EFFECT OF BLUEBERRY POLYPHENOLS ON OSTEOCLAST DIFFERENTIATION AND ACTIVITY

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ABSTRACT

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Bone is a dynamic tissue with osteoblasts and osteoclasts participating concomitantly in its remodeling process. Blueberries are a rich source of polyphenolic compounds and have been shown to be effective in decreasing incidence/symptoms of chronic inflammatory conditions. The purpose of this study was to assess the effect of blueberry polyphenols as a whole on osteoclasts by assessing markers for activity and differentiation. RAW 264.7 murine macrophage cells were cultured, differentiated into osteoclasts and treated with varying blueberry polyphenols doses. Levels of Tartrate Resistant Acid Phosphatases (TRAP), Nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) were determined. Blueberry polyphenols were shown to be effective by decreasing iNOS, COX-2 and TRAP levels. This study indicates that blueberry polyphenols decrease osteoclast activity through multiple mechanisms such as inflammatory mediators and key bone resorption enzymes which in turn cause a decrease in osteoclast function.

Keywords Bone; Osteoclasts; Osteoporosis; RAW 264.7; Macrophage; RANKL

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CHAPTER I

INTRODUCTION

"Good health" is a term that represents many factors to individuals. It not only involves physical health but embodies spiritual, mental, emotional health etc. Above all, humans strive for quality of life with a key aspect of that being physical health. More than 75% of healthcare costs in the United States are due to chronic diseases (Centers for Disease Control and Prevention, 2009). Whereas the previous focus on healthcare was treatment, the new trends are addressing prevention for long term increased quality of life and decreased financial burden. As the year 2014 proceeds, the Patient Protection and Affordable Care Act has been incorporated in the United States. The Act among many other things focuses on preventative services. One of the main strategies of the Affordable Care Act is to "to evaluate, disseminate, and promote effective clinical preventive services" (U.S. Department of Health and Human Services). Therefore, research in lifestyle behaviors that may curtail development of disease is vital to the goals of this Act.

This study addresses prevention of osteoporosis in regards to blueberry polyphenols. Greater than 75 million people are diagnosed with osteoporosis in the United States, Europe and Japan (World Health Organization, 2004). By 2020, it is estimated that 1 in 2 Americans 50 years or older will be at risk for osteoporotic fractures

(Centers for Disease Control and Prevention, 2011). Current U.S. healthcare costs for this condition are estimated to be $19 billion per year (National Osteoporosis Foundation, 2012). Therefore, preventative measures are key to decreasing risk of this devastating disease.

Inflammation and chronic disease go hand in hand. A longstanding association has been identified. Signs of inflammation were first described in 35 AD by Aulus Celsus, a Roman writer, as redness, warmth, swelling, and pain (Granger, Senchenkova, 2010). The definition has now progressed into an acute immune response and a complex system of detrimental long term effects through cytokines, reactive oxygen species and oxidation that lead to chronic diseases. Nutrition, in particular "good nutrition," has become one the most controversial and high topic discussions in relation to disease prevention. Certain foods provide antioxidant/anti-inflammatory effects. Therefore, consumption of these foods is surmised to be vital in disease treatment and prevention.

The polyphenolic content of blueberry polyphenols contains but is not limited to: anthocyanins (malvidin, delphinidin, cyaniding, petunidin, and pelargonidin), chlorogenic acid and quercertin. Various plant polyphenols have been shown to have anti-inflammatory effects such as flavonoids, cathecins and caffeic acids (Quan et al. 2006; Bu et al. 2008). In addition, they have been shown to be beneficial in attenuating factors involved in bone breakdown, the key to development of osteoporosis. Based on this data, it was hypothesized that blueberry polyphenols will dose-dependently decrease Tartrate

Resistant Acid Phosphatase expression in multinucleated cells which will indicate a decrease in osteoclast differentiation and activity. Blueberry polyphenols will also dose-dependently downregulate Nuclear factor of activated T-cells, a key transcription factor in osteoclastogenesis, which will also signify a decrease in osteoclast differentiation. Lastly, blueberry polyphenols will dose-dependently decrease in osteoclasts and osteoclast pre-cursor cells, cyclooxygenase-2 and inducible nitric oxide synthase levels, inflammatory markers of osteoclasts. This will suggest a decrease in osteoclast activity.

CHAPTER II

REVIEW OF LITERATURE

OSTEOPOROSIS

Incidence

Osteoporosis or "porous bone," is characterized as an imbalance in skeletal turnover in which bone resorption exceeds bone formation resulting in skeletal instability (Centers for Disease Control and Prevention, 2011; Teitelbaum, 2000). According to the World Health Organization (WHO), greater than 75 million people in the United States, Europe and Japan are afflicted with osteoporosis (World Health Organization, 2004). It causes more than 8.9 million fractures worldwide per year which represents billions of dollars in rehabilitation and health care cost. Over half of those fractures occur in the Americas and Europe. In June 2014, the National Osteoporosis Foundation released updated data that 10.2 million Americans are afflicted with osteoporosis and 43.4 million have low bone mass (National Osteoporosis Foundation, 2014). The disease is most common in Western society with the highest prevalence among postmenopausal females who are at a 40% risk of osteoporotic fractures, which can often be life threatening (World Health Organization, 2004). The risk for males is approximately half of women's. The Center for Disease Control and Prevention estimates as of 2020, 1 in 2 Americans 50 Years of age or older will be at risk for osteoporotic fractures. This is due to the increase of aged Americans in the United States. By 2020, 25% of the population is projected to

be 65 years or older (Centers for Disease Control and Prevention, 2011). The most common locations for fractures are the hip, spine, forearm and proximal humerus (World Health Organization, 2004). Most countries including the Americas, Europe and South-East Asia have the greatest percentage of fractures in the hip followed by spine. Hip fractures account for the highest morbidity and mortality. In the United States, 20% of seniors who have hip fractures will die within one year due to complications (National Osteoporosis Foundation, 2012). Osteoporosis is considered a "silent disease" because there are no overt symptoms associated with bone loss (Bindu, Banji, Kumar, 2011). Most individuals do not know they have the condition until a fracture occurs. This leaves an individual bedridden or disabled for an extended amount of time inflicting a social and economic burden which results in high costs for treatment and rehabilitation. In Europe and the Americas, it annually accounts for 2.8 million disability-adjusted life years (DALYs) (World Health Organization, 2004). In the United States, osteoporosis is responsible for $19 billion in healthcare costs per year (National Osteoporosis Foundation, 2012). The projected cost for 2025 is $25.3 billion per year. Therefore, it is essential to assess and utilize preventative measures in averting this debilitating disease.

Risk Factors

The lifestyle factors that pose the greatest risk of developing osteoporosis include: excessive alcohol intake, cigarette smoking, certain medication use, physical inactivity and diets low in calcium and Vitamin D (WebMD, 2010). General guidelines for prevention of bone disease include at least 30 minutes of physical activity per day which

incorporates weight bearing exercises. Weight-bearing exercises provide mechanical stress allowing bones to alter their strength by increasing mineral salt deposition and collagen fiber production. Certain nutrients may increase the risk of osteoporosis. High caffeine has recently been shown to increase risk (Choi et al. 2013). Also, a high phosphorus and low calcium diet contributes to decreased bone formation (Linder, 1991). Additional nutritional factors are discussed further in this paper.

Detection

Diagnosis of osteoporosis occurs by measuring bone mineral density (BMD) and is defined as a BMD greater than 2.5 standard deviations from the average value of healthy individuals based on sex (Centers for Disease Control and Prevention, 2011). Osteopenia is considered a milder reduction in BMD of 1 to 2.5 standard deviations (Bu et al. 2008). BMD is most commonly measured using dual energy X-ray absorptiometry (DXA) with the T-score for diagnosis of osteoporosis (World Health Organization, 2004). Ideal DXA is taken of the hip or spine, but the forearm is also appropriate depending on equipment availability (National Osteoporosis Foundation, 2012). DEXA provides low energy radiation, approximately one-tenth of a chest ray (WebMD, 2010). Most standard DXA machines cannot measure BMD of individuals greater than 300 pounds (National Osteoporosis Foundation, 2012). Specialized machines are available for individuals up to 400 pounds but are not readily accessible. Additional screening tests include peripheral analysis such as peripheral dual energy x-ray absorptiometry (pDXA), quantitative ultrasound (QUS) and peripheral quantitative computed tomography

(pQCT). Though an effective method, DXA is not readily available to some areas due to the high cost and has a low sensitivity regarding risk of fractures (World Health Organization, 2004). Fracture risk increases significantly with low BMD but fractures can also occur to those with normal BMD. Additional factors should be taken into account when determining risk of osteoporosis and fractures such as age, sex, prior fracture, family history of fractures, degree of bone turnover and certain lifestyle factors (i.e. physical inactivity, smoking, use of certain medications, diet low in calcium and excessive alcohol intake) (Centers for Disease Control and Prevention, 2011). A common calculation method is the FRAX tool developed by the WHO which estimates an individual's 10 year risk based not only on DXA but also additional risk factors such as lifestyle factors and family history (FRAX). Other methods to detect bone loss include laboratory tests such as serum calcium, thyroid function tests, Parathyroid (PTH) levels and serum Vitamin D (National Osteoporosis Foundation, 2012). These laboratory tests can be used to assess rate of bone loss.

There are two types of osteoporosis, primary and secondary (WebMD, 2010). Primary, often referred to as type 1, is most common and occurs as a result of gradual bone loss with age. Individuals reach peak bone mass at age 30 with a decline occurring after this age. Accelerated bone loss occurs after age 45-50 for males and for women after menstruation discontinues. Though accelerated bone loss occurs earlier for men, once women are post-menopausal the rate will be more rapid putting them at greater risk. In addition, females typically have lower bone mass which results in increased probability

of osteoporosis. Secondary osteoporosis, or type 2, occurs as a result of a medical condition. For example, hyperparathyroidism, hyperthyroidism and leukemia can accelerate bone loss. In addition, long-term use of certain medications can affect bone loss. Medications that can play a role in increasing bone loss include steroids, certain chemotherapeutic drugs, proton pump inhibitors and anti-seizure medications (Centers for Disease Control and Prevention, 2011).

Treatment

Treatment of osteoporosis is based on a number of factors including sex, amount of bone loss and age (National Osteoporosis Foundation, 2012). Two categories of medication are used for treatment: anabolic drugs (increase bone formation) and antiresorptive medications (slow bone breakdown). Anabolic drugs include PTH. Antiresorptive medications include Calcitonin, Estrogen agonists/antagonists and bisphosphonates. Hormone replacement therapy is controversial and no longer being considered primary treatment due to increased risk of breast cancer, cardiovascular disease and stroke in women (Farley, Blalock, 2009). Bisphosphonates are now the main form of treatment; their mechanism of action is through decreasing bone resorption (Kwun, Laufgraben, Gopalakrishnan, 2012). The most commonly available bisphosphonates include alendronate, risedronate, ibandronate and zoledronic acid (intravenous form) (Farley, Blalock, 2009). Aldendronate is the most commonly used because it is inexpensive and effective up to 5 years following cessation of treatment (Kwun, Laufgraben, Gopalakrishnan, 2012). The most common side effects to these

medications include gastrointestinal distress (nausea, vomiting) and esophageal inflammation (Mayo Clinic, 2013). The second line of treatment for those who may not tolerate bisphosphonates are nasal calcitonin and the PTH, teriparatide (Farley, Blalock, 2009). The third line of treatment includes the estrogen receptor modulator, raloxifene for post-menopausal women, and testosterone therapy for males. Raloxifene is associated with increased risk of blood clots (Mayo Clinic, 2013).

In addition to medication, individuals with osteoporosis who have experienced fractures require physical therapy as well as daily resistance exercises to protect the spine, improve posture and balance. Patients are also recommended to increase intake of calcium and Vitamin D through use of supplements (Tortora, Derrickson, 2009; WebMD, 2010). Adherence to medication use has been a topic that has recently been studied; a U.S. study showed only 26% adherence to medication use by patients following diagnosis. Therefore, without proper medication use, therapy and lifestyle changes individuals will not experience positive results in disease treatment.

BONE MORPHOLOGY

Structure

The adult human skeletal system contains 206 bones composing 18% of a human's weight (Tortora, Derrickson, 2009). Bone not only provides structure and support, it also stores minerals (calcium and phosphorus), produces erythrocytes within the red marrow and stores triglycerides in the yellow marrow. Bone is connective tissue that contains an extracellular matrix surrounding cells. The matrix is composed of 25%

collagen fibers, 25% water and 50% crystallized mineral salts. The crystals are formed by hydroxyapatite, the combination of calcium phosphate and calcium hydroxide. Bones store 99% of the body's calcium and aid in maintaining homeostasis by releasing calcium into the blood. The crystals combine with other ions such as magnesium, fluoride, potassium and sulfate, as well as mineral salts (i.e. calcium carbonate). The crystals of the bone provide hardness while the flexibility is based on the amount of collagen. A majority of bone is compact (80%) providing support while some bones also have spongy tissue to provide protection to the bone marrow to allow for hemopoiesis.

Bone tissue is dynamic and constantly in a state of change. Bone modeling is growth until mature height (Mahan, Escott-Stump, 2008). During this time, long bones lengthen and widen. Bone modeling is usually completed in females at age 16-18 and males ages 18-20. Once mature height is reached, bone remodeling begins. Bone remodeling occurs in two phases: bone resorption and bone deposition/formation. There are two main cells involved, osteoblasts and osteoclasts. Osteoblasts are bone-forming cells. Osteoclasts are the multinucleated cells principal in bone resorption (Nesbitt, Horton, 1997).

Osteoblasts

Osteoblasts are formed from mesenchymal stem cells (Ducy, Schinke, Karsenty, 2000). The mesenchymal cells are found within the periosteum and canals that contain blood vessels within the bone (Tortora, Derrickson, 2009). They are similar to fibroblasts and express the same genes. However, one distinctive transcription factor is unique to

osteoblasts, core binding factor alpha 1 (*Cbfa1)* (Pan et al. 2009). It induces osteoblast gene expression in fibroblasts and myoblasts (Ducy, Schinke, Karsenty, 2000). Mice lacking *Cbfa1* not only are deficient of osteoblasts but also osteoclasts because cells of osteoblast lineage are required for osteoclast differentiation (Pan et al. 2009). *Cbfa1* plays a role in osteoblast differentiation and also in the rate of bone formation by controlling expression of *osteocalcin* (Ducy, Schinke, Karsenty, 2000). *Osteocalcin* inhibits osteoblast function and is only expressed in differentiated osteoblasts (Vallet et al. 2011). Osteoblasts produce numerous growth factors that affect differentiation such as insulin-like growth factors (IGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor-beta (TGF-β) and the bone morphogenetic proteins (BMP). There is also evidence of endocrine control by Leptin which plays a role in enhancing bone formation by acting on differentiated osteoblasts (Ducy, Schinke, Karsenty, 2000). Additional hormones that affect osteoblasts include thyroid hormone, insulin, prolactin, estrogen and Vitamin D3.

Osteoclasts

As stated earlier, osteoclasts are the key cells involved in bone breakdown; osteoporosis results from enhanced bone resorption. Therefore, understanding osteoclast formation and mechanism of function is key to determining the disease progression, prevention and treatment. Osteoclasts have hematopoietic origin and form through differentiation of macrophages/monocytes (Teitelbaum, 2000). Osteoblasts or stromal cells must first make contact with macrophages and secrete two molecules essential for

differentiation: macrophage colony-stimulating factor (M-CSF) and RANKL. The receptor for M-CSF on macrophages is c-Fms. c-Fms transcription is regulated by the transcription factor PU.1 contained within all macrophages (Tondravi et al. 1997). PU.1 knockout mice have macrophages but do not foster osteoclasts, which leads to osteopetrosis, enhanced bone development and lack of bone resorption (osteosclerosis) (Teitelbaum, 2000). Though rare in humans, it can occur. In vitro studies require solely RANKL for osteoclast differentiation (Bu et al. 2008). Therefore, RANKL is the primary protein that controls osteoclastogenesis.

BONE REMODELING

Bone Resorption

Bone remodeling begins with bone resorption. Osteoclasts attach to the bone matrix where their plasma membrane folds over to form a "ruffled membrane" (Teitelbaum, 2000). This allows for the transport of vesicles that contain acidifying material. Surrounding the "ruffled membrane" is an extracellular environment composed of various proteins, mainly filamentous actin. This creates an isolated area, or sealed zone, between the osteoclast and the bone which allows for resorption. The area is sealed by filamentous protein (Väänänen, Horton, 1995). Actin, the main protein, forms a band around the ruffled membrane anchored in by podosomes which allow for movement (Chellaiah, Fitzgerald, Alvarez, Hruska, 1998). Other structural proteins that create this isolated environment are vinculin, talin and α-actinin (Teitelbaum, 2000). In order for the osteoclast to attach to bone matrix, integrins on the osteoclast surface must be present

(Ross et al. 1993). Particularly, $\alpha_v\beta_3$ is the predominant integrin on the cell surface that works with binding proteins (i.e. fibrinogen, thrombospondin) to fuse the bone matrix proteins (osteopontin and fibronectin). Osteopontin is a phosphorylated protein of the bone extracellular matrix that is key to anchoring the osteoclast to the matrix (Abu-Amer, Teitelbaum, Chappel, Schlesinger, 1999). In addition, recent studies have revealed another binding protein, Dendritic cell-specific transmembrane protein (D-STAMP) (Choi et al. 2013). Collagen is the main matrix protein. Acidification and collagenases secreted from osteoclasts cause denaturing of the molecule and the $\alpha_v\beta_3$ binding site is exposed (Zhao, Byrne, Boyce, Krane, 1999).

The first step in degradation involves the proton pumps (H^+-ATPase) alongside a Cl^- channel to maintain electroneutrality between the intra and extracellular environment. These channels result in secretion of hydrochloric acid into the bone matrix. H^+-ATPase is a proton pump enzyme that provides trafficking of proteins and vesicles (Voronov et al. 2013). It is composed of 14 known subunits. In vitro studies have shown that a point mutation of the a3 subunit results in increased lysosomal pH causing impaired osteoclastogenesis due to decreased Nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) nuclear translocation. Therefore, H^+-ATPase is key in controlling the intracellular pH which can affect not only osteoclast function but differentiation.

The antiresportive surface on the other side of the cell has an energy-independent $Cl^-/HCO3^-$ exchanger to also maintain intracellular pH (Teitelbaum, 2000). Acidification (pH ~4.5) of the bone matrix is essential to the breakdown of the bone mineral,

hydroxyapatite, an alkaline salt (Blair, Teitelbaum, 1989). The exocytosis of the vesicles is regulated by Rab3, a GTPase located at the periphery of the osteoclast (Abu-Amer, Teitelbaum, Chappel, Schlesinger, 1999). This causes mobilization of bone mineral which allows proteolytic enzymes, such as cysteine proteases, to break down the bone (Teitelbaum, 2000, Inui). The primary proteases are Cathepsin K (cysteine protease) and Tartrate Resistant Acid Phosphatase (TRAP). The principle products of resorption are calcium and type I collagen fragments (Nesbitt, Horton, 1997 , Salo). When the bone matrix is broken down, they are endocytosed into the ruffled membrane and transported to the basolateral membrane/antiresorption surface that faces the extracellular space on the opposite side of the cell (Baron et al. 1988). The route for both inorganic and organic degradation products is the same within the osteoclast. The products then diffuse into blood capillaries.

Bone Deposition

Once the osteoclast has resorbed an area of bone matrix, a lacuna is formed that osteoblasts enters to restore new bone (Teitelbaum, 2000). This process takes approximately ten times longer than bone resorption (Ryser, Komarova, Nigam, 2010). The first step is formation of the osteoid matrix by secretion of collagen fibers (Hadjidakis, Androulakis, 2006). Collagen polymerizes to form fibers and bind other matrix proteins (Mahan, Escott-Stump, 2008). Approximately 15% of the osteoblasts remain trapped in the osteoid to form osteocytes that develop canaliculi to permeate the matrix. The final phase is mineralization of the osteoid. Through calcification, the

mineral salts crystallize and the tissue hardens. Osteoblasts also line the bone as flat lining cells.

At a given time, 5% of total bone mass is being remodeled (Tortora, Derrickson, 2009). The turnover for compact bone is 4% annually and 20% for spongy bone. In young individuals, the bone that is resorbed is completely restored. With age, the amount of bone that is replaced is less than the amount that is resorbed. This negative balance is key to bone loss and development of osteoporosis. **KEY FACTORS ASSOCIATED WITH OSTEOCLAST DIFFERENTIATION AND ACTIVITY**

Receptor for Activation of Nuclear Factor Kappa B Ligand

In order for osteoclast differentiation to begin, the RANK/RANKL pathway must be initiated (Zhang, Heulsmann, Tondravi, Mukherjee, Abu-Amer, 2001). The receptor for RANKL on macrophages is RANK which belongs to the TNF receptor family (Teitelbaum, 2000). Receptors of this family recruit TNF receptor-associated factor (TRAF) proteins that activate transcription factors. In particular, RANKL activates TRAF6, which initiates signaling pathways for NF-κB and c-jun N-terminal kinase (JNK) (Ikeda et al. 2004). JNK activates the transcription factor c-Jun. c-Jun then forms complexes with c-Fos called Activator Protein (AP-1) complexes. These complexes are essential for osteoclast formation including activation of the key transcription factor NFATc1 which is discussed below. RANKL also functions to activate phospholipase C (PLC) causing elevation of cystolic free Ca^{2+} concentrations, key to osteoclast differentiation (Komarova, Pereverzev, Shum, Sims, Dixon, 2005). Osteoclasts

intracellular Ca^{2+} levels increase not only through RANKL but also through ovarian cancer G protein-coupled receptor 1 (OGR1), which links extracellular Ca^{2+} to increase intracellular levels. Cytosolic calcium levels also increase by release of intracellular stores such as in the endoplasmic reticulum (Cary et al. 2013).

RANKL is not only secreted by osteoblasts but also T helper 1 lymphocytes (Ikeda et al. 2004). These cells are responsible for secretion of cytokines such as TNF and IL-1. TNF has been shown to increase osteoclastogenesis in RANKL induced cell through the receptor TNFr1 which increases TRAF proteins and RANK expression on osteoclasts. TNF, an inflammatory marker is present with RANKL in inflammatory bone disease. In contrast, Osteoprotegerin (OPG) is a key protein that competes with RANKL for RANK on the macrophages. OPG is produced by osteoblasts and is a glycoprotein and member of the TNF receptor family (Suda et al. 2004; Kutlucan et al. 2012). OPG binds RANKL inhibiting it from binding to RANK on osteoclasts (Aoki, 2013). Therefore, osteoclastogenesis is inhibited. Some factors can increase RANKL production relative to OPG such as acidosis (due to inflammation), PTH and 1,25-dihydroxyvitamin D_3 (Suda et al. 2004; Komarova, Pereverzev, Shum, Sims, Dixon, 2005).

Nuclear Factor of Activated T-Cells, Cytoplasmic 1

As stated earlier, transcription factors for osteoclastogenesis include PU.1, c-fos, c-jun, NF-κB and NFATc1 (Quan et al. 2006). NFATc1 in particular is the key and most highly induced transcription factor in initiation of osteoclastogenesis (Komarova, Pereverzev, Shum, Sims, Dixon, 2005). Believed to be the master gene of osteoclast

transcription, NFATc1 regulates gene expression for various factors such as TRAP, cathepsin K and other proteins involved in osteoclast function (Quan et al. 2006, Voronov et al. 2013). NFATc1 is controlled by Ca^{2+} -activated phosphatase, calcineurin, which dephosphorylates NFATc1 so it can translocate to the nucleus to promote gene expression. As stated earlier, RANKL and OGR1 increase intracellular calcium levels. The elevation of Ca^{2+} results in activation of calcineurin necessary for NFATc1 translocation (Komarova, Pereverzev, Shum, Sims, Dixon, 2005). Calcineurin is stimulated by calmodulin, a group of calcium receptors kinases (Voronov et al. 2013). The genetic regulator of calcineurin and therefore inhibitor of NFATc1 is *Rcan1*. *Rcan1*, or Down syndrome critical region 1 (DSCR1), is an endogenous protein and is upregulated during osteoclastogenesis. This gene has been shown to be elevated in individuals with Down syndrome resulting in craniofacial and skeletal irregularities. In certain conditions, an increase in translocation of NFATc1 to the nucleus can occur, such as extracellular acidosis due to inflammation and the presence of PTH and 1,25-dihydroxyvitamin D_3 (Suda et al. 2004; Komarova, Pereverzev, Shum, Sims, Dixon, 2005).

Tartrate Resistant Acid Phosphatase

TRAP is an iron-containing metalloenzyme in various tissues and is prevalent in osteoclasts because it catalyzes the hydrolysis of bone in acidic conditions (Bu et al. 2008; Nakayama et al. 2011). TRAP belongs to the purple acid phosphatase family and contains a di-iron center (Hollberg, Hultenby, Hayman, Cox, Andersson, 2002).

Particularly, it targets phosphoproteins (Ek-Rylander, Flores, Wendel, Heinegard, Andersson, 1994). Bone phosphoproteins include osteopontin and sialoprotein, which bind the osteoclast to the bone matrix are dephosphorylated in the presence of TRAP. Therefore, TRAP plays a regulatory role as attacher to the bone and migration to the cell surface. TRAP activity is enhanced by capthesin K and can function to produce ROS in a neutral pH (Nakayama et al. 2011; Hollberg, Hultenby, Hayman, Cox, Andersson, 2002). It is unknown if this function plays any role in bone degradation. There are two theories as to osteoclast release of TRAP. It is hypothesized that the TRAP is either secreted directly into the "ruffled membrane" or through transcytotic vesicles. Recent studies support the latter.

Hormones

As previously listed, osteoclast differentiation can be enhanced by a number of factors such as PTH and 1,25-dihydroxyvitamin D_3. PTH plays a role by increasing blood calcium levels causing hypercalcemia (Zhao, Byrne, Boyce, Krane, 1999, Tortora, Derrickson, 2009). PTH also increases calcium resorption in the kidneys and increases formation of calcitriol. These all allow for an increase in blood calcium levels. The counter regulatory hormone to PTH is calcitonin which inhibits osteoclasts and increases bone uptake of calcium. The mechanisms of action of PTH are through osteoblasts to increase osteoclast differentiation, increase production of collagenases and decrease synthesis of collagen (Zhao, Byrne, Boyce, Krane, 1999). The collagenases work on

hypomineralized collagen to prepare for osteoclast attachment and allow proteases from the osteoclast to breakdown the collagen.

Postmenopausal osteoporosis results from estrogen deficiency causing an increase in osteoclast function and decrease in osteoblast activity (Jagger, Lean, Davies, Chambers, 2005). Estrogen is key in females for bone protection. It not only promotes osteoblast differentiation and proliferation, but also affects osteoclasts (Garcia et al. 2013). Estrogen inhibits bone resorption by decreasing resorptive enzymes and promoting osteoclast apoptosis (Sunyer, Lewis, Collin-Osdoby, Osdoby, 1999). The mechanism of action is by upregulating Fas ligan (FasL) and the protein MMP3 on osteoblast to induce osteoclast apoptosis (Garcia et al. 2013). It also plays an indirect role by inhibiting inflammatory cytokines (Sunyer, Lewis, Collin-Osdoby, Osdoby, 1999). Therefore, postmenopausal women lose the bone sparing effects of estrogen contributing to increased risk of osteoporosis.

Inflammation

The inflammatory process results as a natural response to stimuli such as infection or trauma to form an immune response (Sagin, Sozmen, 2004). This can be beneficial, but can also be destructive when the response is prolonged and reactive oxygen species (ROS) and cytokine production is increased. This contributes to the development of chronic diseases such as osteoporosis, cancer, diabetes and cardiac disease (Libby, 2007). Inflammation causes the buildup of ROS and depletion of major antioxidants such as Glutathione (GSH) resulting in inflammatory markers. The main ROS is hydrogen

peroxide (HP) due to its long half-life and that it is membrane permeable. HP has been shown to increase levels of RANKL and M-CSF in human studies (Baek et al. 2010). Glutathione peroxidase 1 (GPX) is the major antioxidant enzyme in osteoclasts involved in the breakdown of HP (Lean, Jagger, Kirstein, Fuller, Chambers, 2004). It is increased by RANKL and estrogen. Therefore, post-menopausal women with estrogen deficiency have resulting bone loss due to a decrease in GPX causing increased inflammation. Low levels of GSH have been shown to cause activation of NFκB and an increase in HP. Administration of antioxidants (GSH and ascorbate) can restore levels of GSH (Martensson et al. 1991). Therefore, it is thought that foods which contain compounds that act as antioxidants (i.e. Polyphenols) can work to decrease inflammatory markers and prevent or retard bone loss.

Inflammatory Cytokines

Various inflammatory markers such as IL-1, IL-6, NO, tumor necrosis factor (TNF), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) can also increase formation of osteoclast during chronic inflammation and expression of Rab3 (Bu et al. 2008, Abu-Amer). TNF is believed to act on osteoblast cells to increase production of RANKL (Teitelbaum, 2000). However, it is also hypothesized that TNF can induce macrophages to produce differentiation by causing upregulation of NFATc1 (Bu et al. 2008). IL-1 has been shown to increase production of osteoclasts and inflammatory cell recruitment while decreasing OPG (Herrera et al. 2011).

COX-2 is an inducible pro-inflammatory prostaglandin enzyme that stimulates production of other prostaglandins from arachidonic acid (Linder, 1991). Prostaglandins regulate various functions within the body such as hormone regulation, calcium movement, fracture healing (increasing osteoblast and osteoclasts), smooth muscle contraction and kidney filtration rate (Linder, 1991; Hossein-Nezhad, Mirzaei, Birami Jamal, Mirfakhraeir, 2010). COX-2's mechanism of action is through Prostaglandin E_2 (PGE_2). PGE_2 has been shown to stimulate osteoblast activity important in bone fracture healing (Lau, Kothari, Das, Zhang, Baylink, 2013). However, PGE_2 also increases osteoclast differentiation by increasing RANKL and M-CSF production (Kaneko et al. 2007). In addition, PGE_2 stimulates COX-2 activity which further promotes osteoclast activity. COX-2 inhibitors have recently been a drug target on decreasing osteoclast production (Geng et al. 2011). COX-2 is the rate-limiting step in PGE_2 production. Therefore, targeting COX-2 activity is key in decreasing osteoclast production.

iNOS is one of the key enzymes involved in production of the free radical nitric oxide (NO) (Herrera et al. 2011). iNOS also plays a critical role in the differentiation of osteoclasts and leads to accumulation of NO in the bone environment (Oktem et al. 2006). iNOS, like PGE_2, increases production of RANKL (Herrera et al. 2011). This enzyme is a key signaling molecule that contributes to the imbalance between bone formation and breakdown. NO accumulation in the bone can lead to an increase and activation of other pro-inflammatory cytokine such as IL-1 and interferon (IFN)-γ. It plays a major role in development of osteoporosis in postmenopausal women because

iNOS can be inhibited by estrogen (Oktem et al. 2006). Therefore, products that are able to impede this enzyme are essential.

NUTRITION

Micronutrients

As stated earlier, diets low in calcium and Vitamin D have been associated with increased risk of osteoporosis (Centers for Disease Control and Prevention, 2011). Dietary prevention includes adequate intake of calcium (1000-1200 mg/day) and vitamin D (600-800 IU/day). Individuals are recommended to consume foods high in calcium such as: dairy products, calcium fortified foods (orange juice, cereal), soy products, almonds, dark green leafy vegetables and sardines. Calcium supplementation is available in two main forms: calcium citrate and carbonate. Absorption is the same when both forms are taken with meals (Kenny et al. 2004). However, studies are conflicting regarding absorption in the fasting state. There is significant data regarding calcium supplementation and prevention of bone loss and decreased risk of fractures. However, there are limited studies as to which form is optimal in prevention.

Vitamin D is another key nutrient in prevention of bone loss. Not only do certain foods provide Vitamin D but the body also produces Vitamin D from exposure to sunlight (National Institute of Health, 2012). Foods high in Vitamin D are: fortified milk, salmon, tuna, sardines, fortified cereals and juices (WebMD, 2010). Supplementation is available in two main forms: D2 (ergocalciferol) and D3 (cholecalciferol) (National Institute of Health, 2012). Recent studies have shown that D3 is more effective in

increasing serum levels of 1,25-dihydroxyvitamin D_3 (Nimitphong, Saetung, Chanprasertyotin, Chailurkit, Ongphiphadhanakul, 2013).

Additional micronutrients have been shown to be effective in prevention of bone loss. Fluoride increases bone strength and stimulate bone formation. Fluoride supplementation was often used in the past to stimulate bone formation and decrease bone mineral loss. However, recent studies are controversial as to its effectiveness. Adequate Vitamin A intake is important for activation of osteoblasts but has also been shown to activate cathepsin K (Tortora, Derrickson, 2009). Vitamin C plays a role in collagen synthesis and increases calcium absorption in the gastrointestinal tract. In addition, Vitamin K and B_{12} are needed for synthesis of bone proteins. Phosphorus plays a key role in bone structure as part of mineral salts (Mahan, Escott-Stump, 2008). Phosphorus and calcium ratios should be 1:1 for proper mineralization. However, with excessive phosphorus and low calcium intake, serum levels of calcium decrease which leads to PTH stimulation causing bone loss. Vitamin K is essential for bone health because it plays a role in formation of osteocalcin, a bone protein produced by osteoblasts that aids in mineralization. Studies show Vitamin K can decrease bone loss. Additional minerals that are part of the structure of bone are magnesium and manganese.

Dietary Polyphenols

Epidemiological studies have shown a correlation with high consumption of fruits and vegetables and maintenance of bone health (Bu et al. 2008). Plant products such as green tea and dried fruits provide polyphenolic compounds. Therefore, recent in vitro

studies have addressed particular polyphenols in order to target the specific mechanism that aids in skeletal health. Indirectly, polyphenols can have a significant impact on inflammatory markers because they act as antioxidants, scavenging free radicals. Antioxidants counteract inflammation by interacting with ROS and cytokine activity as discussed earlier. Particularly, they prevent the oxidation of substrates.

Dried plum polyphenols have been shown to increase bone strength in animal models that have undergone ovariectomy and orchidectomy and reverse any damaging effects on BMD (Bu et al. 2008). The mechanism of action is by enhanced markers of bone formation such as Insulin-like Growth Factor and a decrease in bone resorption. In addition, plum polyphenols have shown a decrease in RANKL expression in osteoblasts. They have also been shown to downregulate NFATc1 in osteoclast. COX-2 and iNOS are also decreased in lipopolysaccharide induced osteoclast pre-cursor cells.

Simon Extract which comes from the Simon wheat potato contains caffeic acid, chlorogenic acids and isochlorogenic acids (Quan et al. 2006). This has been shown to inhibit osteoclastogenesis. Certain polyphenols such as flavonoids, cathecins and caffeic acids act as antioxidants and have been shown to decrease inflammatory mediators which are known to increase osteoclast activity and formation. For example, kaempferol and quercertin (flavonoids) can inhibit osteoclastogenesis differentiation and activity by suppressing formation of NO and COX-2 (Bu et al. 2008). Dried plum polyphenols such as hydroxycinnamic acids (chlorogenic acid and caffeic acid) and anthocyanins directly inhibit osteoclast differentiation by suppression of NFATc1. In

addition, they downregulate RANKL production and inflammatory mediators such as TNF-α. Blueberry polyphenols are hypothesized to yield similar results because they contain high amounts of the polyphenols given above.

Blueberry Polyphenols

Blueberry polyphenols have been shown to have various anti-inflammatory effects (Giacalone et al. 2011). Neuroprotective properties include improvement of motor and cognitive function on the cellular level through improvement of synaptic plasticity and signaling pathways. In addition, motor function and long term memory are improved in rats. One study has shown an increased life span, slowed age-related declines and increased survival during acute heat distress in nematodes (Wilson et al. 2006).

Blueberry polyphenols have also been shown to decrease inflammation in rats with colon cancer and protect against hepatic damage (Håkansson et al. 2012). In addition, blueberry supplementation has protection against oxidative stress through decreasing levels of pro-inflammatory hormones in rats with breast cancer (Aiyer, Srinivasan, Gupta, 2008). Therefore, evidence indicates various roles of blueberry polyphenols in protecting the body against oxidative stress.

Blueberries have also shown cardiovascular and diabetic protection (Roopchand, Kuhn, Rojo, Lila, Raskin, 2013). Recent studies with rats have shown blueberry increases insulin sensitivity in insulin-resistant rats and improves hypertension. In addition, blood glucose levels have been improved diabetic and obese rats. The most recent study

released shows the beneficial effects of blueberry polyphenol enriched defatted soybean flour on blood glucose, body weight and serum cholesterol to hyperglycemic, obese mice.

Blueberry has been termed a "superfruit" due to its high polyphenolic content (Stevenson, Scalzo, 2012). The polyphenolic content contains but is not limited to: anthocyanins (malvidin, delphinidin, cyaniding, petunidin, and pelargonidin), chlorogenic acid and quercertin. Anthocyanins are the majority of polyphenols with malvidin being the highest of this group in blueberry. As stated earlier, specific polyphenols have been shown to have protective effects. Quercertin has been shown to inhibit inflammatory markers (Bu et al. 2008). Anthocyanins have strong antioxidant properties. Lastly, chlorogenic acid has a direct role in inhibition of osteoclast differentiation and activity. Therefore, the goal of the study is to assess whether blueberry polyphenols as a whole will have an effect on osteoclasts.

In conclusion, osteoporosis inflicts a significant social and economic burden. In the United States, 10 million individuals have been diagnosed with osteoporosis and 34 million are estimated to be at risk. The most accurate method of detection is measuring BMD through DXA. However, many individuals do not realize they have this debilitating disease until a fracture occurs. Treatment costs are high due to need for medication and physical therapy. Osteoporosis occurs when the osteoclast activity outweighs osteoblast action. Various factors can upregulate osteoclast activity including chronic inflammation. Dietary polyphenols and various other micronutrients have been shown to decrease inflammation. Extensive studies have shown that blueberry polyphenols have beneficial

effects on chronic diseases associated with inflammation such as neurological disorders, diabetes, heart disease and cancer. The objective of this study is to assess the effects of blueberry polyphenols on osteoclasts with future implications of decreasing risk of osteoporosis.

CHAPTER III

METHODOLOGY

Cell Culture and Treatment

RAW 264.7 mouse murine macrophage cells were purchased from the American Type Culture Collection (ATCC TIB-71; Rockville, MD). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS) and 1% *Penicillin streptomysin*. DMEM and FBS were purchased from Thermo Scientific (Pittsburgh, PA). Macrophages were incubated at 37 °C and 5% CO_2. Frozen cells were stored in growing media and 10% dimethyl sulfoxide (DMSO) in liquid nitrogen.

Blueberry Polyphenols

Freeze dried blueberry powder was generously provided by the U.S. Highbush Blueberry Council. Polyphenols were lyophilized by use of a Freeze Dry System (Labconco Inc; Kansas City, MO). Polyphenol extraction began with freeze-dried polyphenol powder (10 gm) diluted in 80% methanol (100 mL) and sonication for 20 minutes under pulsated nitrogen gas. The solution was then filtered using a vacuum system with a Buchner funnel and Whatman No. 2 filter paper. The filter was then rinsed with 100% methanol. The residue in the filter repeated the above process and the total filtrate placed in an evaporating flask with 80% methanol (50 mL). Following this, a Rotary Evaporator was used at 40°C to remove the solvent. 100% methanol (50 mL) was then be added to the phenolic concentrate followed by deionized water to bring to a final volume of 100 mL. Lastly, the solution was centrifuged at 12000g for 20 minutes at -20°C. Polyphenol concentrations were determined by the Folin-Calteau assay using gallic acid as

standards. The plate was then read by the Tecan Infinite 200 PRO series microplate reader at an absorbance of 750 nm.

Cell Viability

The CellTiter 96® colorimetric method assay (Promega; Fitchburg, WI) for proliferative cells was used to determine the number of viable cells and doses of blueberry polyphenols to be used in the experiments. Cells were plated in a 96 well plate. 20 µL of AQueous One Solution Reagent was added to cells in 100 µL DMEM. The plate was then incubated at 37°C for 2 hours. The plate was read by the Tecan Infinite 200 PRO series microplate reader at an absorbance of 490 nm.

Measurement of Inducible Nitric Oxide Synthase in RAW 264.7 Macrophages

Macrophages were assessed for iNOS levels with corresponding blueberry polyphenol dosages. Cells were cultured in 96 well plates (20,000 cells/well). 24 hours later treatment occurred: one group was treated with Lipopolysaccharide (LPS) (1µg/mL) and four with blueberry polyphenols (50, 100, 150 and 200 µg/mL). 24 hours later all groups were treated with Hydrogen Peroxide (1 µg/mL) for 6 hours. Cells were then prepped for Colorimetric In-Cell ELISA (Thermo Scientific). Treatment media was removed and incubation with Formalin (100 µL) at room temperature for 15 minutes occurred. Cells were then washed twice with Tris-buffered saline (TBS), incubated with Permeabilization Buffer (100 µL) for 15 minutes at room temperature followed by one wash with TBS. Blocking buffer (100 µL) was then added at room temperature for 30 minutes. Cells were then incubated in 50 µL diluted iNOS antibody (Santa Cruz Biotechnology; Santa Cruz, CA) overnight at 4ºC. The following day, cells were washed three times with Wash Buffer (100 µL) and incubated at room temperature for 30 minutes with diluted HRP Conjugate Anti-iNOS antibody (Santa Cruz). Cells were washed three times with

Wash Buffer (200 µL). TMB Substrate (100 µL) was then added and plates incubated for 10 minutes protected from light at room temperature. TMB Stop Solution (100 µL) was then added. The plate was read by the Tecan Infinite 200 PRO series microplate reader at 450 nm.

Tartrate Resistant Acid Phosphatase Staining of Multinucleated Osteoclasts

RAW cells were cultured in 96 well plates and 24 hours later RANKL (30 ng/mL) was added. 72 hours later, blueberry polyphenol treatment occurred (50, 100 and 200 µg/mL) with RANKL (30 ng/mL). 6 hours later TRAP levels were determined with a staining kit (Takara Bio; Japan). The media was removed followed by washing with Hank's Balanced Salt Solution (HBSS). Extraction solution (5-50 µL) was added followed by Substrate Solution (50 µL). The plate was then incubated at 37°C for 15-60 minutes followed by Stop Solution (50 µL). The plate was read by the Tecan Infinite 200 PRO series microplate reader at 405 nm. In addition, digital photographs were taken of the stained TRAP.

Western Blot Analysis

Cells were plated in petri dishes at 1.5 million cells per dish. RANKL (30 ng/mL) was added 24 hours later to all group and incubated at 37ºC. 48 hours later, treatment was administered: all groups were treated with RANKL and HP (100 µM/mL), groups with blueberry doses (50, 100, 150 and 200 µg/mL). Treatment lasted 6 hours then media was removed and washed with HBSS. Cells were then lysed by RIPA buffer (Cell signaling, Danvers, MA) and protease inhibitors (FabGennix Inc., Frisco, TX), incubated on ice for five minutes and mechanically stimulated for removal. Samples were then sonicated for 10 seconds, 3 times. Centrifugation occurred for 10 minutes and the supernatant was saved. Protein levels were determined using the BCA Protein Assay Kit (Thermo Scientific) following 30 minutes incubation at 37 °C for 30 min before reading on the microplate reader at 562 nm. Protein was

then reduced with β-mercaptoethanol (2.5%) and bromophenol blue (2.5%) was added as a coloring agent. The cells were then heated for 5 minutes at 70°C and stored at -80°F.

Protein samples were loaded and separated into SDS-polyacrylamide gel (125 volts) using an 8-10% resolving gel and electroblotted to PVDF membranes (70 volts for 90 minutes). Transfer was confirmed using Ponceau S staining (FabGennix). Membranes were then washed in 5% milk Tris-buffered saline with Tween 20 (TBST).

For the COX-2 antibody (Cell signaling), membranes were incubated in a 1:1000 dilution of TBST overnight. Membranes were then washed three times with TBST for 5 minutes. Secondary antibody incubation (1:2000 in TBST) occurred for 1 hour and subsequent washes of TBST (3 times, 5 minutes) and TBS (2 times, 5 minutes). Proteins were detected with Enhanced Chemiluminescence (Bio-Rad; Hercules, CA) and read by the Bio Rad ChemiDoc™ camera and Quantity One software.

For NFATc1 and β-actin antibodies (Santa Cruz), membranes were kept in milk overnight and primary antibody incubation was for 1 hour. The remaining procedure was the same as above.

Statistical Analysis

All experiments were repeated at least 3 times. Statistical analysis was done with SPSS 19.0. Descriptive statistics include means, medians and standard deviations. Cell titer assays, TRAP analysis and Western blot have a one-way analysis of variance (ANOVA) with the Bonferroni procedure to verify differences between treatments. $P<0.05$ is statistically significant and values are expressed as means ± standard error (SE).

CHAPTER IV

EFFECT OF BLUEBERRY POLYPHENOLS ON OSTEOCLAST DIFFERENTIATION AND ACTIVITY

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ABSTRACT

Bone is a dynamic tissue with osteoblasts and osteoclasts participating concomitantly in its remodeling process. These cells are derived from different progenitor pools and under different molecular control. Osteoclast maturation requires stimulation by receptor-activated nuclear kappa ligand (RANKL), a downstream product of inflammation and an important regulator of bone resorption. Blueberries are a rich source of polyphenolic compounds and have been shown to be effective in decreasing incidence/symptoms of chronic inflammatory conditions. Various individual polyphenols found in blueberry decrease osteoclast differentiation and activity. Therefore, the purpose of this study was to assess the effect of blueberry polyphenols as a whole on osteoclasts by assessing markers for activity and differentiation. RAW 264.7 murine macrophage cells were cultured and treated with varying blueberry polyphenols doses (50, 100, 150, 200 μg/mL). Levels of inducible nitric oxide synthase (iNOS) were measured by use of In-Cell ELISA. RAW 264.7 cells were also differentiated into osteoclasts with RANKL. Tartrate Resistant Acid Phosphatases (TRAP), a measure of osteoclast activity, were stained and photographed. Western blot was used to determine protein levels of Nuclear

factor of activated T-cells, cytoplasmic 1 (NFATc1) and cyclooxygenase-2 (COX-2). Blueberry polyphenols were shown to be effective by decreasing iNOS levels in osteoclast precursor cells. COX-2 and TRAP levels were decreased in osteoclasts under inflammatory conditions. However, NFATc1, a key osteoclast transcription factor was increased in correlation to blueberry concentration. This study indicates that blueberry polyphenols decrease osteoclast activity through multiple mechanisms such as inflammatory mediators and key bone resorption enzymes which in turn cause a decrease in osteoclast function.

Keywords Bone; Osteoclasts; Osteoporosis; RAW 264.7; Macrophage; RANKL

INTRODUCTION

Osteoporosis or "porous bone," is characterized as an imbalance in skeletal turnover in which bone resorption exceeds bone formation resulting in skeletal instability (1,2). It causes more than 8.9 million fractures worldwide per year which represents billions of dollars in rehabilitation and health care cost. In the United States, 10 million individuals have been diagnosed with osteoporosis and 34 million are estimated to be at risk of developing this debilitating disease (3). The disease is most common in Western society having the highest prevalence among postmenopausal females who are at a 40% risk of osteoporotic fractures, which can often be life threatening (4).

There are two types of osteoporosis, primary and secondary (5). Primary, often referred to as type 1, is most common and occurs as a result of gradual bone loss with age. Individuals reach peak bone mass at age 30 with a decline occurring after this age.

Accelerated bone loss occurs after age 45-50 for males and for women after menstruation discontinues. Though accelerated bone loss occurs earlier for men, once women are post-menopausal the rate will be more rapid putting them at greater risk. In addition, females typically have lower bone mass which results in increased probability of osteoporosis. Secondary osteoporosis, or type 2, occurs as a result of a medical condition or long-term use of certain medications such as steroids, certain chemotherapeutic drugs, proton pump inhibitors and anti-seizure medications (1).

Osteoclasts are the key cells involved in bone breakdown; osteoporosis results from enhanced bone resorption. Therefore, understanding osteoclast formation and mechanism of function is key to determining the disease progression, prevention and treatment. Osteoclasts have hematopoietic origin and form through differentiation of macrophages/monocytes (2). Osteoblasts or stromal cells must first make contact with the macrophages and secrete two molecules essential for differentiation: macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor kappa B ligand (RANKL). In vitro studies require solely RANKL for osteoclast differentiation (6). Therefore, RANKL is the primary protein that controls osteoclastogenesis.

Transcription factors for osteoclastogenesis include PU.1, c-fos, c-jun, NF-κB and NFATc1 (7). NFATc1 in particular is the key and most highly induced transcription factor in initiation of osteoclastogenesis (8). Believed to be the master gene of osteoclast transcription, NFATc1 regulates gene expression for various factors such as TRAP, cathepsin K and other proteins involved in osteoclast function (7,9). NFATc1 is

controlled by Ca^{2+} -activated phosphatase, calcineurin, which dephosphorylates NFATc1 enabling it to translocate to the nucleus to promote gene expression. RANKL initiates the increase in intracellular calcium levels. The elevation of Ca^{2+} results in activation of calcineuron necessary for NFATc1 translocation (8). In certain conditions, an increase in translocation of NFATc1 to the nucleus can occur, such as extracellular acidosis due to inflammation and the presence of PTH and 1,25-dihydroxyvitamin D_3 (10,8).

Bone remodeling begins with bone resorption. Osteoclasts attach to the bone matrix where their plasma membrane folds over to form a "ruffled membrane" (2). This allows for the transport of vesicles that contain acidifying material. Surrounding the "ruffled membrane" is an extracellular environment composed of various proteins, mainly filamentous actin. This creates an isolated area, or sealed zone, between the osteoclast and the bone which allows for resorption. Acidification and collagenases secreted from osteoclasts cause denaturing of the molecule (11). The first step in degradation involves the proton pumps (H^+-ATPase) alongside a Cl^- channel to maintain electroneutrality between the intra and extracellular environment. These channels result in secretion of hydrochloric acid into the bone matrix. H^+-ATPase is a proton pump enzyme that provides trafficking of proteins and vesicles (9). Acidification (pH ~4.5) of the bone matrix is essential to the breakdown of the bone mineral, hydroxyapatite, an alkaline salt (12). The primary proteases secreted by osteoclasts are Cathepsin K (cysteine protease) and TRAP (2). The principle products of resorption are calcium and type I collagen fragments (13,14). When the bone matrix is broken down, they are

endocytosed into the ruffled membrane and transported to the basolateral membrane/antiresorption surface that faces the extracellular space on the opposite side of the cell (15). The products then diffuse into blood capillaries. Once this process is complete, osteoblasts enter for bone deposition. This process takes approximately ten times longer than bone resorption (16). At a given time, 5% of total bone mass is being remodeled (17). In young individuals, the bone that is resorbed is completely restored. With age, the amount of bone that is replaced is less than the amount that is resorbed. This negative balance is key to bone loss and development of osteoporosis.

The inflammatory process results as a natural response to stimuli such as infection or trauma to form an immune response (18). This can be beneficial, but can also be destructive when the response is prolonged and reactive oxygen species (ROS) and cytokine production is increased. This is another contributing factor to the development of chronic diseases such as osteoporosis (19). The main ROS is hydrogen peroxide (HP) due to its long half-life and that it is membrane permeable. HP has been shown to increase levels of RANKL and M-CSF in human studies (20). iNOS is one of the key enzymes involved in production of the free radical nitric oxide (NO) (21). iNOS also plays a critical role in the differentiation of osteoclasts and leads to accumulation of NO in the bone environment (22). In addition, iNOS increases production of RANKL (21). This enzyme is a key signaling molecule that contributes to the imbalance between bone formation and breakdown. NO accumulation in the bone can lead to an increase and activation of other pro-inflammatory cytokine such as IL-1 and interferon (IFN)-γ. It also

plays a major role in development of osteoporosis in postmenopausal women because iNOS can be inhibited by estrogen (22).

Various inflammatory markers such as IL-1, IL-6, Nitric Oxide (NO), tumor necrosis factor (TNF) and COX-2 can also increase formation of osteoclasts (6,23). COX-2's mechanism of action is through Prostaglandin E_2 (PGE_2) (24). PGE_2 has been shown to stimulate osteoblast activity important in bone fracture healing (25). However, PGE_2 also increases osteoclast differentiation by increasing RANKL and M-CSF production (26). In addition, PGE_2 stimulates COX-2 activity which further promotes osteoclast activity. COX-2 inhibitors have recently been a drug target on decreasing osteoclast production (27). COX-2 is the rate-limiting step in PGE_2 production. Therefore, targeting this enzyme's activity is key in decreasing osteoclast production.

Epidemiological studies have shown a correlation with high consumption of fruits and vegetables and maintenance of bone health (6). Certain polyphenols such as flavonoids, cathecins and caffeic acids act as antioxidants and have been shown to decrease inflammatory mediators which are known to increase osteoclast activity and formation (7). For example, kaempferol and quercertin (flavonoids) can inhibit osteoclast differentiation and activity by suppressing formation of NO and COX-2. In addition, they downregulate RANKL production and inflammatory mediators such as TNF-α (6).

Plant products such as green tea and dried fruits provide polyphenolic compounds(6). Recent in vitro studies have addressed particular polyphenols in order to target the specific mechanism that aids in skeletal health. Dried plum polyphenols have

been shown to decrease RANKL expression in osteoblasts. They have also been shown to downregulate NFATc1 in osteoclasts. COX-2 and iNOS are also decreased in lipopolysaccharide induced osteoclast pre-cursor cells. Simon Extract which comes from the Simon wheat potato contains caffeic acid, chlorogenic acids and isochlorogenic acids (7). This has been shown to inhibit osteoclastogenesis.

Blueberry polyphenols have been shown to have various anti-inflammatory effects (28). Blueberry is now considered a "superfruit" due to its high polyphenolic content (29). The polyphenolic content contains but is not limited to: anthocyanins (malvidin, delphinidin, cyaniding, petunidin, and pelargonidin), chlorogenic acid and quercertin. Anthocyanins are the majority of polyphenols with malvidin being the highest of this group in blueberry. As stated earlier, specific polyphenols have been shown to have protective effects. Quercertin acts as an inhibitor to inflammatory markers (6). Anthocyanins have strong antioxidant properties. Lastly, chlorogenic acid has a direct role in inhibition of osteoclast differentiation and activity. This study hypothesized that blueberry polyphenols would dose-dependently decrease osteoclast activity by inhibiting inflammatory markers involved in activity. The benefits of this study are an assessment of the mechanism of action of the polyphenols and an initial step to recommendation of blueberry as a preventative factor and possible therapy for bone health.

MATERIALS AND METHODS

Cell Culture and Treatment

RAW 264.7 mouse murine macrophage cells were purchased from the American Type Culture Collection (ATCC TIB-71; Rockville, MD). Cells were cultured in Dulbecco's Modified Eagle

Medium (DMEM), 10% Fetal Bovine Serum (FBS) and 1% *Penicillin streptomysin*. DMEM and FBS were purchased from Thermo Scientific (Pittsburgh, PA). Macrophages were incubated at 37 °C and 5% CO_2. Frozen cells were stored in growing media and 10% dimethyl sulfoxide (DMSO) in liquid nitrogen.

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RESULTS

Cell Viability

Cell viability was based on the standard curve quantified by the polyphenolic concentration using the slope formula determined by the Folin-Calteau assay. This was followed by cell viability assay to determine the lethal doses of blueberry polyphenols to use for all of our experiments. Appropriate levels were determined to be 50, 100, 150 and 200 µg/mL.

Inducible Nitric Oxide Synthase in RAW 264.7 Macrophages

iNOS levels were highest in the LPS stimulated macrophages and lowest in the highest blueberry treatment of 200 μg/mL (Figure 1A). Blueberry doses of 100, 150 and 200 were statistically significant from LPS stimulated cells. The percentage differences are also shown with LPS stimulated cells as control (Figure 1B). This data indicates that under inflammatory conditions blueberry polyphenols can dose dependently decrease iNOS activity.

Osteoclast Activity as Determined by Tartrate Resistant Acid Phosphatase

RAW 264.7 macrophages, osteoclast progenitor cells, were stimulated with RANKL (30 ng/mL) for 72 hours (Figure 2). To confirm osteoclast differentiation and activity, TRAP levels were measured. TRAP activity was decreased by 13.5% in the 100 μg/mL dose and 34% in the 200 μg/mL dose of blueberry polyphenols (Figure 3A). TRAP staining is also provided (Figure 3B). This indicates that blueberry polyphenols can dose dependently decrease osteoclast activity through attenuation of TRAP.

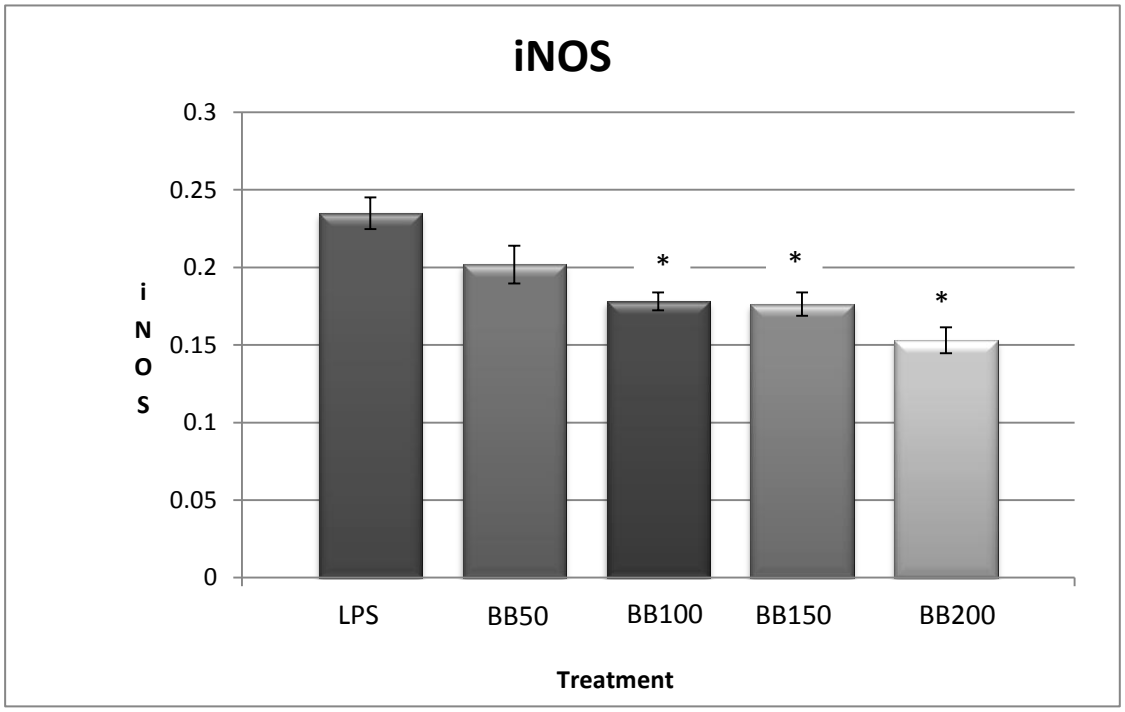
Western Blot Analysis

Macrophages were differentiated into osteoclasts followed by inflammatory stimulation with HP and blueberry treatments. The results of the Western blot showed an inverse relationship between COX-2 expression and blueberry polyphenol dosage (Figure 4A). Percent density was corrected for β-actin (Figure 4B). Using the control as a reference of 100% protein density, corresponding blueberry doses showed a decrease of 28% (50 μg/mL), 51% (100 μg/mL), 74% (150 μg/mL), 81% (200 μg/mL). However, no doses were statistically significant ($p<0.05$). P values did decrease with increasing dosage

and the highest dosage of blueberry had a p value of 0.696. Lack of statistical significance was most likely due to the low count (n=8). However, the results indicate that under inflammatory conditions, blueberry polyphenols can decrease inflammatory cytokines such as COX-2. In the future, a higher level of blueberry dosage or increase in the number of blots may prove to yield statistically significant results.

There were some unexpected results; NFATc1 doses increased as blueberry concentration increased (Figure 5A). Percent density was corrected for β-actin (Figure 5B). Using the control as a reference of 100% protein density, corresponding blueberry doses showed an increase of 33% (50 µg/mL), 11% (100 µg/mL), 79% (150 µg/mL), 60% (200 µg/mL). However, no doses were statistically significant ($p<0.05$). The implications of the results indicate that osteoclast differentiation, in relation to NFATc1, is not decreased with blueberry polyphenols in this particular treatment.

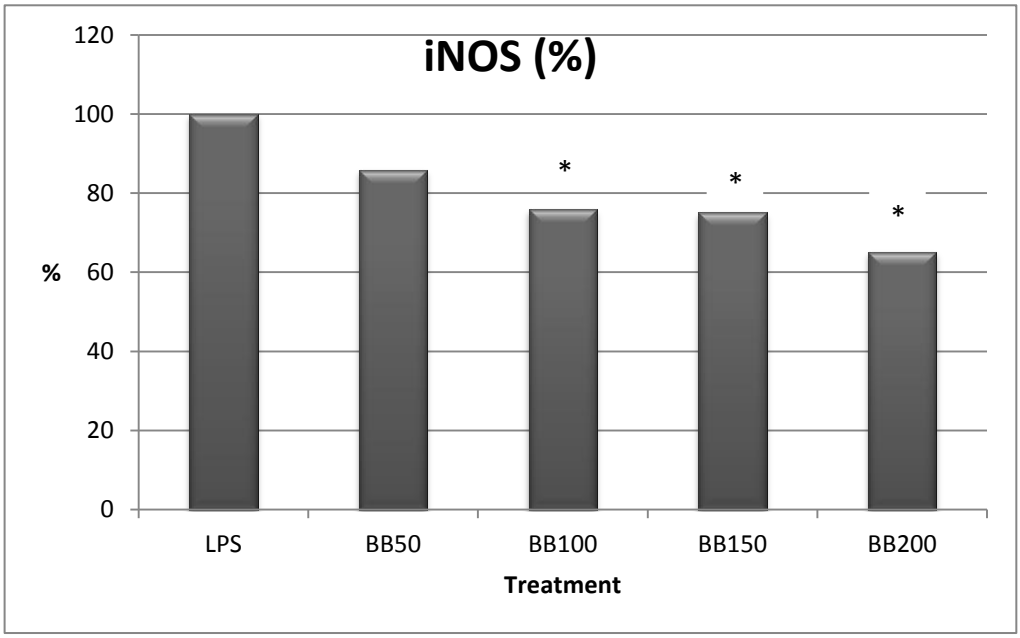
Figure 1A. Dose Dependent Effect of Blueberry Polyphenols on iNOS in Murine Macrophage Cells



* p<0.05

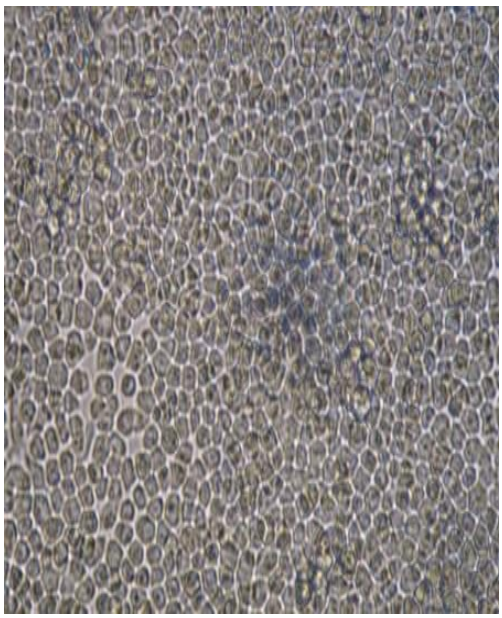
iNOS	LPS	BB50	BB100	BB150	BB200
Mean	0.234845	0.201761	0.178147	0.1763	0.153037
Median	0.223	0.2015	0.1809	0.17195	0.1496
Standard Deviation	0.04570497	0.051424	0.025291	0.033889	0.036071
Standard Error	0.01021994	0.012121	0.005802	0.007578	0.008275

Figure 1B
Dose Dependent Effect of Blueberry Polyphenols on %iNOS using LPS as control

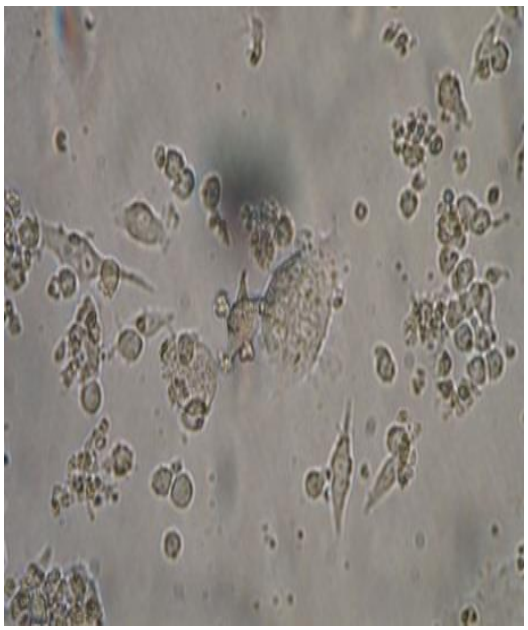


* $p<0.05$

Figure 2
Murine RAW 264.7 Macrophage Cells Stimulated With and Without RANKL



Non-Stimulated



Stimulated

Figure 3A
Dose Dependent effect of Blueberry Polyphenols on TRAP Activity in RANKL Mediated Murine Macrophages

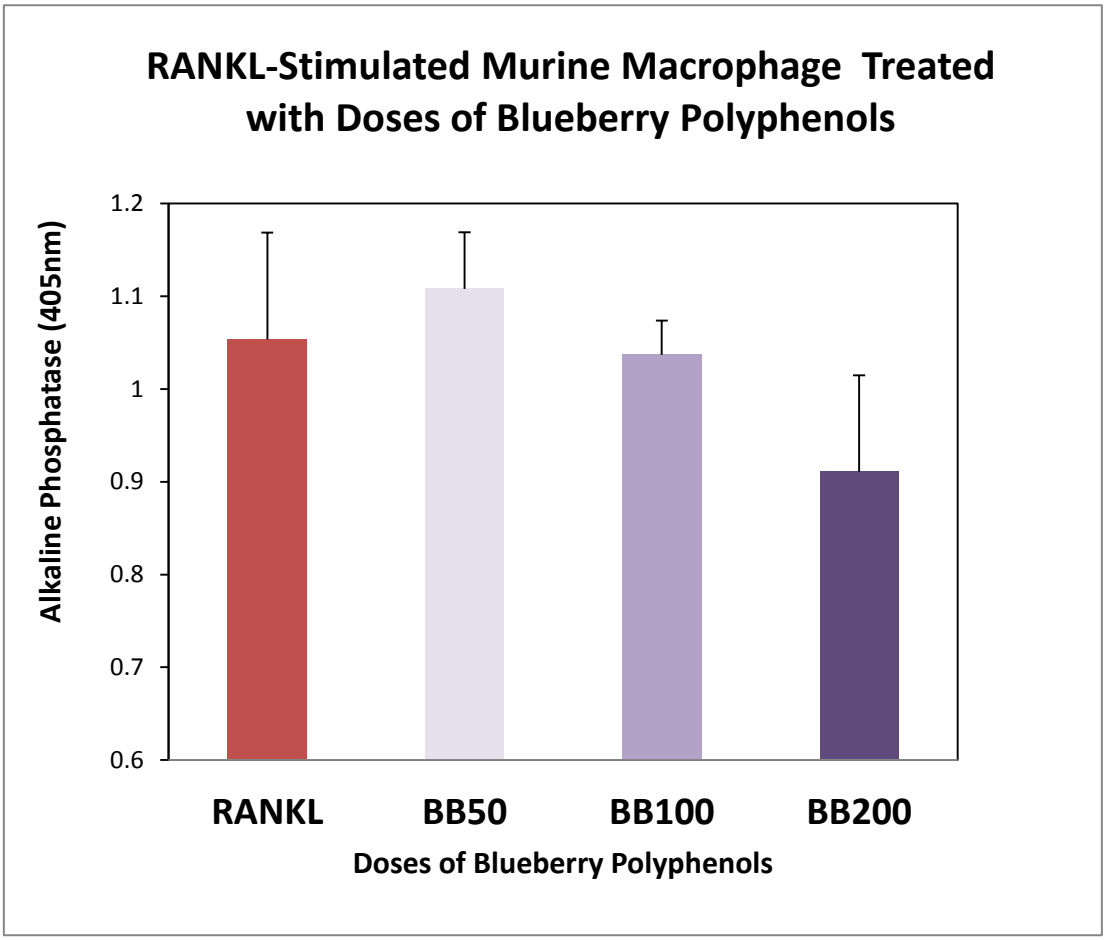


Figure 3B. TRAP Staining of RAW 264.7 Cells

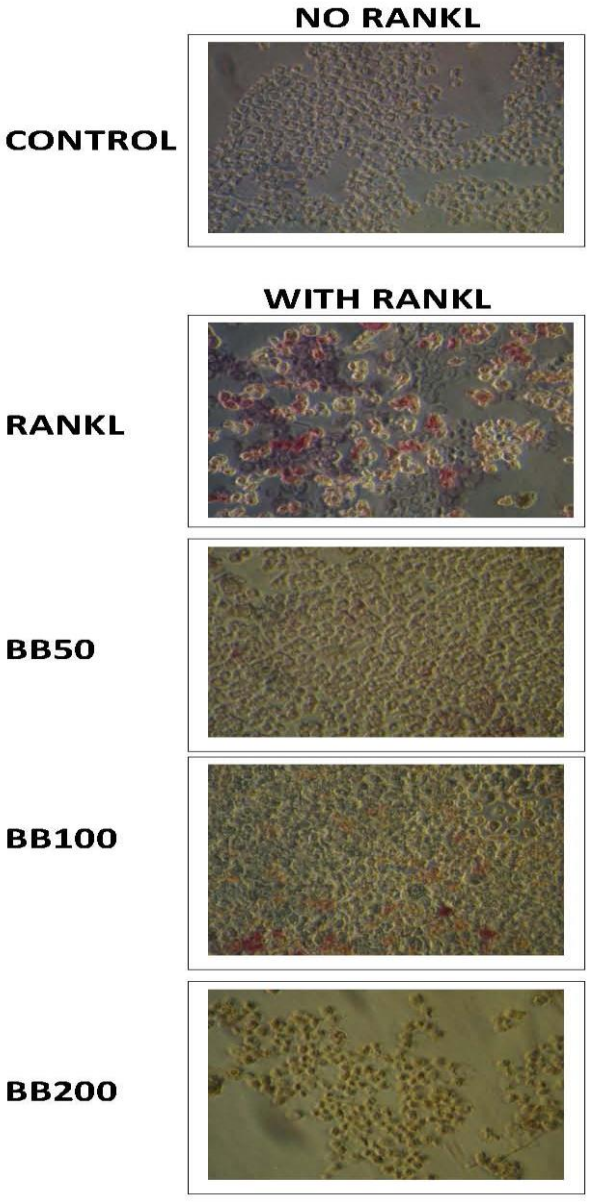
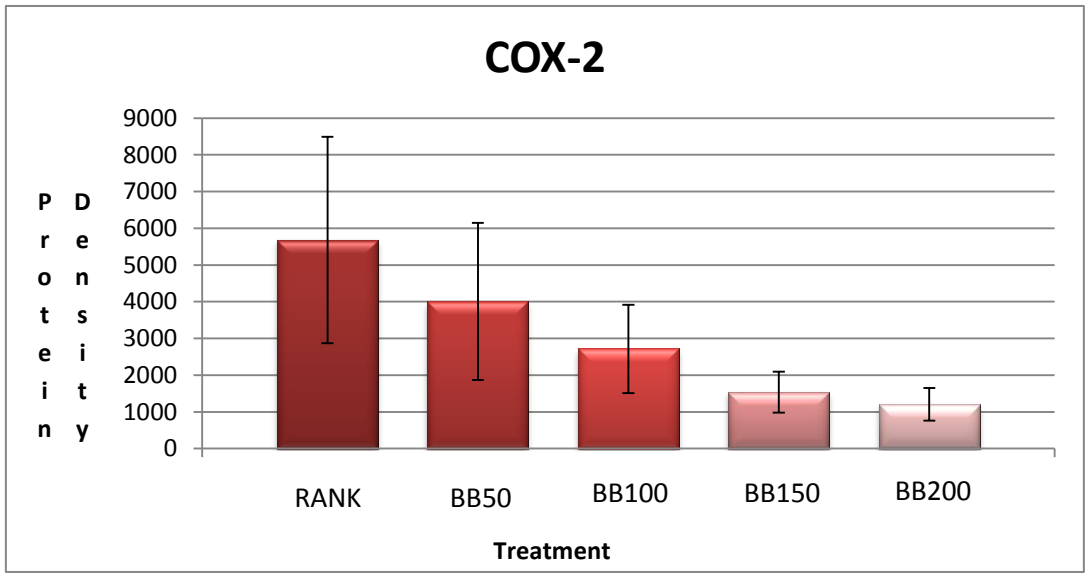


Figure 4A
Dose Dependent effect of Blueberry Polyphenols on COX-2 in RANKL Mediated Murine Macrophages (n=8)*

* n representative of number of experiments



COX-2	RANKL	BB50	BB100	BB150	BB200
Mean	5682.8889	4005	2716.4	1537.4889	1203.9333
Median	2508.8	1450	1512.4	1172.2	279.8
Standard Deviation	8427.4490	6416.0512	3610.3239	1678.1431	1339.3208
Standard Error	2809.150	2138.684	1203.4412	559.381023	446.440256

Figure 4B
%Density of COX-2 with β-Actin corrected

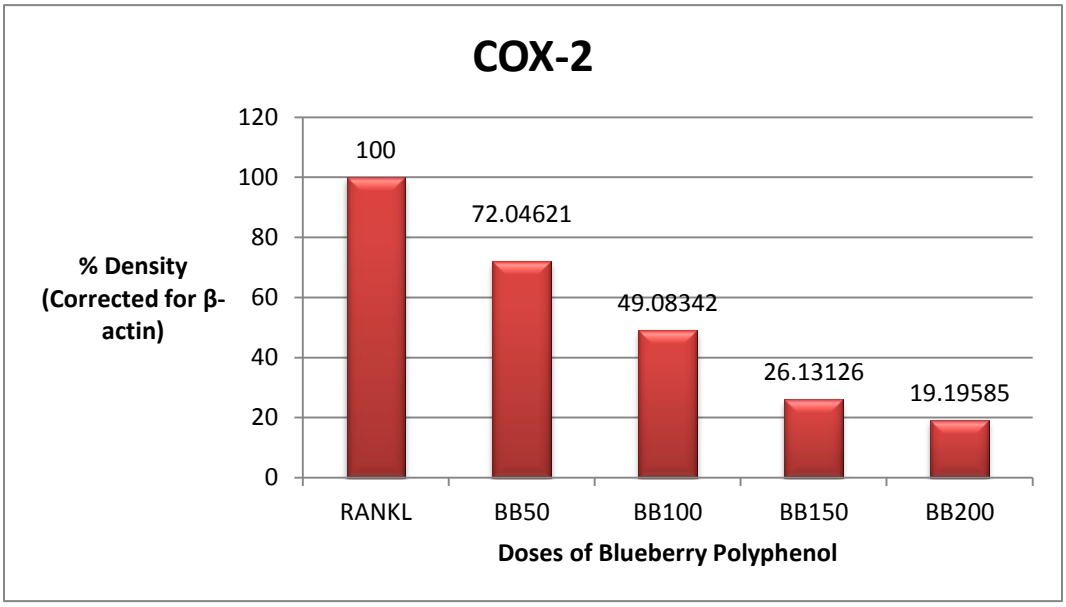
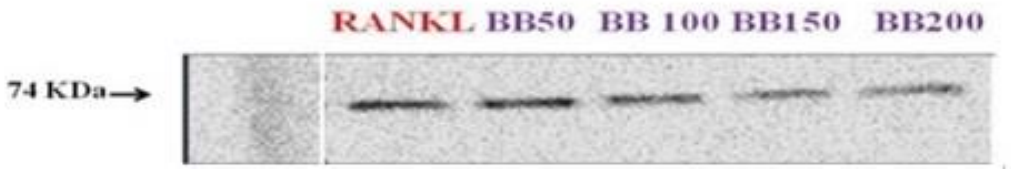


Figure 4C
Representative Immunoblots of COX-2 and β-Actin



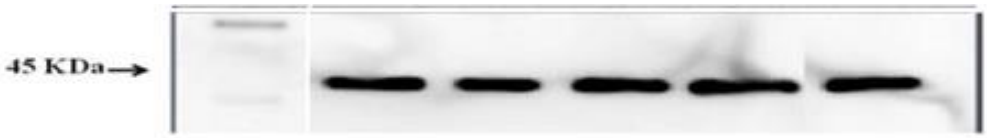
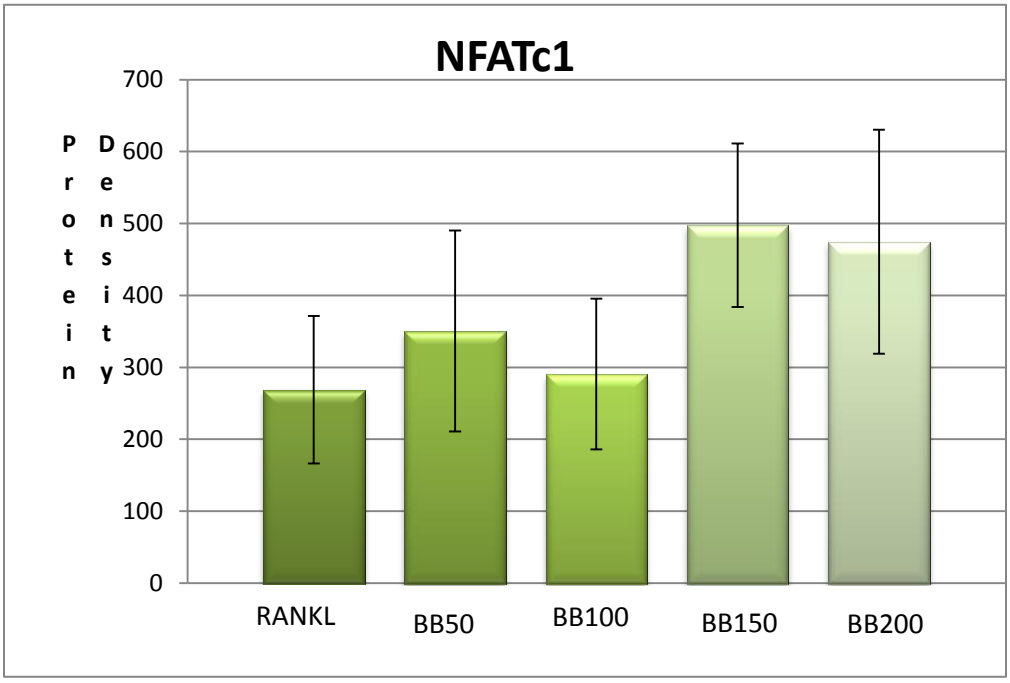


Figure 5A
Dose Dependent effect of Blueberry Polyphenols on NFATc1 in RANKL Mediated Murine Macrophages (n=3)*

* n representative of number of experiments



NFATc1	RANKL	BB50	BB100	BB150	BB200
Mean	5682.8889	4005	2716.4	1537.4889	1203.9333
Median	2508.8	1450	1512.4	1172.2	279.8
Standard Deviation	8427.4489	6416.051	3610.323	1678.143	1339.321
Standard Error	2809.1497	2138.6837	1203.4413	559.38102	446.44026

Figure 5B
%Density of NFATc1 with β-Actin corrected

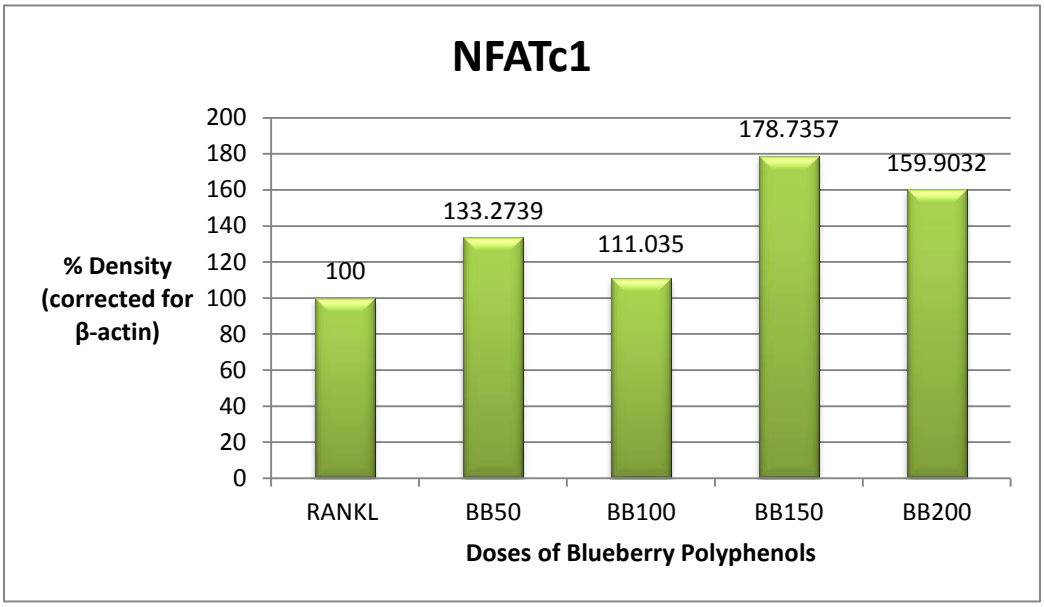
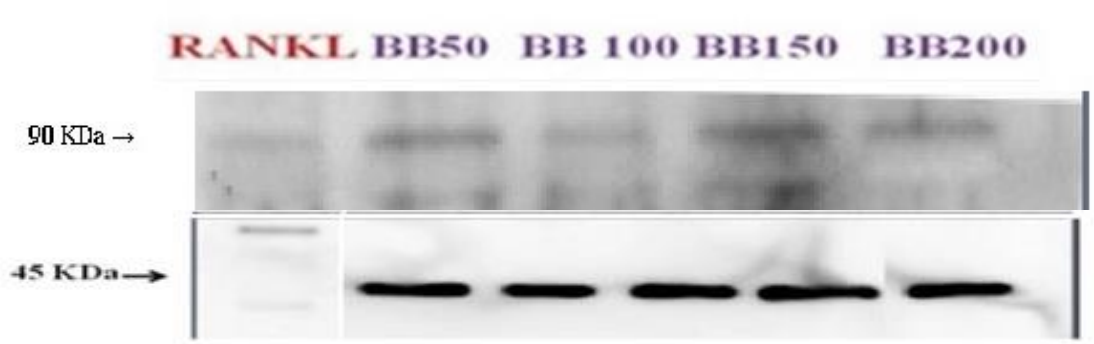


Figure 5C
Representative Immunoblot of NFATc1 and β-Actin



DISCUSSION

Our present study shows that blueberry polyphenols as a whole display anti-inflammatory effects and decreased activity in osteoclasts and osteoclast pre-cursor cell. Blueberry polyphenols have been shown to have anti-inflammatory effects through prevention or attenuating the detrimental effects of certain diseases. Giacalone researched neuroprotective properties including improvement of motor and cognitive function on the cellular level through enhancement of synaptic plasticity and signaling pathways (28). In addition, motor function and long term memory improved in rats. Håkansson showed that blueberry polyphenols decreased inflammation in rats with colon cancer and protected against hepatic damage (30). In addition, blueberry supplementation has been

shown to be protective against oxidative stress through decreasing levels of pro-inflammatory hormones in rats with breast cancer (31). Lastly, various studies have been conducted concerning blueberries in relation to cardiovascular and endocrinological protection (32). Rats have displayed increased insulin sensitivity in those with insulin-resistance and improved hypertension. In addition, blood glucose levels were improved in diabetic and obese rats. The most recent study released shows the beneficial effects of blueberry polyphenol enriched defatted soybean flour on blood glucose, body weight and serum cholesterol to hyperglycemic, obese mice. Ours is the first known study to show the anti-inflammatory effects of blueberry polyphenols on osteoclasts.

Osteoclast precursor cells, RAW 264.7 macrophages were experimented under inflammatory conditions. NO levels were found to be decreased by 35% in the highest blueberry dosage versus the control. This is consistent with anti-inflammatory effects that have been shown by blueberry polyphenols. Bu displayed the same effects with plum polyphenols which have a similar content to blueberry (6). ČÍŽ also showed decreased iNOS levels in RAW 264.7 cells with quercertin that was extracted from wine (33)**.** The implications of these results are a decrease in pro-inflammatory cytokines which contribute to increased osteoclast differentiation. Therefore, targeting the precursor cells indicates a possible decrease in subsequent osteoclast differentiation.

Osteoclast activity was addressed in regards to TRAP and corresponding blueberry polyphenols. TRAP activity was decreased with increasing levels of blueberry polyphenols similar to the findings of Bu with regards to plum polyphenols (6). TRAP

activity decreased by 13.5% in the 100 µg/mL dose and 34% in the 200 µg/mL dose of blueberry polyphenols. Le´otoing also found that TRAP activity was decreased by fisetin, a flavonoid found in blueberries and other fruits (34). Yun showed that epigallocatechin, another flavonoid found in blueberry, decreased TRAP activity as well (35). TRAP is an iron-containing metalloenzyme in various tissues and is prevalent in osteoclasts because it catalyzes the hydrolysis of bone in acidic conditions (6,36). TRAP also plays a regulatory role as attacher to the bone and migration to the cell surface (37). TRAP activity is enhanced by capthesin K and can function to produce ROS in a neutral pH (36,38). Therefore, with a decrease in this enzyme not only will be there be a decrease in osteoclast resorption processes but also a possible decrease in ROS production.

Osteoclast inflammatory marker, COX-2, and differentiation factor, NFATc1, were also studied under inflammatory conditions. As stated earlier, COX-2 as a pro-inflammatory enzyme, increases osteoclast activity (26). Flavonoids, hydroxycinnamic acids and anthocyanins have been individually studied and shown to suppress COX-2 in osteoclasts (6,7). The highest blueberry polyphenol concentration showed an 80% decrease in comparison to osteoclasts with no treatment. The implications of this data are significant in regards to blueberry's anti-inflammatory effects.

The results for NFATc1 did not align with the results seen for iNOS and TRAP. The highest level of blueberry polyphenol had a 60% higher protein density than the untreated osteoclast; the highest percentage increase was at 150 µg/mL dosage with a 78% increase. This is the first know experiment to look at NFATc1 and blueberry

polyphenols. Quan found that the individual polyphenol, caffeic acid suppressed NFATc1 with western blot (7). However, no other studies have used polyphenols as a whole to determine NFATc1 using western blot. A limitation of this study was when the blueberry was added to the experiment. A likely improvement would be to add the blueberry with the RANKL to stop initial differentiation and not of those continuing during inflammatory conditions. Inflammation has been shown to increase NFATc1 activity (10). However, this does not address the increased activity with blueberry polyphenols. An additional limitation is the low number of experiments (n=3). Suggestions for improvement in the study would be earlier addition of blueberry polyphenol to the treatment group and increasing the number of experiments.

In conclusion, various factors can upregulate osteoclast activity including chronic inflammation. Dietary polyphenols and various other micronutrients have been shown to decrease inflammation. Extensive studies have shown that blueberry polyphenols yield beneficial effects on chronic diseases associated with inflammation such as neurological disorders, diabetes, heart disease and cancer. This study shows that blueberry polyphenols have anti-inflammatory effects on osteoclast precursor cells and osteoclasts. In addition, osteoclast activity is dose-dependently decreased with blueberry polyphenol treatment. However, inconclusive evidence was shown as to the effect on differentiation factors. Future studies should look further at differentiation and the effect of blueberry polyphenol supplementation in vivo in regards to bone health.

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