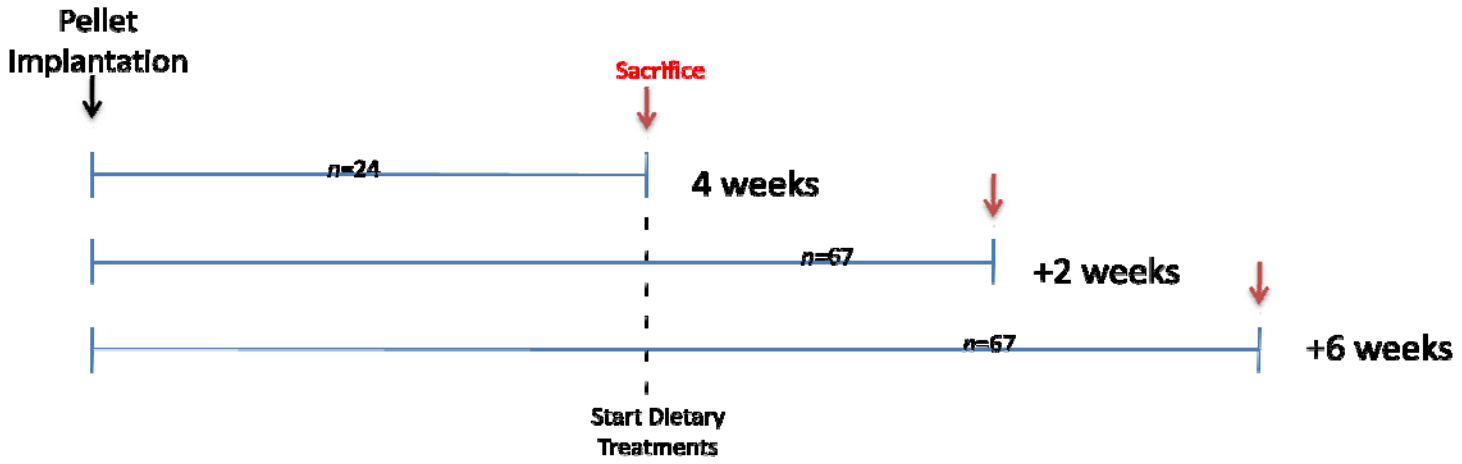
Dietary treatments were: control diet, control diet with low (LDP) or high (HDP) dose dried plum added (low = 5% or high = 25%, w/w), control diet with comparable dose of polyphenolic compounds as low (LPP) and high (HPP) dose dried plum, or simvastatin (Statin) as a positive control (25 mg/kg diet).

Table 3. List of Primer Sequences Used for Real Time-PCR

Symbol	Name	Accession #	Sequence
ALP	Alkaline phosphatase		QF 5'- GGT ATG GGC GTC TCC ACA GT -3'
		NM_007431.2	QR 5'- GCC CGT GTT GTG GTG TAG CT -3'
HPRT1	Hypoxanthine guanine phosphoribosyltransferase 1		QF 5'- GCC TAA GAT GAG CGC AAG TTG -3'
		NM_013556.2	QR 5'- TAC TAG GCA GAT GGC CAC AGG -3'
IGF-1	Insulin-like growth factor 1		QF 5'- CCA CAC TGA CAT GCC CAA GA -3'
		NM_184052.2	QR 5'- CTC CTT TGC AGC TTC GTT TTC T -3'
IL-6	Interleukin 6		QF 5'- GAG GAT ACC ACT CCC AAC AGA CC -3'
		NM_031168.1	QR 5'- AAG TGC ATC ATC GTT GTT CAT ACA -3'
IL-10	Interleukin 10		QF 5'- GGT TGC CAA GCC TTA TCG GA -3'
		NM_010548.1	QR 5'- ACC TGC TCC ACT GCC TTG CT -3'
OPG	Osteoprotegerin		QF 5'- TCC TGG CAC CTA CCT AAA ACA GCA -3'
		NM_008764.3	QR 5'- ACA CTG GGC TGC AAT ACA CA -3'
RANKL	Receptor activator for nuclear factor κ B ligand		QF 5'- CTG ATG AAA GGA GGG AGC AC -3'
		NM_011613.3	QR 5'- GAA GGG TTG GAC ACC TGA ATG -3'
TNF-α	Tumor necrosis factor-alpha		QF 5'- CTG AGG TCA ATC TGC CCA AGT AC -3'
		NM_013693.2	QR 5'- CCT CAC AGA GCA ATG ACT CCA AAG -3'
TRAP	Tartrate resistant acid phosphatase		QF 5'- CTG CAC AGA TTG CAT ACT CTA AGA TCT-3'
		NM_007388.3	QR 5'- TTT GAA GCG CAA ACG GTA GTA A -3'

The criteria used for primer design/validation is the amplicon must span an intron, template titration must have an efficiency slope of -3.3, and demonstrate the formation of a single dissociation curve.

Figure 1. Experimental design



Placebo or LPS pellets were implanted on day 0 and baseline sacrifice was performed 4 weeks later on one Placebo and one LPS group. During the first 4 weeks all animals received AIN-93M (control) diet. Dietary treatments were: control diet, control diet with low (LDP) or high (HDP) dose dried plum added (low = 5% or high = 25%, w/w), control diet with comparable dose of polyphenolic compounds as low (LPP) and high (HPP) dose dried plum, or simvastatin (Statin) as a positive control (25 mg/kg diet). Dietary treatments started after the baseline sacrifice (4 weeks) and was maintained for 2 or 6 weeks.

CHAPTER IV

FINDINGS

Bone Mass and Densitometry

To determine the alterations in bone density in response to LPS and treatment, excised tibias were scanned using dual-energy X-ray absorptiometry**.** There was no significant difference in tibial BMC, BMA **(Table 4)**, or BMD **(Figure 2)** between the placebo or LPS pellet group's tibia on control diet at baseline. After 2 and 6 weeks on dietary treatments, no differences were observed in tibial BMC, BMA **(Table 4)**, or BMD **(Figure 2)** in any of the treatment groups or between the placebos versus LPS treated groups on control diet. These findings suggest that no alterations occurred in tibial bone mass and density in response to LPS or treatment over the course of the study.

Proximal Tibia Trabecular and Cortical Bone Microarchitecture

Analysis of trabecular bone of the proximal tibia at baseline revealed that though there were no alterations in BV/TV **(Figure 3A)**, but there was a significant increase in trabecular separation (TbSp) in the mice exposed to LPS compared to the placebo group **(Figure 3D)**. No other alterations in trabecular bone morphometric parameters at the proximal tibia were observed at baseline, including trabecular number (TbN) and trabecular thickness (TbTh) **(Figure 3B and 3C)**. Trabecular bone connectivity density,

which indicates the number of trabecular connections in a given volume of interest, tended to be lower in the LPS group compared to placebo group **(Table 5)**. As expected, LPS did not significantly alter any of the cortical parameters at the tibial midshaft at baseline **(Table 6)**. At 2 weeks and 6 weeks no trabecular (**Figures 4 & 5**) or cortical bone parameters (**Table 7**) were altered by LPS or dietary treatments.

Biomechanical Properties of Trabecular Bone

Due to the lack of an effect on trabecular bone microarchitecture, finite element analysis revealed that there were no differences in total force or size independent stiffness between groups at baseline or after 2 weeks and 6 weeks of treatments **(Table 8)**.

Gene Expression of Whole Femurs

In contrast to our expectation, at baseline there were no statistically significant differences in gene expression of the pro-inflammatory mediators TNF-α and IL-6, or in the anti-inflammatory mediator IL-10 **(Figure 6A-C)**. There was also no observed difference ($P<0.05$) in genes associated with bone resorption including RANKL, OPG, TRAP, and the RANKL/OPG ratio **(Figure 7A-D)**. Likewise, at baseline there were no alterations in the bone ALP, which is an indicator of bone formation **(Figure 8)**.

Following two weeks of treatment, there continued to be no statistically significant differences in TNF-α, IL-6, and IL-10 gene expression in the femur of the LPS group compared to the placebo **(Figure 9A-C)**. No alterations bewteen any of the treatment groups were observed in IL-6 or IL-10, but the high dose of dried plum polyphenols (HPP) did down-regulate ($P<0.05$) the relative gene expression of TNF-α,

compared to the placebo and LPS control groups **(Figure 9A)**. Expression of other target genes involved in bone resorption, RANKL, TRAP, OPG and the RANKL/OPG ratio were not statistically altered by the LPS compared to placebo, or within any of the treatment groups at 2 weeks **(Figure 10A-D)**. At two weeks post dietary treatment, ALP gene expression had not been significantly altered, by LPS or any of the treatment groups **(Figure 11A)**. IGF-1 gene expression was significantly decreased in the LPS cohort on control diet compared to the placebo group, however, none of the dietary treatments were able to increase IGF-1 expression **(Figure 11B)**.

After 6 weeks of treatment, there were no statistically significant differences observed in bone TNFα, IL-6, or IL-10 relative gene expression **(Figure 12A-C)**. RANKL gene expression was significantly up-regulated by LPS in the group on the control diet compared to the placebo group (**Figure 13A)**. HPP and LDP down-regulated RANKL gene expression in mice with the LPS pellet similar to that of the mice implanted with the placebo pellet **(Figure 13A)**. However, there was no statistically significant difference in gene expression associated with bone resorption **(Figure 13D)** or bone formation **(Figure 14)** at six weeks.

Our positive control, simvastatin had no significant effect on any parameters tested other than IGF-1 at 2 weeks **(Figure 11B)** and RANKL at 6 weeks **(Figure 13A)**. Statin therapy was able to down-regulate IGF-1 compared to placebo and LPS controls after 2 weeks on dietary treatment. RANKL gene expression was also significantly lowered compared to LPS control group after 6 weeks of treatment. Based on previous reports that statin drugs have anti-inflammatory and potential anabolic effects on bone, simvastain was included in the study designed as a positive control. After 2 and 6 weeks

of statin treatment, animals exhibited no response in the expression of TNF-α, IL-6 or IL-10 mRNA **(Figure 9 and 13)**.

Histological Evaluation of the Myocardium and Aorta

Representative micrographs of the heart (**Figure 15**) and aorta (**Figure 16**) are shown. After 2 weeks of treatment the LPS pellet had not altered narrowing of large or small artery or arterioles compared to the placebo group **(Table 9).** Lymphocyte infiltration of the heart had significantly increased in the LPS control group compared to the placebo after 2 weeks dietary treatment, the high dose of dried plum and statin therapy were able to decrease this response **(Table 10)**. Mast cell and PMN infiltration frequency were not statistically altered by the LPS after 2 weeks of dietary treatment (6 weeks after pellet implantation) **(Table 10)**.

At 6 weeks post dietary treatment (10 weeks after placebo or LPS pellet implantation), the LPS group on control diet had a significantly higher degree of large and small artery narrowing, however, all of the dietary treatments were able to reverse these vascular changes **(Table 11)**. All treatment groups with exception of the low dose of dried plum,were ble to decrease the f narrowing of large arterioles. No treatments were able to impact the frequency of small arteriole narrowing **(Table 11)**. The LPS pellet increased the degree of lymphocyte infiltrate of the heart, and all of the treatments attenuated this cellular response to a similar degree **(Table 12)**. Although PMN infiltration was observed in 83% of the LPS treated heart specimens, this was not observed in any of the LPS groups on the dietary treatments **(Table 12)**. The LPS group on control diet had a statistically higher number of mast cells in the heart compared to

placebo and the high dose of dried plum **(Table 12)**. At 9 weeks post dietary treatments or control diets, no animals had an observed infarction **(Table 12)**. There were no statistical differences in aortas at 2 weeks post dietary treatment **(Table 13)**. After 6 weeks of dietary treatment, the LPS had induced vascular changes leading to higher pathology scores in the aorta compared to the placebo control. All treatments were able to reduce these negative effects on the aorta **(Table 13).**

Table 4. Bone Mineral Area (BMA) and Content (BMC) of the Tibia at Baseline and Following 2 and 6 Weeks of Dietary Treatments

	Placebo	LPS	LPS/LPP	LPS/HPP	LPS/LDP	LPS/HDP	LPS/Statin
Baseline							
BMA (cm^2)	0.480 ± 0.01	0.470 ± 0.01					
BMC (g)	0.0242 ± 0.001	0.0238 ± 0.001					
2 weeks							
BMA (cm^2)	0.456 ± 0.04	0.490 ± 0.01	0.502 ± 0.01	0.510 ± 0.01	0.511 ± 0.01	0.501 ± 0.01	0.488 ± 0.010
BMC (g)	0.0267 ± 0.001	0.0263 ± 0.001	0.0273 ± 0.001	0.0285 ± 0.001	0.0284 ± 0.001	0.0276 ± 0.001	0.0263 ± 0.001
6 weeks							
BMA (cm^2)	0.512 ± 0.01	0.527 ± 0.01	0.512 ± 0.01	0.513 ± 0.01	0.520 ± 0.01	0.519 ± 0.01	0.499 ± 0.01
BMC (g)	0.0289 ± 0.0007	0.0305 ± 0.001	0.0291 ± 0.001	0.0283 ± .001	0.0301 ± 0.001	0.0299 ± 0.001	0.0271 ± 0.001

Slow release pellets delivered 0 (Placebo) or 0.1 mg LPS/kg bw/d (LPS). Baseline animals received only AIN-93M (control) diet. Dietary treatments were: control diet, control diet with low (LPS/LDP) or high (LPS/HDP) dose dried plum added (low = 5% or high = 25%, w/w), control diet with comparable dose of polyphenolic compounds as low (LPS/LPP) and high (LPS/HPP) dose dried plum, or simvastatin (LPS/Statin) as a positive control (25 mg/kg diet). Dietary treatments started after the baseline sacrifice (4 weeks post pellet implantation) and were maintained for 2 or 6 weeks.

Table 5. Tibia Trabecular Bone Microarchitectural Parameters at Baseline and Following 2 or 6 Weeks of Dietary Treatments

	Placebo	LPS	LPS/LPP	LPS/HPP	LPS/LDP	LPS/HDP	LPS/Statin
Baseline							
ConnDens	341.09 ± 23.2	276.92 ± 22.3					
SMI	1.39 ± 0.1	1.63 ± 0.1					
2 weeks							
ConnDens	304.28 ± 21.4	377.49 ± 25.9	281.13 ± 21.2	270.59 ± 31.3	293.00 ± 15.6	301.72 ± 27.9	349.17 ± 34.8
SMI	1.24 ± 0.2	1.30 ± 0.1	1.28 ± 0.2	1.41 ± 0.1	1.34 ± 0.1	1.28 ± 0.2	1.22 ± 0.2
6 weeks							
ConnDens	272.6 ± 32.6	274.4 ± 41.5	369.9 ± 93.4	248.3 ± 6.1	241.7 ± 32.7	281.6 ± 12.2	226.5 ± 18.6
SMI	0.87±0.2	1.00 ± 0.2	0.85 ± 0.2	1.04 ± 0.2	0.92 ± 0.2	0.77 ± 0.2	0.94 ± 0.2

Connectivity density (ConnDens) and structural model index (SMI) as assed by μCT. Slow release pellets delivered 0 (Placebo) or 0.1 mg LPS/kg bw/d (LPS). Baseline animals received only AIN-93M (control) diet. Dietary treatments were: control diet, control diet with low (LPS/LDP) or high (LPS/HDP) dose dried plum added (low = 5% or high = 25%, w/w), control diet with comparable dose of polyphenolic compounds as low (LPS/LPP) and high (LPS/HPP) dose dried plum, or simvastatin (LPS/Statin) as a positive control (25 mg/kg diet). Dietary treatments started after the baseline sacrifice (4 weeks post pellet implantation) and were maintained for 2 or 6 weeks.

Table 6. Cortical Bone Morphometry at the Tibial Mid-diaphysis.

	Placebo	**LPS**
Baseline		
Cortical Thickness (mm)	0.239 ±0.001	0.241±0.01
Cortical Area (mm^2)	0.82 ±0.04	0.83 ±0.02
Medullary Area (mm^2)	0.0117 ±0.00	0.0109±0.00
Porosity (%)	1.39 ±0.07	1.31 ±0.03

Baseline animals had either placebo pellet (0.0 μg LPS/day) or an LPS pellet delivering 0.1 mg LPS/kg/d, and maintained on control diet for 4 weeks.

Table 7. Cortical Bone Morphometry at the Tibial Mid-diaphysis After 2 or 6 Weeks of Dietary Treatments

	Placebo	LPS	LPS/LPP	LPS/HPP	LPS/LDP	LPS/HDP	LPS/Statin
2 weeks							
Cortical Thickness (mm)	0.236 ± 0.00	0.240 ± 0.01	0.257 ± 0.01	0.246 ± 0.01	0.248 ± 0.01	0.251 ± 0.01	0.243 ± 0.01
Cortical Area (mm^2)	0.815 ± 0.02	0.796 ± 0.03	0.914 ± 0.03	0.837 ± 0.03	0.865 ± 0.03	0.874 ± 0.04	0.860 ± 0.03
Medullary Area (mm^2)	1.071 ± 0.01	1.070 ± 0.07	1.190 ± 0.06	1.143 ± 0.09	1.219 ± 0.13	1.083±0.05	1.131 ± 0.02
Porosity (%)	1.31 ± 0.04	1.36 ± 0.13	1.31± 0.07	1.36 ± 0.08	1.42 ± 0.14	1.25 ± 0.064	1.32 ± 0.08
6 weeks							
Cortical Thickness (mm)	0.254 ± 0.01	0.259 ± 0.01	0.268 ± 0.00	0.246 ± 0.01	0.258 ± 0.01	0.256 ± 0.01	0.238 ± 0.01
Cortical Area (mm^2)	0.844 ± 0.05	0.935 ± 0.11	0.952 ± 0.08	0.804 ± 0.05	0.864 ± 0.07	0.939 ± 0.01	0.862 ± 0.04
Medullary Area (mm^2)	1.035 ± 0.10	1.060 ± 0.11	1.039 ± 0.11	1.062 ± 0.06	0.897 ± 0.07	1.29 ± 0.06	1.19 ± 0.14
Porosity (%)	0.55 ± 0.02	0.53 ± 0.01	0.52 ± 0.01	0.57 ± 0.01	0.54 ± 0.02	0.51 ± 0.01	0.58 ± 0.02

Slow release pellets delivered 0 (Placebo) or 0.1 mg LPS/kg bw/d (LPS). Baseline animals received only AIN-93M (control) diet. Dietary treatments were: control diet, control diet with low (LPS/LDP) or high (LPS/HDP) dose dried plum added (low = 5% or high = 25%, w/w), control diet with comparable dose of polyphenolic compounds as low (LPS/LPP) and high (LPS/HPP) dose dried plum, or simvastatin (LPS/Statin) as a positive control (25 mg/kg diet). Dietary treatments started after the baseline sacrifice (4 weeks post pellet implantation) and were maintained for 2 or 6 weeks.

Table 8. Biomechanical Properties of the Tibia as Assessed by Finite Element (FE) Analysis at Baseline and Following 2 or 6 Week of Dietary Treatments

	Placebo	**LPS**	**LPS/LPP**	**LPS/HPP**	**LPS/LDP**	**LPS/HDP**	**LPS/Statin**
Baseline							
Total Force (N)	5.83 ± 0.9	4.97 ±0.5					
Size Independent Stiffness (N/m)	714.71 ±94.5	601.35 ±67.7					
2 weeks							
Total Force (N)	6.07 ±0.7	6.78 ±1.0	6.49 ±0.8	5.09 ±0.5	5.83 ±0.7	6.03 ±0.9	5.99 ±0.8
Size Independent Stiffness (N/m)	727.57 ±69.9	841.56 ±132.7	814.92 ±98.9	650.08 ±62.2	732.90 ±81.8	816.42 ±118.9	724.62±93.1
6 weeks							
Total Force (N)	7.48 ±0.4	9.22 ±1.2	10.65 ±2.7	8.09 ±1.3	8.46 ±1.7	10.45 ±1.8	9.33 ±1.4
Size Independent Stiffness (N/m)	819.45±48.2	1023.32±129.4	1146.34±235.1	956±170.5	1006±206.9	1154.23±203.0	963±134.0

Slow release pellets delivered 0 (Placebo) or 0.1 mg LPS/kg bw/d (LPS). Baseline animals received only AIN-93M (control) diet. Dietary treatments were: control diet, control diet with low (LPS/LDP) or high (LPS/HDP) dose dried plum added (low = 5% or high = 25%, w/w), control diet with comparable dose of polyphenolic compounds as low (LPS/LPP) and high (LPS/HPP) dose dried plum, or simvastatin (LPS/Statin) as a positive control (25 mg/kg diet). Dietary treatments started after the baseline sacrifice (4 weeks post pellet implantation) and were maintained for 2 or 6 weeks.

Table 9. Histological Scoring of the Myocardium 2 Weeks Post Dietary Treatment of the Large and Small Arteries and Arterioles

	Large Artery				Small Artery				Large Arteriole				Small Arteriole			
Scoring Category	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
Treatment Groups																
Placebo (%)	100	_	_	_	100	_	_	_	100	_	_		100	_	_	_
LPS (%)	100	_	_	_	100	_	_	_	100	_	_	_	100	_	_	_
LPS+LPP (%)	100	_	_	_	100	_	_	_	100	_	_	_	100	_	_	_
LPS+HPP (%)	100	_	_	_	100	_	_	_	100	_	_	_	100	_	_	_
LPS+LDP (%)	100	_	_	_	100	_	_	_	100	_	_	_	100	_	_	_
LPS+HDP (%)	100	_	_	_	100	_	_	_	100	_	_	_	100	_	_	_
LPS+Statin (%)	100	_	_	_	100	_	_	_	100	_	_	_	100	_	_	_

Large and small arteries and arterioles (n=6) were scored 0 through 3 based on severity of narrowing of the vessel (0 indicated no observed narrow and 3 was severe).

Table 10. Histological Scoring of the Myocardium 2 Weeks Post Dietary Treatment for Cellular Infiltration and Infarction

	Lymphocyte Infiltration				PMN Infiltration				Infarction		Mast Cells			
Scoring Category	0	1	2	3	0	1	2	3	0	1	0	1	2	3
Treatment Groups														
Placebo (%)	100	_	_	_	100	_	_	_	100	_	67	33	_	_
LPS (%)	_	67	33	_	100	_	_	_	100	_	17	50	33	_
LPS+LPP (%)	_	83	17	_	100	_	_	_	100	_	17	83	_	_
LPS+HPP (%)	_	33	67	_	100	_	_	_	100	_	_	67	33	_
LPS+LDP (%)	17	50	33	_	100	_	_	_	100	_	17	83	_	_
LPS+HDP (%)	67	33	_	_	100	_	_	_	100	_	50	50	_	_
LPS+Statin (%)	67	33	_	_	100	_	_	_	100	_	33	67	_	_

Infiltration was scored based on a 0-3 scale with 0 indicating no infiltration and 3 significant infiltration of lymphocytes and polymorphonuclear cell PMN. Infarction was scored based on the absence (0) or presence (1) of incident (*n*=6).

Lymphocyte Infiltration:
Placebo vs. LPS (p=0.0025)
LPS vs. HDP (p=0.0357); LPS vs. Statin (p=0.0357)

Table 11. Histological Scoring of the Myocardium 6 Weeks Post Dietary Treatment of the Large and Small Artery and Arteriole

	Large Artery				Small Artery				Large Arteriole				Small Arteriole			
Scoring Category	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
Treatment Groups																
Placebo (%)	100	_	_	_	100	_	_	_	100	_	_	_	100	_	_	_
LPS (%)	_	100	_	_	_	100	_	_	_	50	50	_	_	50	33	17
LPS/LPP (%)	100	_	_	_	100	_	_	_	66	17	17	_	50	33	17	_
LPS/HPP (%)	100	_	_	_	100	_	_	_	66	33	_	_	17	83	_	_
LPS/LDP (%)	100	_	_	_	100	_	_	_	17	83	_	_	_	100	_	_
LPS/HDP (%)	100	_	_	_	100	_	_	_	100	_	_	_	50	50	_	_
LPS/Statin (%)	100	_	_	_	100	_	_	_	100	_	_	_	50	50	_	_

Large and small arteries and arterioles (*n*=6) were scored 0 through 3 based on severity of narrowing of the vessel (0 indicated no observed narrow and 3 was severe).

Large and Small Artery:
Placebo vs. LPS (p=0.0025)
LPS vs. LPP (p=0.0025); LPS vs. HPP (p=0.0025); LPS vs. LDP (p=0.0025); LPS vs. HDP (p=0.0025); LPS vs. Statin (p=0.0025)
Large Arteriole:
Placebo vs. LPS (p=0.0025)
LPS vs. LPP (p=0.0498); LPS vs. HPP (p=0.0273); LPS vs. HDP (p=0.0025); LPS vs. Statin (p=0.0025)
Small Arteriole:
Placebo vs. LPS (0.0025)

Table 12. Histological Scoring of the Myocardium 6 Weeks Post Dietary Treatment for Cellular Infiltration and Infarction

	Lymphocyte Infiltration				PMN Infiltration				Infarction		Mast Cells			
Scoring Category	0	1	2	3	0	1	2	3	0	1	0	1	2	3
Treatment Groups														
Placebo (%)	100	_	_	_	100	_	_	_	100	_	100	_	_	_
LPS (%)	_	_	17	83	17	83	_	_	100	_	_	_	100	_
LPS+LPP (%)	17	50	33	_	100	_	_	_	100	_	_	50	50	_
LPS+HPP (%)	17	50	33	_	100	_	_	_	100	_	17	17	66	_
LPS+LDP (%)	_	67	33	_	100	_	_	_	100	_	_	67	33	_
LPS+HDP (%)	33	50	17	_	100	_	_	_	100	_	17	66	17	_
LPS+Statin (%)	_	17	83	_	100	_	_	_	100	_	_	33	67	_

Infiltration was scored based on a 0-3 scale with 0 indicated no observed infiltration and 3 indicative of many cells. Infarction was scored based on the absence (0) or presence (1) of incident (n=6).

Lymphocyte Infiltration:

Placebo vs. LPS (p=0.0025)
LPS vs. LPP (p=0.0252); LPS vs. HPP (p=0.0252); LPS vs. LDP (p=0.0094); LPS vs. HDP (p=0.0112); LPS vs. Statin (p=0.0131)
Mast Cells:
Placebo vs. LPS (p=0.0025)
LPS vs. HDP (p=0.0138)

Table 13. Histological Scoring of the Aorta Following 2 or 6 Weeks of Dietary Treatment

Aorta	**Mean Score**	
	2 weeks	*6 weeks*
Placebo	0.00 ± 0.00	0.00 ± 0.00[a]
LPS	0.17 ± 0.17	6.83 ± 0.75[b]
LPS+LPP	0.00 ± 0.00	2.17 ± 0.40[c]
LPS+HPP	0.00 ± 0.00	1.50 ± 0.62[c]
LPS+LDP	0.00 ± 0.00	2.33 ± 0.42[cd]
LPS+HDP	0.00 ± 0.00	0.83 ± 0.48[ac]
LPS+Statin	0.00 ± 0.00	0.00 ± 0.00[ac]

Scores of the aorta (n=6) were calculated by taking the mean of 3 parameters; thickening of the wall (0-3), thinning of the wall (0-3), and arthermatous plaque (0-3). In all cases 0 indicated no observed event and 3 indicated the severity of observed event. Within a column, values that do not share the same superscript are significantly different ($p<0.05$) from each other.

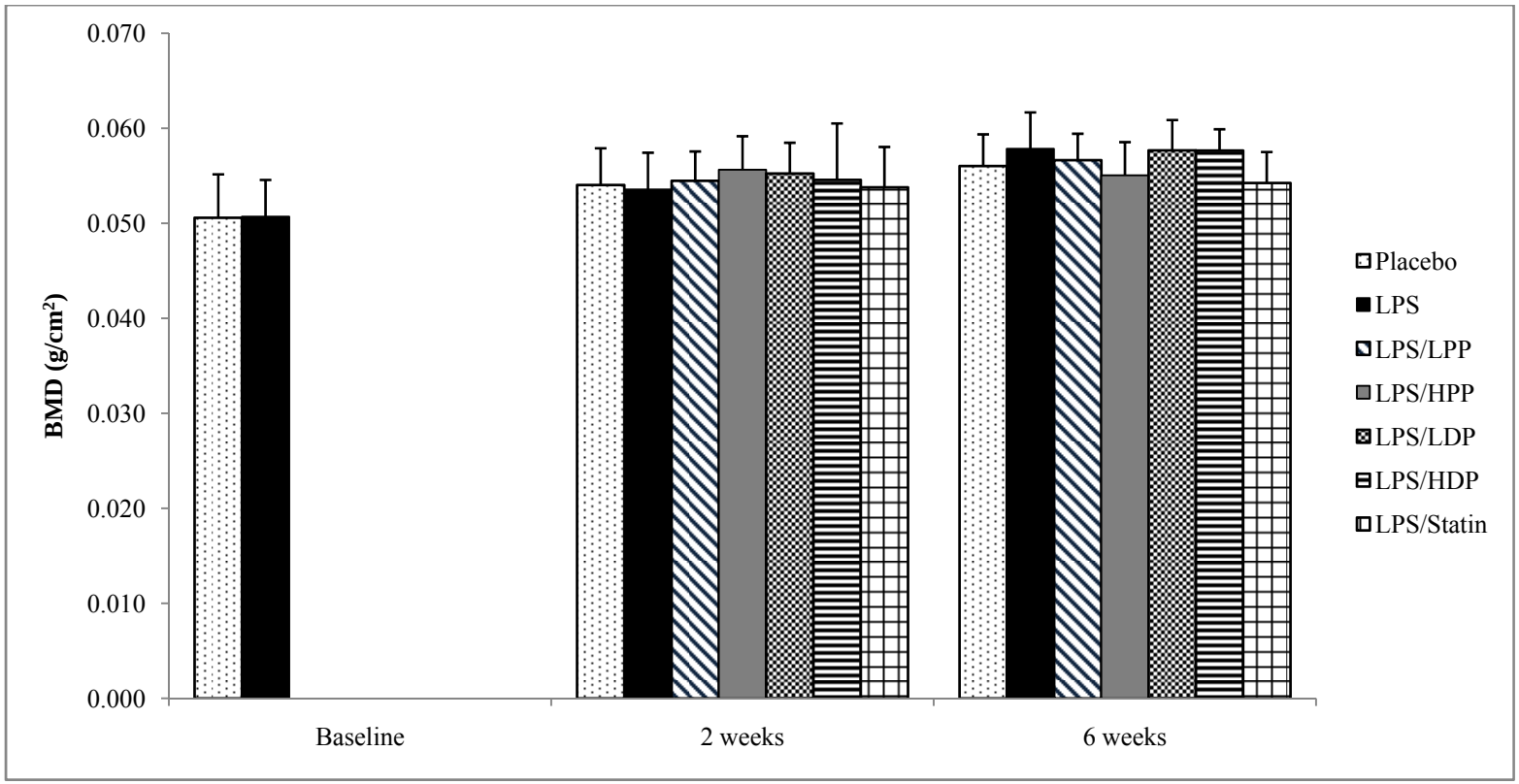


Figure 2. Bone mineral density (BMD) of excised tibia. Baseline animals had either placebo pellet (0.0 μg LPS/day) or an LPS pellet delivering 0.1 mg LPS/kg/d, and maintained on control diet for 4 weeks. Dietary treatments started after baseline and were maintained on control AIN-93 (Control), control diet with low (LDP) or high dose dried plum (HDP) added (low = 5% or high = 25%, w/w), control diet with comparable dose of polyphenolic compounds as low (LPP) and high dose dried plum (HPP), or simvastatin (Statin) as a positive control (25 mg/kg diet) for 2 or 6 weeks.

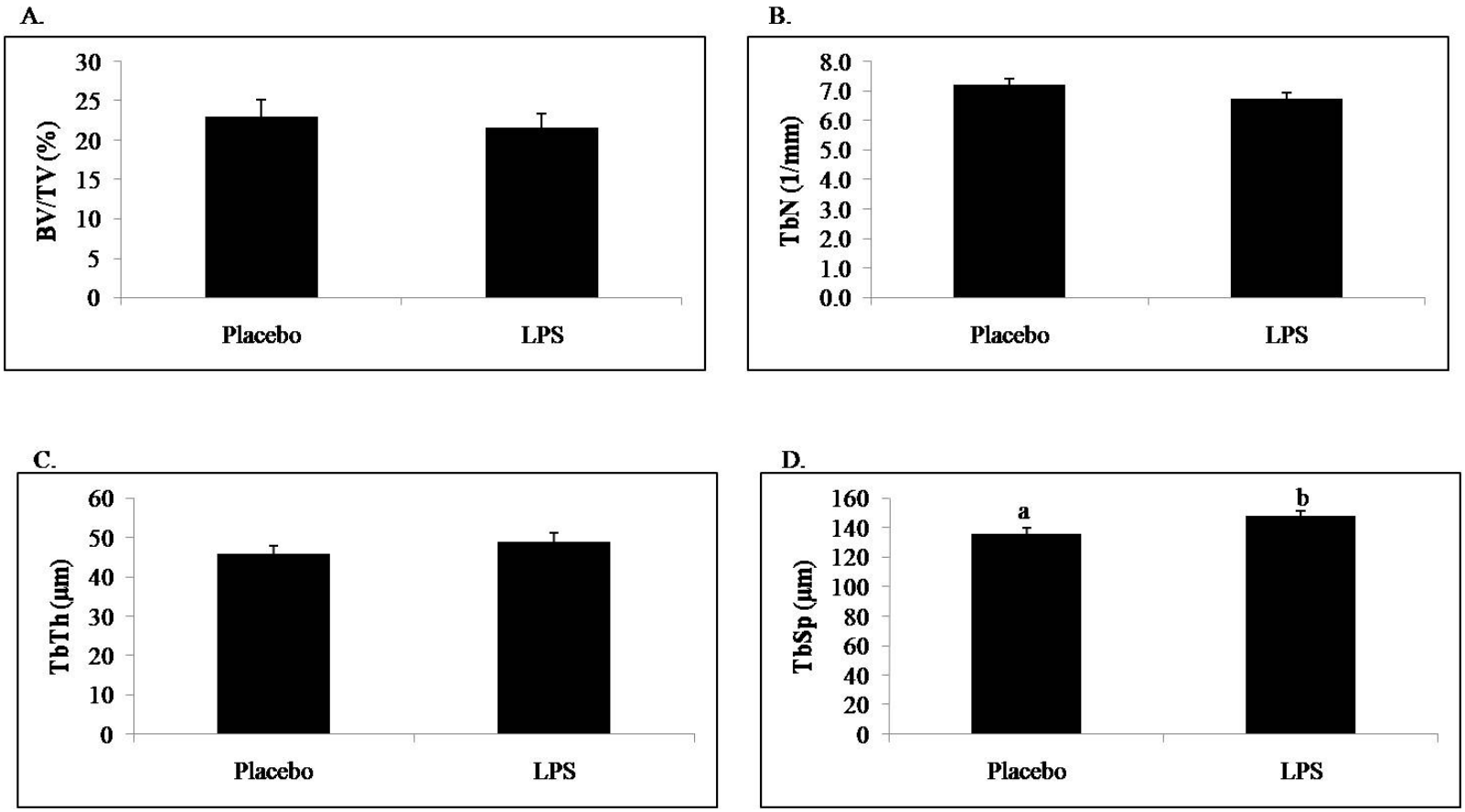


Figure 3. Baseline microarchitectural parameters of trabecular bone in the proximal tibial metaphysis. Parameters assessed by micro-CT were (A) bone volume/total volume (BV/TV), (B) trabecular number (TbN), (C) thickness (TbTh), and (D) separation. Bars represent the mean $\pm$ SE for each treatment group. Bars that share the same superscript letter are not statistically different from each other ($p<0.05$).

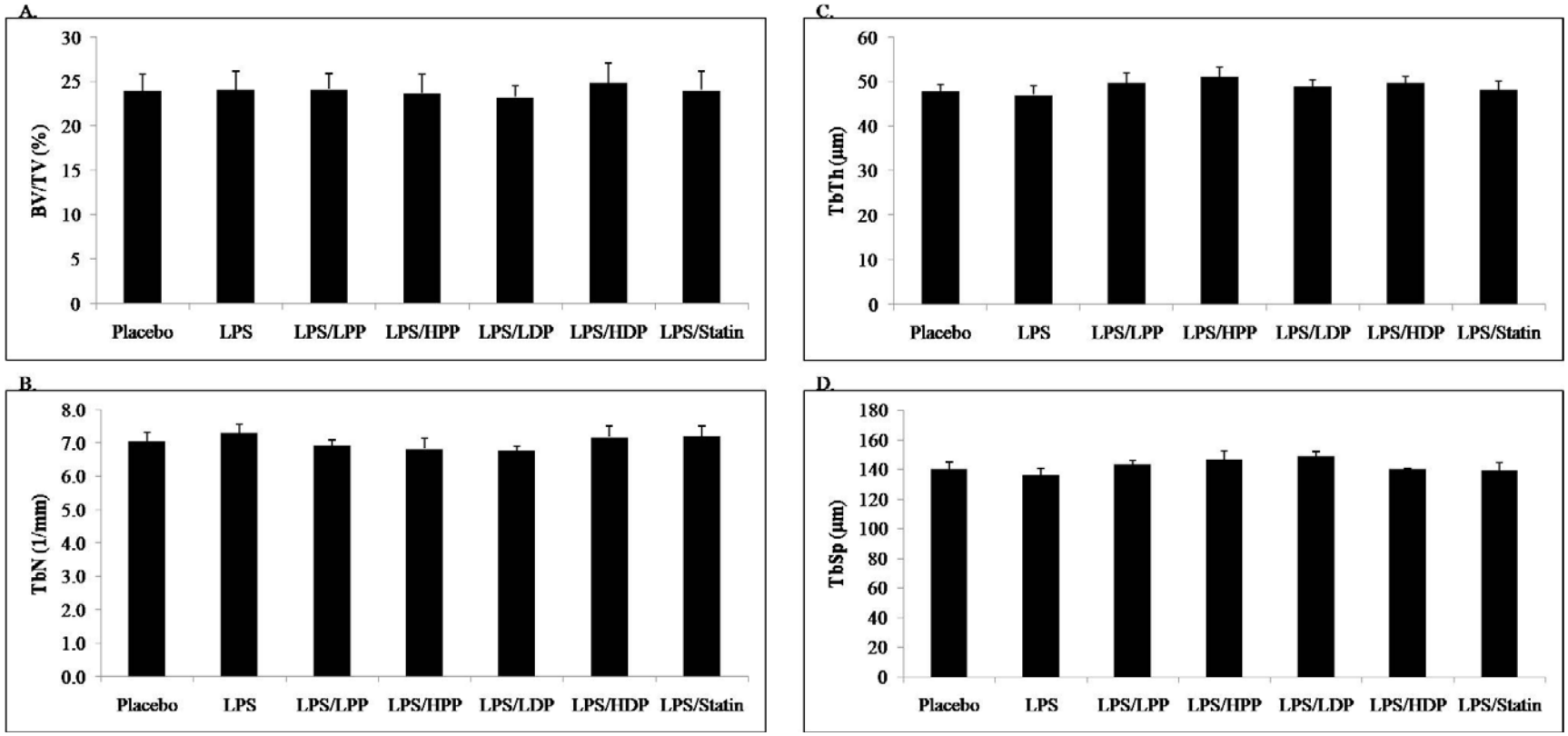


Figure 4. 2 week microarchitectural parameters of trabecular bone in the proximal tibial metaphysic. Trabecular bone parameters include (A) bone volume/total volume (BV/TV), (B) trabecular number (TbN), (C) trabecular thickness (TbTh) and (D) trabecular separation.

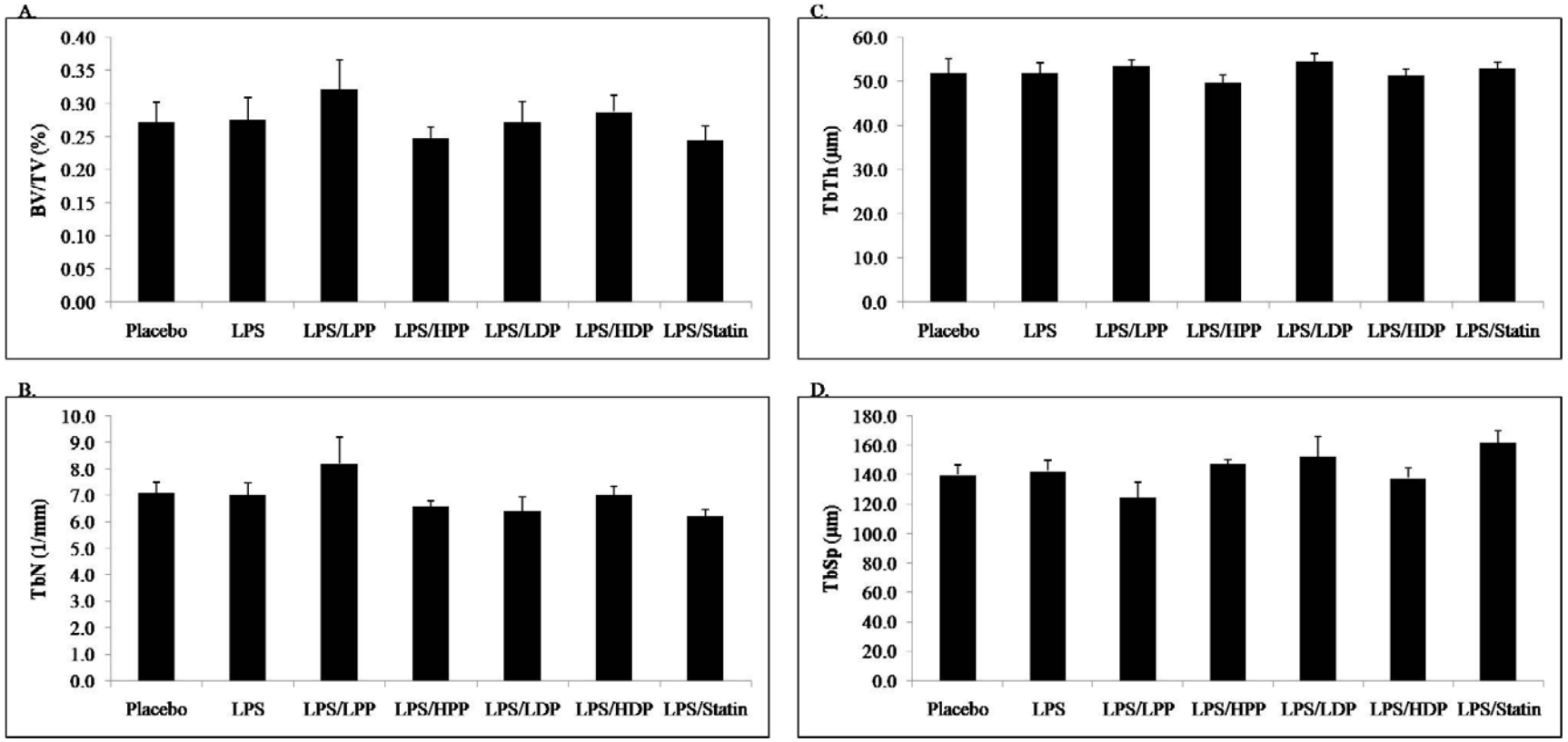


Figure 5. 6 week microarchitectural parameters of trabecular bone in the proximal tibial metaphysisSix week microarchitecture parameters of trabecular bone observed in the proximal tibia including (A) bone volume/total volume (BV/TV), (B) trabecular number (TbN), (C) thickness (TbTh), and (D) separation.

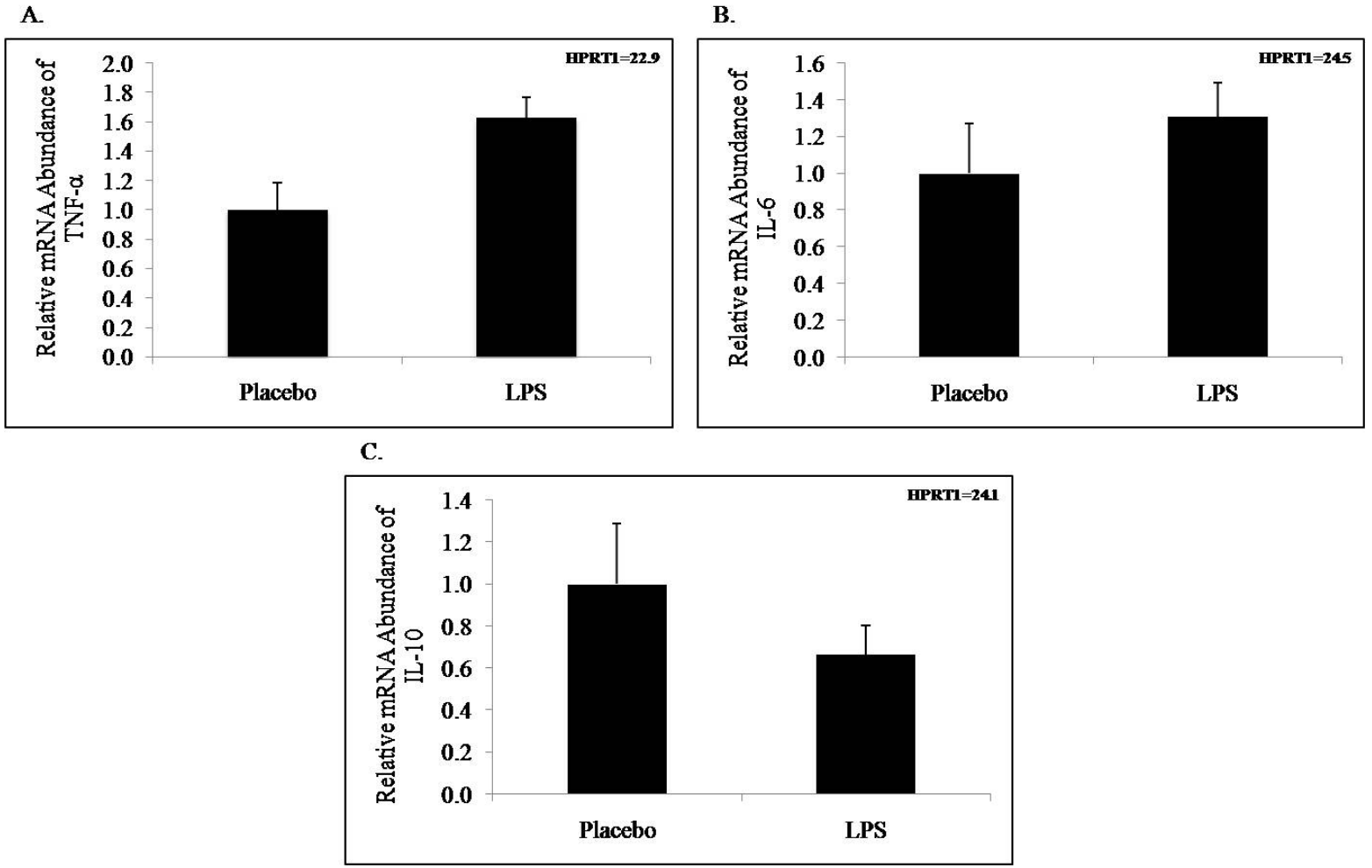


Figure 6. Gene expression of inflammatory mediators gene expression of the pro-inflammatory mediators (A) tumor necrosis factor (TNF)-α, and (B) interleukin (IL)-6, as well as the anti-inflammatory cytokine (C) IL-10 at baseline. Bars represent the mean SE for each treatment group.

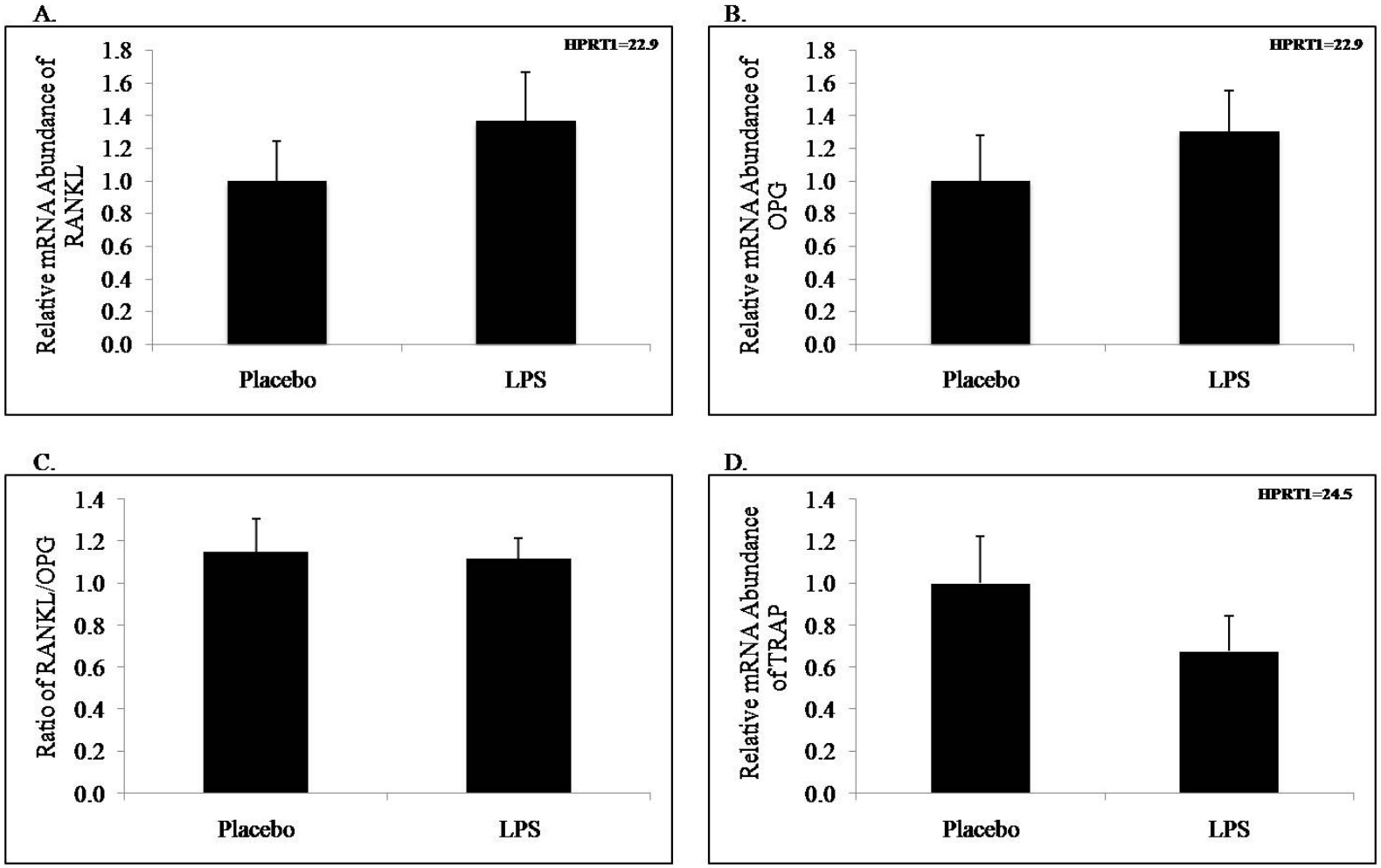


Figure 7. Relative gene expression of key genes involved in bone resorption; (A) receptor activator of NF-κB ligand (RANKL), the decoy of RANKL, (B) osteoprotegerin (OPG), the ratio of (C) RANKL/OPG, and (D) tartrate resistant acid phosphatase (TRAP). Bars represent the mean SE for each treatment group.

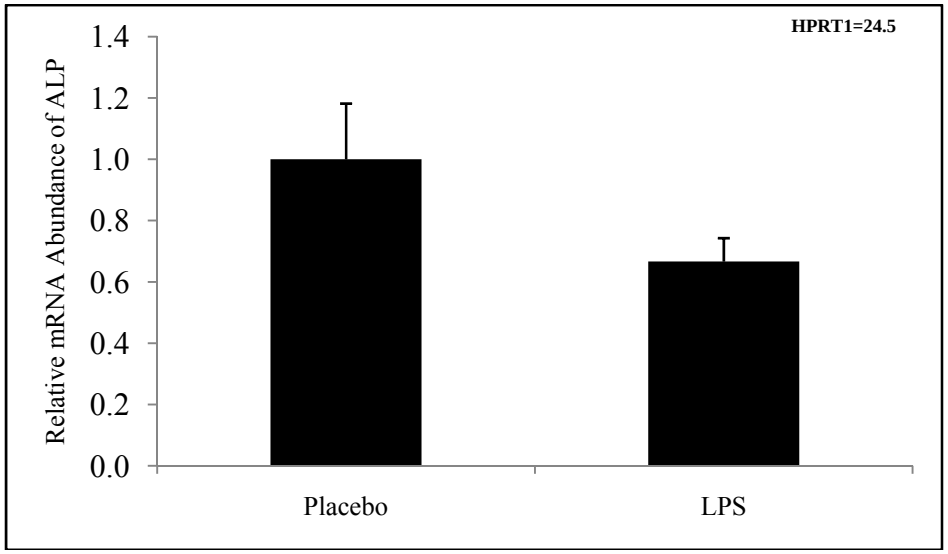


Figure 8. Bone formation was analyzed at baseline by assessing the gene expression of alkaline specific phosphatase (ALP). Bars represent the mean SE for each treatment group.

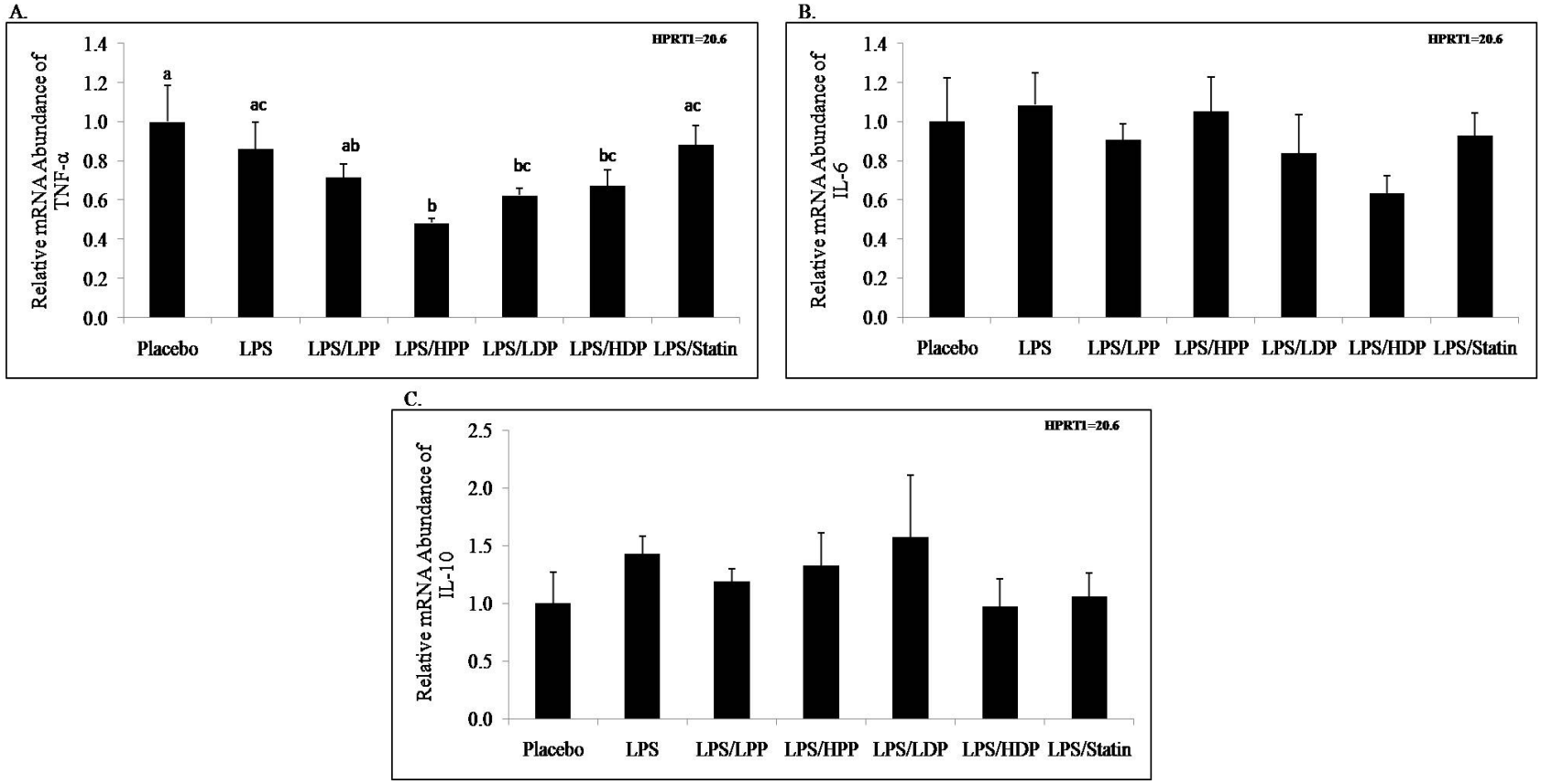


Figure 9. Inflammatory mediators at 2 weeks post dietary treatments, gene expression of common mediators involved in inflammation, (A) tumor necrosis factor (TNF)-α, and (B) interleukin (IL)-6, and (C) IL-10. Bars represent the mean SE for each treatment group. Bars that share the same superscript letter are not statistically different from each other ($p<0.05$).

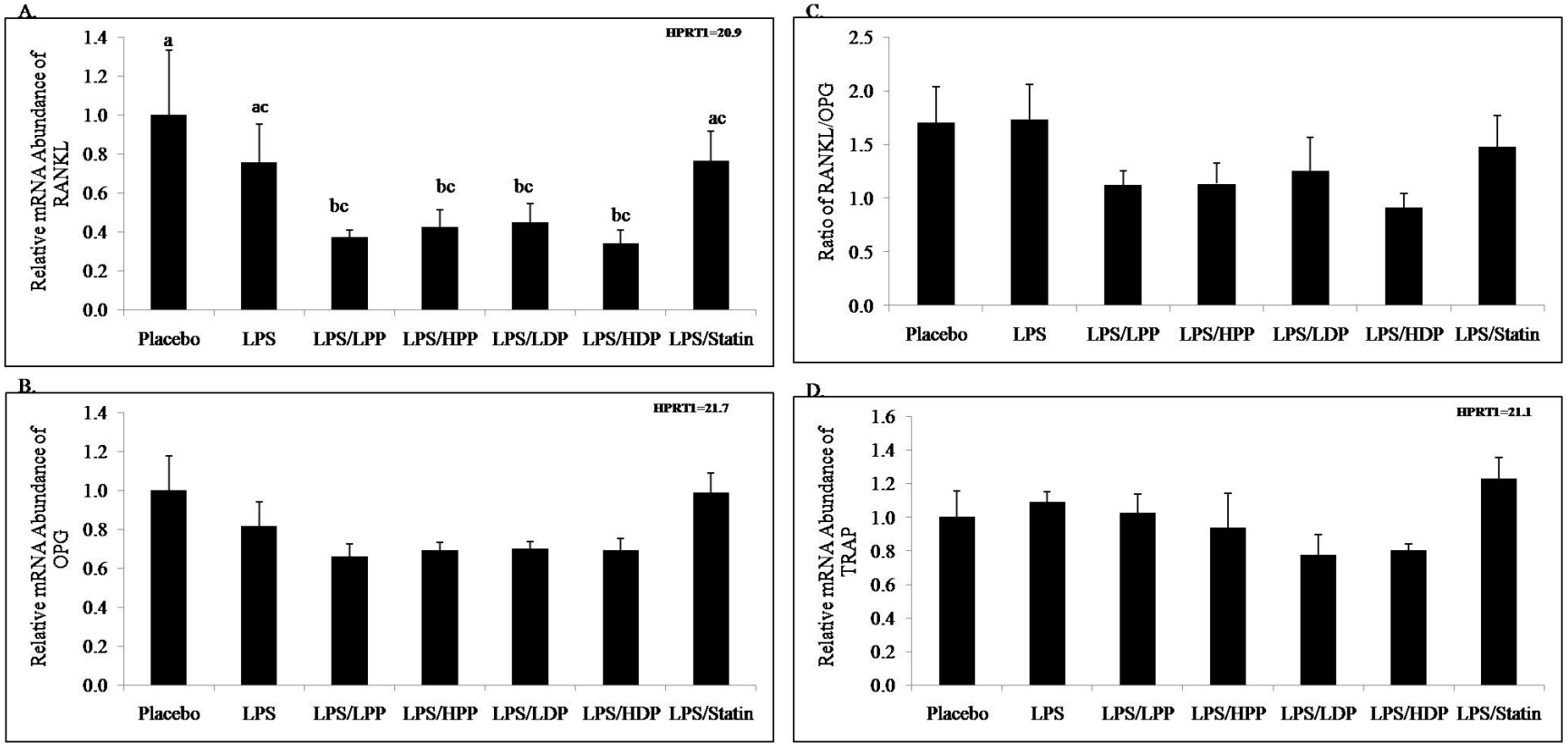


Figure 10. Alterations in gene expression following 2 weeks of dietary treatment; (A) receptor activator of NF-κB ligand (RANKL), the decoy of RANKL, (B) osteoprotegerin (OPG), the ratio of (C) RANKL/OPG, and (D) tartrate resistant acid phosphatase (TRAP). Bars represent the mean SE for each treatment group. Bars that share the same superscript letter are not statistically different from each other ($p<0.05$).

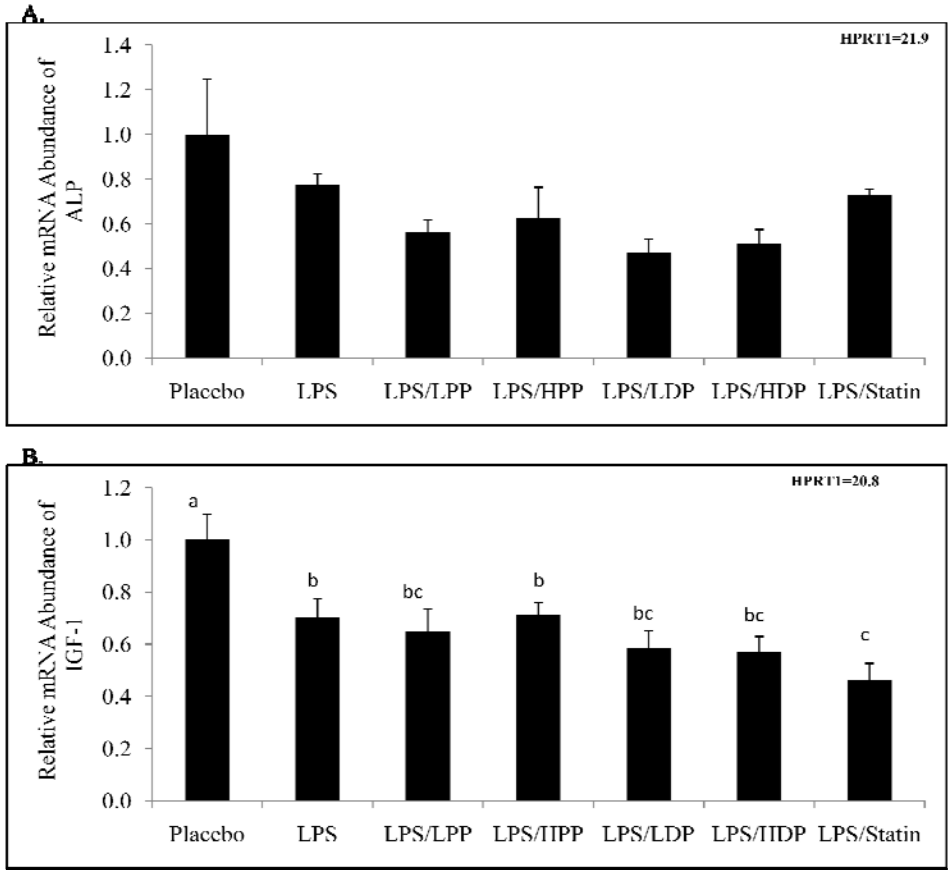


Figure 11. Expression of key genes in bone formation at 2 weeks following dietary treatments by assessing the gene expression of (A) alkaline specific phosphatase (ALP) and (B) insulin like growth factor (IGF)-1. Bars represent the mean SE for each treatment group. Bars that share the same superscript letter are not statistically different from each other ($p<0.05$).

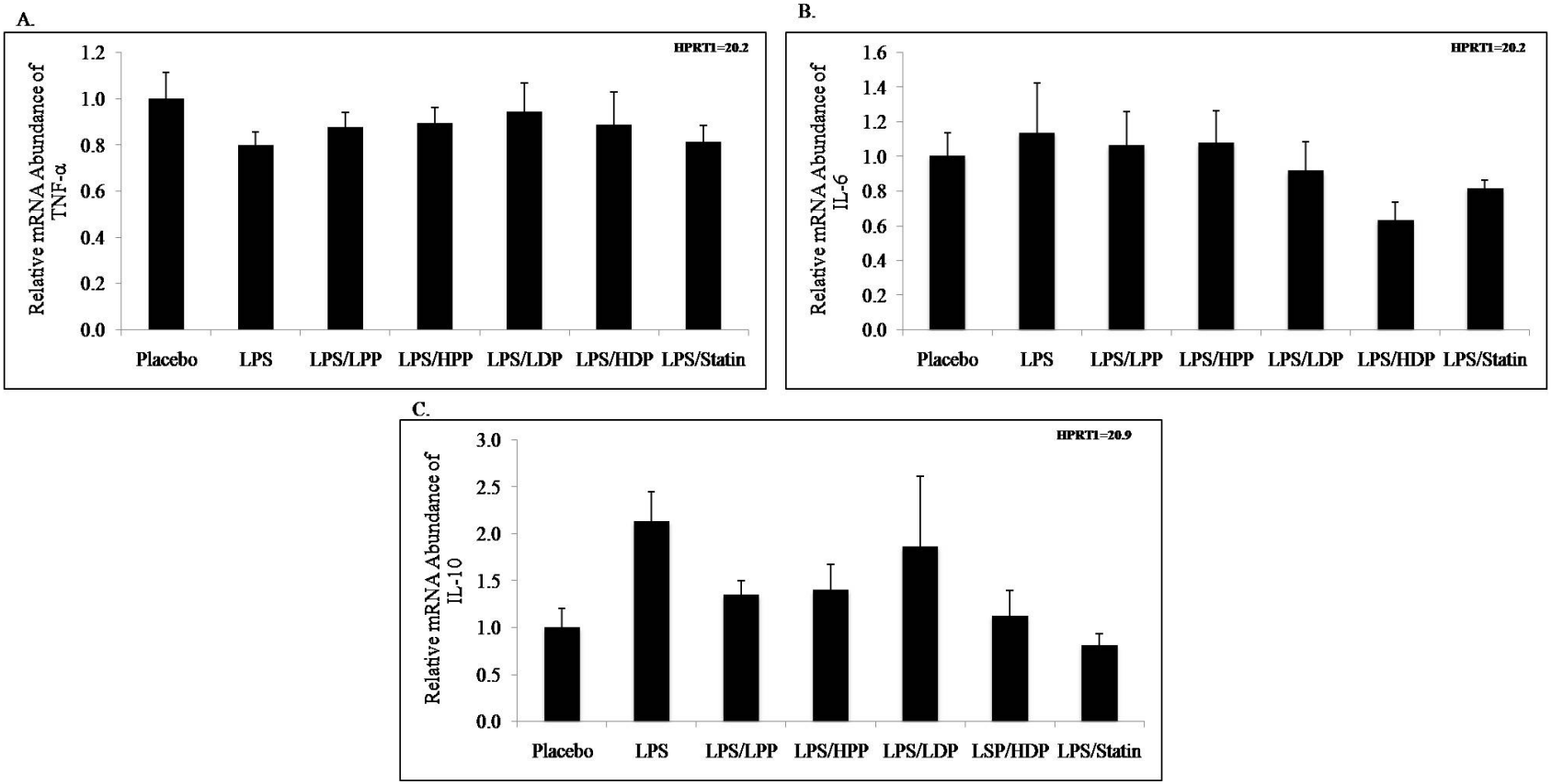


Figure 12. Inflammatory mediators at 6 weeks post dietary treatments, gene expression of common mediators involved in inflammation, (A) tumor necrosis factor (TNF)-α, and (B) interleukin (IL)-6, and (C) IL-10. Bars represent the mean SE for each treatment group

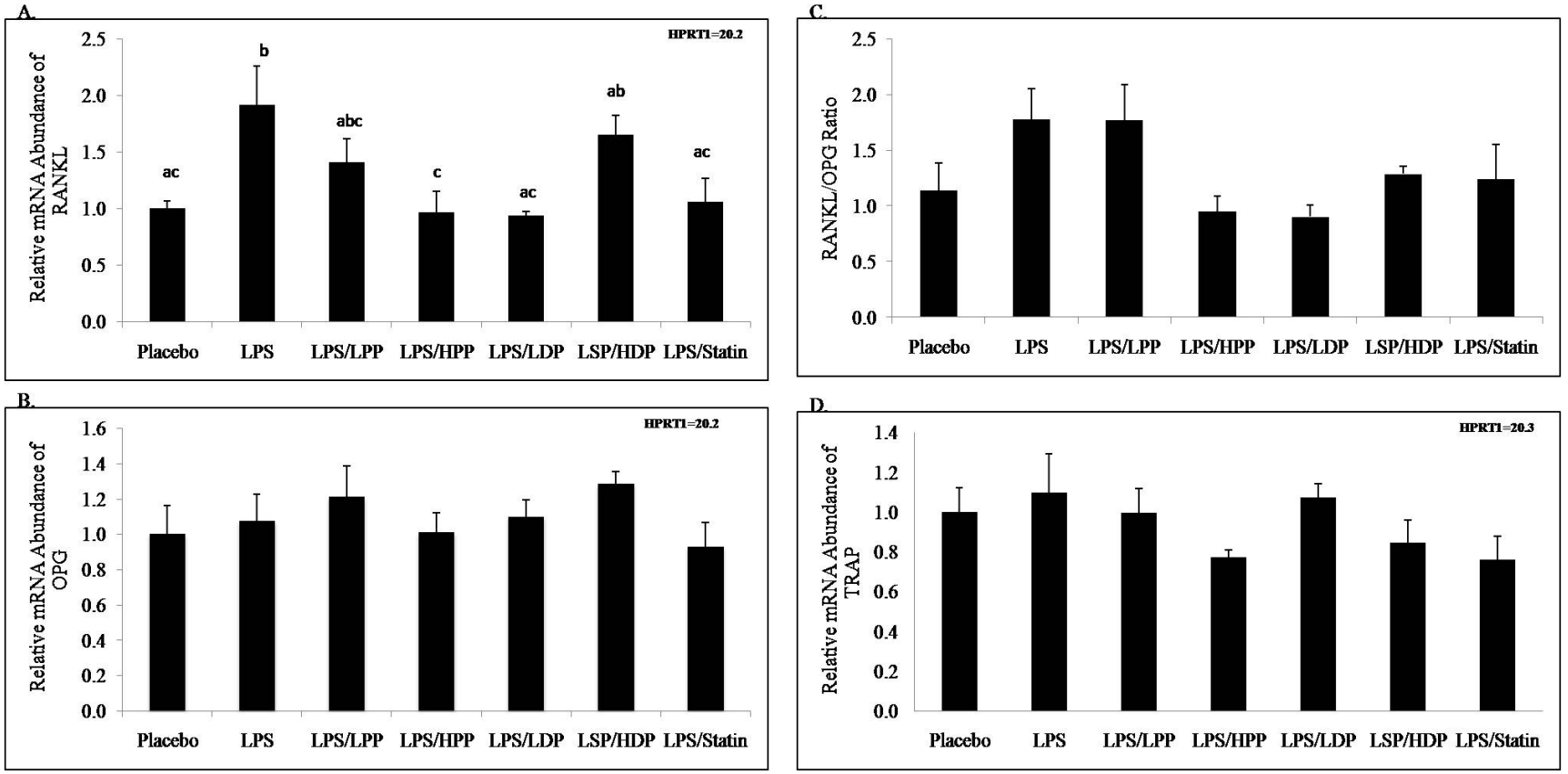


Figure 13. Alterations in gene expression following 6 weeks of dietary treatment; (A) receptor activator of NF-κB ligand (RANKL), the decoy of RANKL, (B) osteoprotegerin (OPG), the ratio of (C) RANKL/OPG, and (D) tartrate resistant acid phosphatase (TRAP). Bars represent the mean SE for each treatment group. Bars that share the same superscript letter are not statistically different from each other ($p<0.05$).

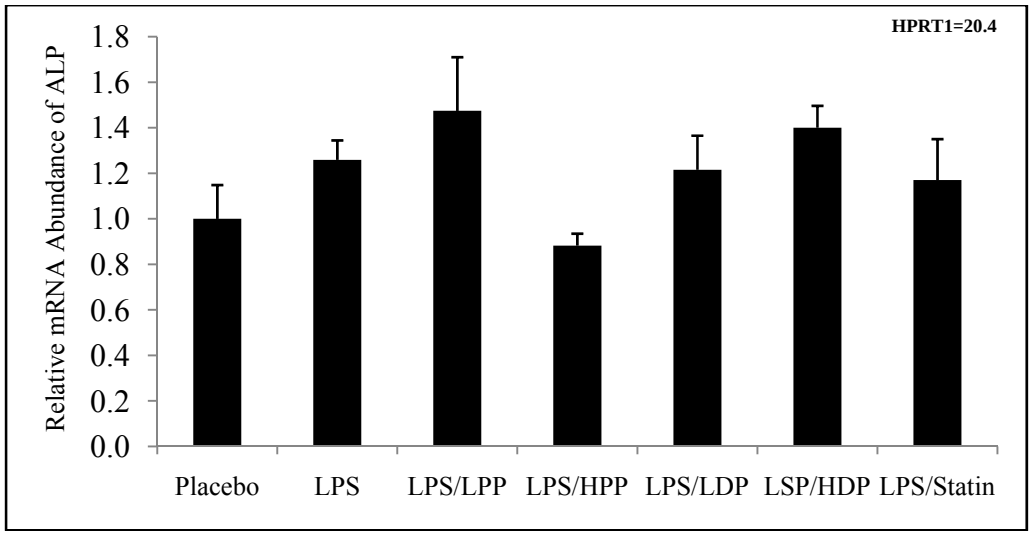


Figure 14. Gene expression of alkaline phosphatase (ALP) following 6 Weeks of dietary treatment. Bars represent the mean SE for each treatment group.

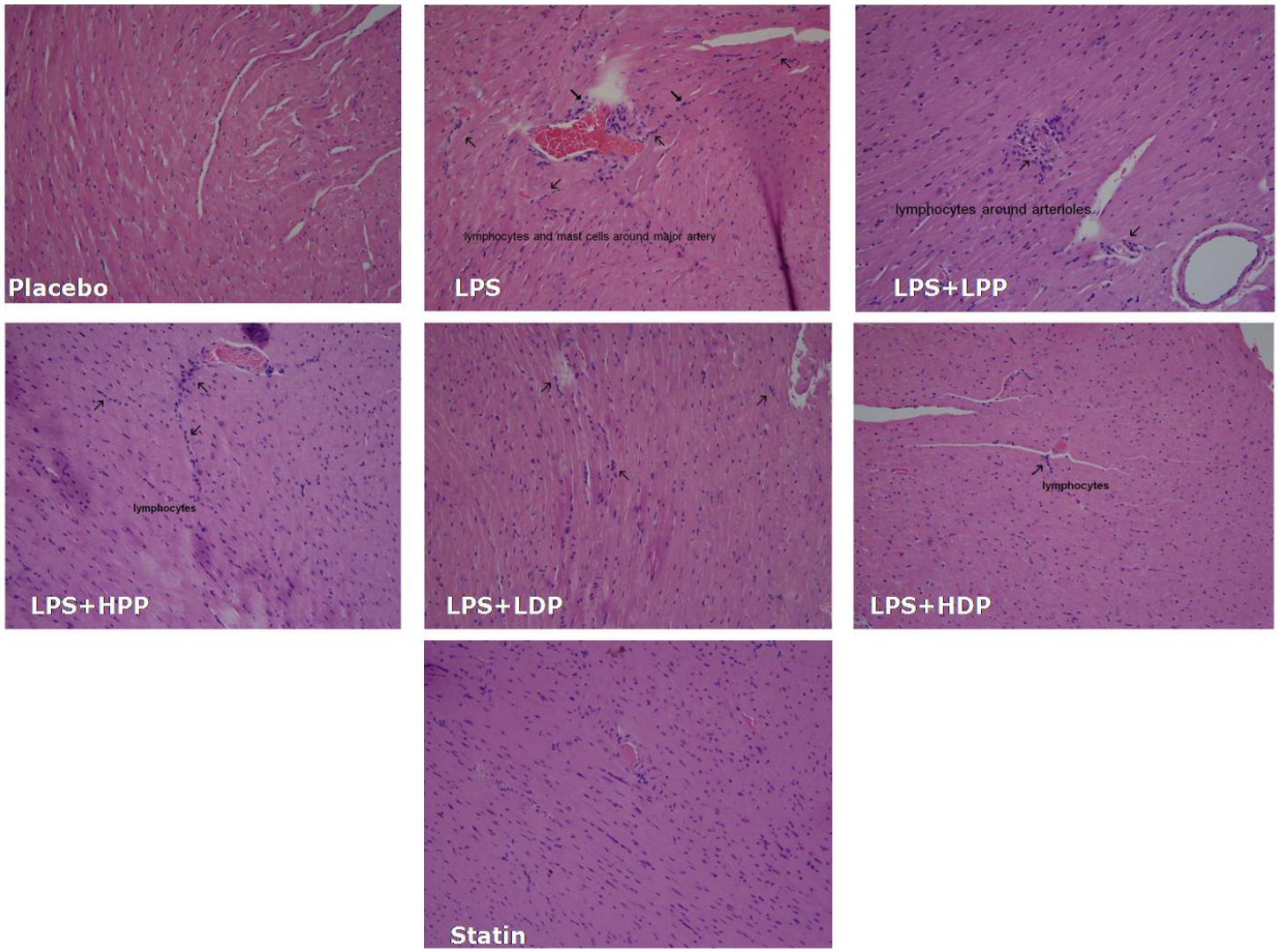


Figure 15. Histological evaluation of cellular infiltration of the myocardium 6 weeks after dietary treatments

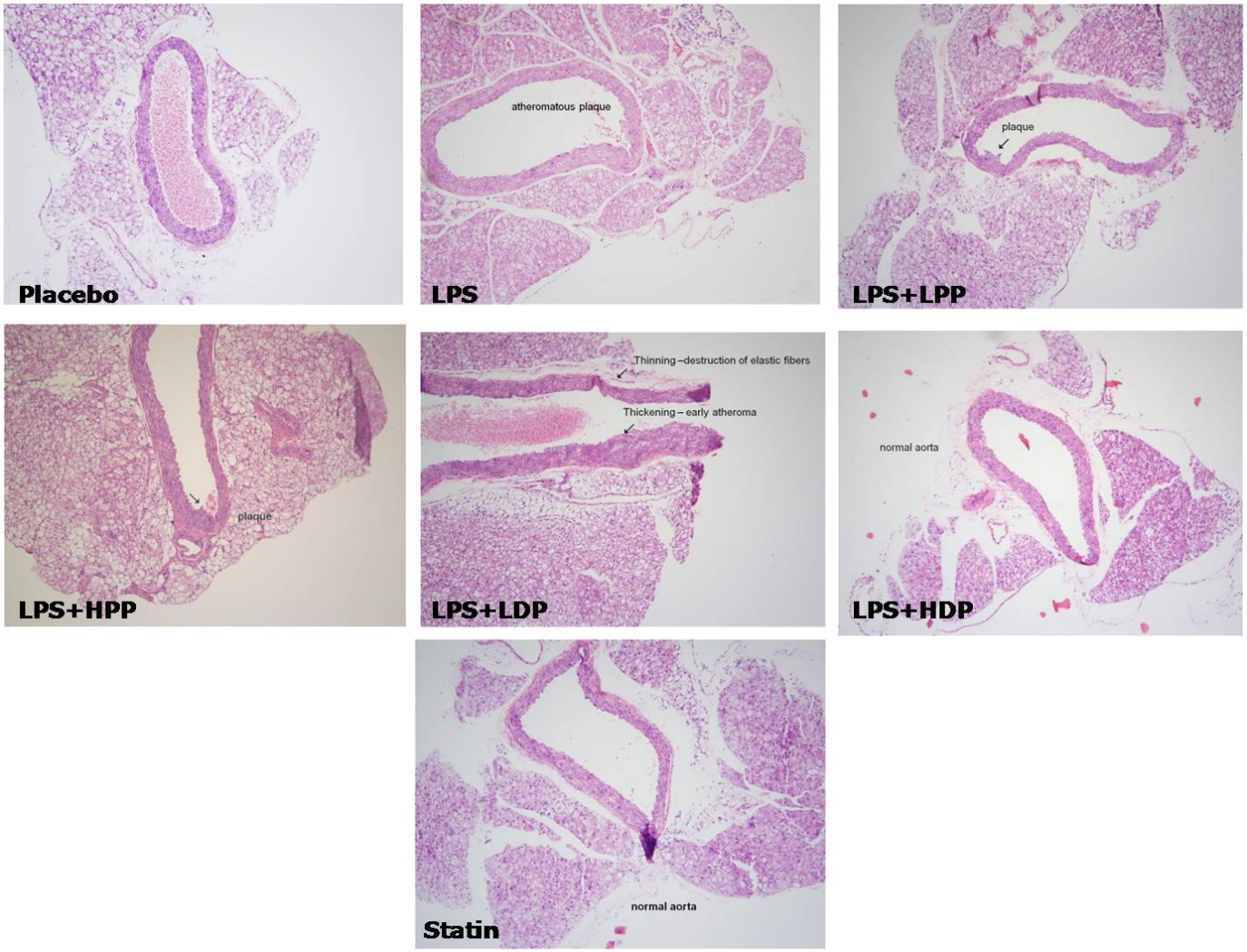


Figure 16. Histological evaluation of the aorta 6 weeks after dietary treatments.

CHAPTER V

DISCUSSION

The purpose of the current study was to determine if dried plum's polyphenols are able to reverse the skeletal and vascular pathology previously observed in a model of chronic low-grade inflammation (21, 22). We have demonstrated that this model of chronic low grade inflammation induces bone loss of the tibia and femur, as indicated by decreased BMD, and compromised trabecular bone microarchitecture (21, 46). In the present study, however, we were unable to reproduce these changes in tibial BMD, or trabecular microarchitectural parameters at baseline, and 2 and 6 weeks after dietary treatments. Based on these results, we conclude that the LPS pellet did not produce inflammation-induced bone loss at the skeletal site tested as previously indicated. A potential explanation for this is the fact that in this study only one skeletal site (i.e. the tibia), was used for the assessment of bone quality, as compared to the other studies where multiple sites had been used (21, 46). Based on the results of previous studies (21, 46), we anticipated that the tibia was a reasonable site to assess the skeletal changes, and had allocated other long bone specimens for other analyses. By limiting ourselves to this one site, we may have induced an inflammatory more apparent responses at other sites (i.e. femur or vertebra), but were unable to evaluate.

In the current study one of the objectives was to determine the mechanism by

which chronic low grade inflammation induces bone loss and cardiovascular pathology. In a prior study (23), 30 days after pellet implantation produced a significant increase in the pro-inflammatory cytokine, TNF-α protein expression of the tibia. Therefore, we expected that in the current study that LPS-treated animals' TNF-α expression would be increased in the bone at the transcriptional level. However, at baseline (i.e. 4 weeks post LPS pellet implant) there was no change in the relative TNF-α RNA abundance compared to the placebo group. Gene expression of IL-6 was also assessed, given that this pro-inflammatory cytokine is up-regulated during states of inflammation and may remain up for extended periods of time (84), however, no differences were observed in groups receiving the LPS compared to the placebo pellets. At 2 weeks post dietary treatment, the placebo and LPS control did not have different levels of TNF-α gene expression. Even though similar levels of TNF-α in the placebo and LPS group was not expected, the high dose of polyphenols was able to attenuate TNF-α expression compared to placebo and LPS controls. Both doses of dried plum decreased the gene expression of TNF-α compared to the placebo control. Due to dried plum's high polyphenol content (31) and the fact that polyphenols are known to possess potent anti-inflammatory properties (151), the dietary treatment's ability to down-regulate TNF-α production seems in line with previous findings (23).

This trend did not continue at 6 weeks as there was no difference in TNF-α expression, or any other cytokines. Although we did not observe the expected results with regards to the LPS pellet, dried plum's polyphenols and the whole fruit were able to down-regulate TNF-α to some extent, which further supports the potential potency of this dietary intervention.

To determine if dried plum's polyphenols were acting via anti-resorptive pathways, RANKL gene expression was studied at 2 weeks post treatment. The placebo and LPS groups on the control diet demonstrated no alterations in RANKL gene expression. However, the low and high dose of dried plum's polyphenols and the low and high doses of whole fruit dried plum tended to decrease RANKL expression compared to the placebo control. Although this observation did not reach the level of statistical significance, it seemed as though dried plum and its polyphenols were able to exert some beneficial effect on RANKL gene expression. After 6 weeks of dietary treatment dried plum and its polyphenols did exert beneficial effects on RANKL gene expression. In the LPS-control cohort, RANKL gene expression was up-regulated compared to the placebo controls. The groups receiving the high dose of dried plum's polyphenols and the low dose of dried plum were able down-regulate this response. Previous studies have suggested that dried plum's polyphenols can attenuate RANKL gene expression (34, 36), and our results not only support this conclusion, but also indicate the possibility that dried plum's polyphenols are able to suppress bone resorption.

In addition to the lack of an effect of LPS on bone structural properties and gene expression and the limited response to dried plum and its polyphenols, it should also be noted that alterations at the tissue level were not observed with the pharmacological agent, simvastatin. We had chosen to use simvastatin as a positive control in this study based on evidence that this pharmacological agent has known anti-inflammatory properties (27) and exerts beneficial effects on the skeletal (26) and cardiovascular systems. There were no alterations observed in the statin group other than its ability to

down-regulate IGF-1, which was not expected based on previous studies (27). We anticipated that simvastatin would increase BMD or BV/TV, and up-regulated ALP which is associated with bone formation (29). At the very least we expected simvastatin to down-regulate TNF-α and IL-6 (27, 30), however this was not observed. Due to the lack of response by the inflammatory mediators, TNF-α and IL-6, it seems as though we were unable to induce chronic-low grade inflammation in the bone to the extent we had in prior studies.

A potential issue that should be addressed is the composition of the bone specimen (i.e. whole bone vs flushed bone or bone marrow) used for the evaluation of mRNA may have impacted the real time-PCR results (152). In the present study we used whole femurs to extract RNA. Some studies (152, 153) have used either the distal metaphyseal region of the femur or flushed bone, while others have used whole bone specimens. Although the same bone is used, it is apparent that the presence of bone marrow may dilute the findings relative to genes expressed specifically by osteoclasts and osteoblasts. However, the fact that gene expression levels for TNF-α and IL-6 were not altered combined with the fact that skeletal changes were not observed suggests that this may not be the case.

Although the skeletal system did not seem to respond as expected, vascular pathology was observed in the aorta and hearts of these specimens. Histological staining of the myocardium after 2 weeks of dietary treatment showed that the high dose of dried plum and statin therapy were able to decrease the frequency of lymphocyte infiltration, which is indicative of microvascular disease. After 6 weeks of treatment all dietary interventions were able to totally prevent the large and small artery narrowing induced by

LPS of such vessels and reduced PMN infiltration. Lymphocyte infiltration was decreased in LPS model after 6 weeks of treatment with all dietary treatments. These results suggest that in this study the cardiovascular system was more responsive to the LPS and thus the dietary treatments compared to the skeletal system

CHAPTERVI

CONCLUSIONS

Summary of Findings

Several population-based studies have indicated that a relationship exists between the pathophysiology of osteoporosis and cardiovascular disease. Chronic elevation of inflammatory mediators, such as tumor necrosis factor (TNF)-α and members of the TNF receptor superfamily of proteins such as the RANKL and its soluble decoy receptor, OPG, have been proposed as playing a pivotal role in concurrent osteoporosis and atherosclerosis. Previously, our laboratory has shown that dietary supplementation with dried plum and its polyphenols down-regulate inflammatory mediators such as TNF-α and RANKL *in vitro* and in models of gonadal hormone deficiency (35, 36). The aim of the current study was to induce simultaneous occurrence of bone loss and vascular pathology by chronic low grade inflammation, and to determine the protective effect dried plum's polyphenols exert *in* vivo. This study utilized 12-wk-old C57BL/6 male mice (n=192) that were implanted with pellets designed to deliver either 0.0 or 0.1 mg LPS/kg bw/d and randomly assigned to one of the following dietary treatments: Placebo-control (AIN-93M) diet, LPS Control , control diet supplemented with low (LDP) or high dose dried plum (HDP) (low = 5% or high = 25%, w/w DP added), control diet with

comparable dose of polyphenolic compounds as low (LPP) and high dose dried plum (HPP), or simvastatin (Statin) as a positive control (25 mg/kg diet). All dietary treatments initiated after the 4 week period to induce bone loss and vascular disease and were maintained for either 2 or six weeks. We have reported that no inflammation-induced changes in bone mass, trabecular or cortical microarchitecture, and bone biomechanical properties at baseline, 2 or 6 weeks were observed in this study. However, in the cardiovascular system we did observe vascular changes indicative of microvascular disease and that after 6 weeks of dietary intervention dried plum's polyphenols were able to protect from observed pathology.

Conclusions

Hypothesis: Polyphenols extracted from dried plum will reverse the bone loss and vascular pathology induced by chronic low grade inflammation by reducing key inflammatory mediators involved in the pathophysiology of osteoporosis and CVD.

Based on the results of this study we reject our proposed hypothesis due to the fact that we were unable to observe bone loss in any of our time points using the LPS pellet model. Our results do suggest, however, that the LPS pellet did induce vascular pathology, and histology revealed that all of our treatment groups were able to protect against this induction. These findings suggest that in this study the cardiovascular system was more responsive to the LPS and thus the dietary treatments compared to the skeletal system.

Recommendations for Further Research

Although we were unable to demonstrate that dried plum's polyphenols reversed skeletal deterioration in response to chronic inflammation *in vivo*, this model may have induced bone loss at another site. A similar study should be designed to test multiple sites at which bone loss may occur (i.e. spine, femur), and could therefore truly determine the degree dried plum's polyphenols had an effect. Furthermore, there remains the possibility that the pellet preparation did not allow for the appropriate delivery of the dose of LPS. This statement is made based on the fact that there was not response in the bone and even the cardiovascular changes were not as pronounced as we have previously observed. However, we are unable to determine if the pellets were problematic at this point. If in fact, there have been alterations in LPS (or other contaminate) dose, this would have dramatically affected our results. A method to pre-test the response may prevent this from occurring in the future. One of the important features of an animal model system is that it provides reproducible results.

The possibility that dried plum's polyphenols are able to protect against vascular pathology may open many doors for additional research to begin to assess the practicality of such alternative treatment options. Different combinations of foods rich in antioxidants, combined with lower doses of pharmacological agents may be achievable, and offer patients the same beneficial effects without undesirable side-effects. Such studies evaluating the efficacy of dried plums and their bioactive components alone and in combination with low dose pharmacological interventions warrant further investigation.

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APPENDICES

The University of Oklahoma
Health Sciences Center
Institutional Animal Care and Use Committee

July 12, 2006

Brenda J. Smith, Ph.D.
Department of Surgery
ORI - 349

Re: IACUC Protocol #: 06-081
Title: Anti-Inflammatory Properties of Compounds in Dried Plums
Funding Source: OCAST

Dear Dr. Smith:

Your revised version of the above mentioned protocol was reviewed and approved by the Institutional Animal Care and Use Committee on July 12, 2006.

Please note that any changes to the protocol must be submitted to the IACUC prior to being implemented. Also, the addition of any new investigators or animal handlers will need to be communicated to the IACUC in the form of an addendum request.

Sincerely,

Philip A. McHale, Ph.D.
Chairman, Institutional Animal Care and Use Committee

940 Stanton L. Young Blvd., Room 207 · Oklahoma City, Oklahoma 73190 · (405) 271-7381 · Fax (405) 271-7382

Figure 16. The bone turnover mediator TRAP was assessed using Elisa kit on mouse serum (R&D Systems and IDS Dianostics) at baseline (A), 2 weeks (B), and 6 weeks (C)

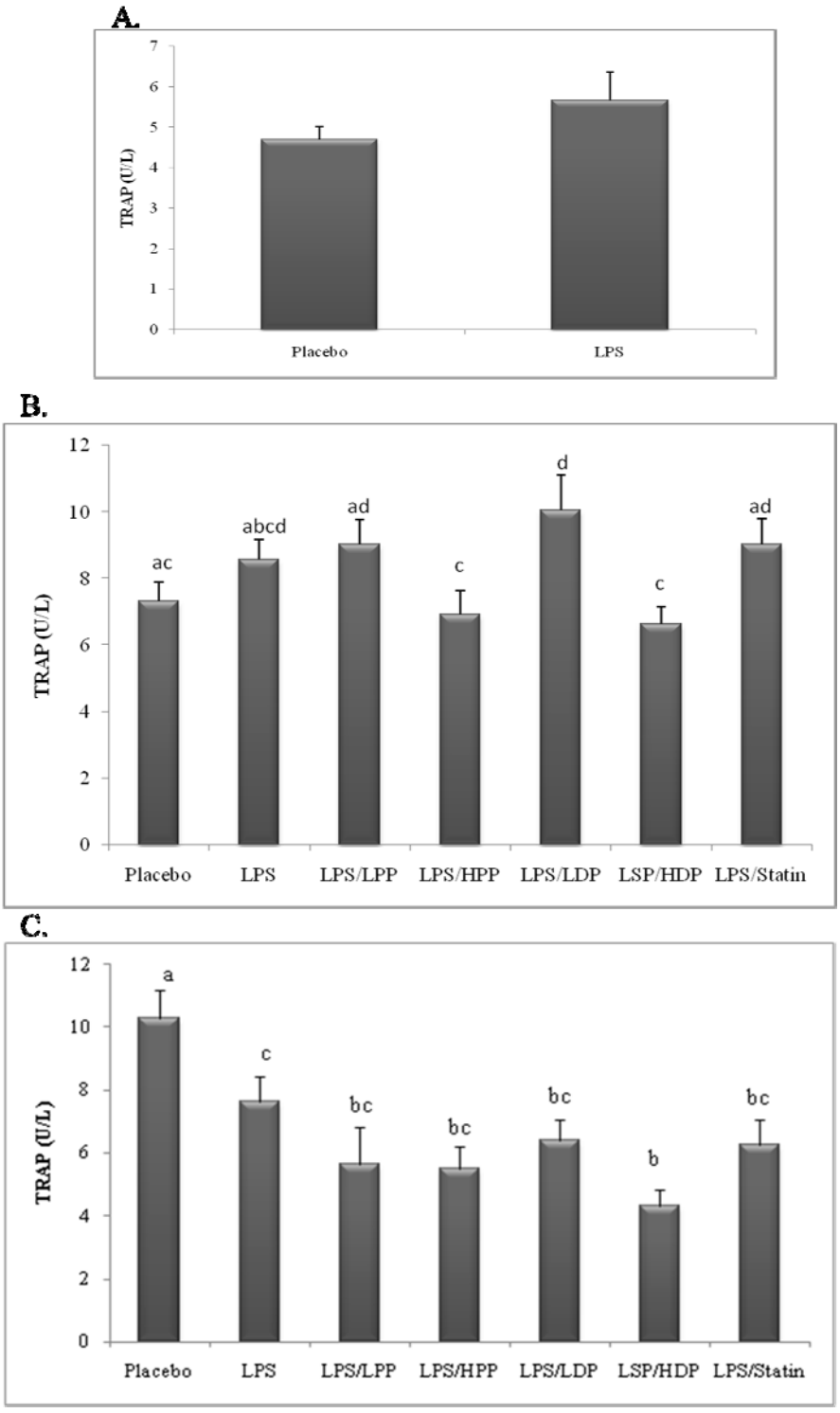
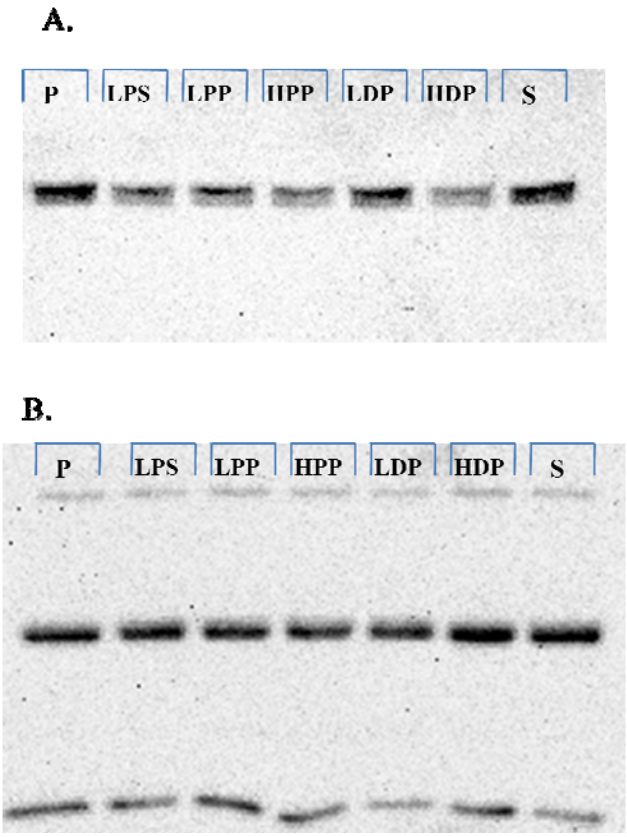


Figure 17. A portion of the liver was used to extract protein to be used to asses any observable alterations in HMG-CoA Reductase. A small portion of the liver was homogenized using a VWR Power MAX homogenizer and lysis buffer. Microsomal protein was isolated by centrifugation. The Beckman Coulter DU800 spectrophotometer was used to determine protein concentration and SDS-PAGE and Immunoblot was performed. Anti-HMG CoA reductase antibody (Millipore, Temecula, CA) was used for incubations to determine protein level of HMG-CoA reducatse among groups (B), anti-actin antibody was used as the control (A).



VITA

Elizabeth Rendina

Candidate for the Degree of

Master of Science

Thesis: REVERSAL OF INFLAMMATION-INDUCED BONE LOSS AND VASCULAR PATHOLOGY BY DRIED PLUM'S POLYPHENOLS

Major Field: Nutritional Sciences

Biographical:

Personal Data: Bone in Germany on October 4, 1984, to Carmela and Joseph Rendina.

Education: Graduated from Eisenhower High School, Lawton, OK in May 2003; received Bachelor of Science degree in Biochemistry from Oklahoma State University, Stillwater, OK in May 2007.

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Name: Elizabeth Rendina Date of Degree: May, 2009

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: REVERSAL OF INFLAMMATION-INDUCED BONE LOSS AND VASCULAR PATHOLOGY BY DRIED PLUM'S POLYPHENOLS

Pages in Study: 97 Candidate for the Degree of Master of Science

Major Field: Nutritional Sciences

Scope and Method of Study:

Recent studies have indicated that a relationship exists between the pathophysiology of osteoporosis and cardiovascular disease. Chronic elevation of inflammatory mediators, such as tumor necrosis factor (TNF)-α and members of the TNF receptor superfamily of proteins such as the ligand for receptor activator for nuclear factor-κB (RANKL) and its soluble decoy receptor, osteoprotegerin (OPG), have been proposed as playing a pivotal role in concurrent osteoporosis and atherosclerosis. Previously, our laboratory has shown that dietary supplementation with dried plum and its polyphenols down-regulate inflammatory mediators such as TNF-α and RANKL *in vitro* and in models of gonadal hormone deficiency. The aim of the current study was to induce simultaneous occurrence of bone loss and vascular pathology by chronic low grade inflammation, and to determine the protective effect dried plum's polyphenols exert *in* vivo. This study utilized 12-wk-old C57BL/6 male mice (n=192) that were implanted with pellets designed to deliver either 0.0 or 0.1 mg LPS/kg body weight/d and randomly assigned to one of the following dietary treatments: Placebo-control (AIN-93M) diet, LPS (Control), control diet supplemented with low (LDP) or high dose dried plum (HDP) (low = 5% or high = 25%, w/w DP added), control diet with comparable dose of polyphenolic compounds as low (LPP) and high dose dried plum (HPP), or simvastatin (Statin) as a positive control (25 mg/kg diet). All dietary treatments started after baseline (4 weeks) and were maintained for either 2 or 6 weeks.

Findings and Conclusions:

We reported no inflammation-induced changes in bone mass, trabecular or cortical microarchitecture, and bone biomechanical properties at baseline, 2 or 6 weeks. However, we did observe cardiovascular pathology indicative of microvascular disease in the animals receiving LPS on the control diet. After 6 weeks of treatment, dried plum and its polyphenols were able to attenuate the vascular pathology. The results obtained in this study suggest that in this study the cardiovascular system was more responsive to the LPS and thus the dietary treatments compared to the skeletal system the cardiovascular system may be more sensitive to chronic-low grade inflammation and dietary responsiveness relative to the skeletal system. Further investigation should be carried out to determine if these results are reproducible, and if so, what these findings reveal about inflammation-induced vascular changes.

ADVISER'S APPROVAL: Dr. Brenda J. Smith