

USE OF PHOTOBIMODULATION IN OSTEOCLAST FORMATION:  
POSSIBLE INTERVENTION FOR THE TREATMENT OF  
OSTEOPOROSIS

by

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## ABSTRACT

### USE OF PHOTOBIMODULATION IN OSTEOCLAST FORMATION: POSSIBLE INTERVENTION FOR THE TREATMENT OF OSTEOPOROSIS

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After critically examining the literature to gain a robust understanding for the pathogenesis of bone loss, specifically osteoporosis, the development of a possible new intervention to prevent or treat osteoporosis was explored. The purpose of this dissertation was to pilot test a new protocol designed to answer the broad research question: Does Near-Infrared Light Emitting Diode (NIR-LED) treatment affect Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) induced osteoclastogenesis in a cell culture model?

Osteoporosis is defined as a disease characterized by low bone mass and structural deterioration of bone tissue, leading to bone fragility and an increased susceptibility to fractures, especially of the hip, spine, and wrist. Specifically, osteoporosis has been shown to increase the risk for fracture resulting from a fall. The World Health Organization reported in 2003 that osteoporosis-induced fractures burden society due to the great health care costs for treatment and rehabilitation. Experts have estimated that, between 2005-2025, the incidence and economic burden to treat osteoporotic related fractures will increase 50% annually (Burge et al., 2007). The development of additional interventions meant to slow the progression of osteoporosis may contribute to decreasing this burden. Near-Infrared Light Emitting Diode (NIR-LED) photobiomodulation has been found to be effective in improving wound healing,

bone regeneration, mitochondrial function, and attenuating cellular oxidative stress. Little is known regarding the use of NIR-LED and the formation of osteoclasts, which break down bone.

This pilot study included two experiments using the WARP™ 75 light source. RAW264.7 cells were cultured for 24 hours and induced to differentiate into osteoclasts, using the cytokine Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL). Cultures were divided into groups according to RANKL dose (0, 2.5, 10, 25, 50 ng/ml) and different energy densities (2.25, 4.5, 45 J/cm<sup>2</sup>), treated with light either once or on four consecutive days using the WARP™ 75 (Quantum Devices, Barneveld, WI). Osteoclast-like cells were stained for Tartrate-Resistant Acid Phosphatase (TRAP). Multinucleated, TRAP<sup>+</sup> cells were scored as osteoclasts, counted manually by microscopy. Results were expressed as means and standard deviations, and groups were compared by one-way ANOVA with posthoc Tukey. RANKL-induced osteoclast formation by RAW264.7 cells occurred as expected in all experiments. Light-treatment alone had no observable effect. A single light-treatment at 4.5 J/cm<sup>2</sup> with RANKL added (10 - 50ng/ml) suggested a biostimulatory effect upon osteoclastogenesis compared to controls, as multiple light-treatments compared to single light-treatment were less stimulatory in the energy density 4.5 J/cm<sup>2</sup> and RANKL dose 25ng/ml group. In conclusion, effects of NIR-LED treatment on osteoclastogenesis are RANKL dose and light-intensity specific. NIR-LED light-treatment affects RANKL-induced osteoclast formation suggestive of a biostimulatory effect, and multiple light treatments compared to single light treatment may biostimulate osteoclastogenesis less.

These experiments provide the foundation to further investigate possible NIR-

LED effects upon bone formation and include alternative bone formation quantification utilizing alternative cellular assays. Contributing to this body of knowledge provides insight for nurses' application of new therapies to prevent and treat bone loss.

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DEDICATION PAGE

To my husband, Shawn.

I thank you for your undying and continual patience and support.

To my Children, Drew and Lauren.

Thank you for supporting your mother as she completed her professional goals.  
You both have incredible resilience.

To Dr. Mary Pat Kunert

Thank you for believing in my creativity, your mentorship and dedication to student success. You are deeply missed.

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## **CHAPTER ONE**

### **Introduction**

This chapter sets the context for the study. The following will be presented in this chapter: the significance, statement of the problem, followed by the study method and purpose, research questions and concluding with the implications for nursing practice, research and policy. Within the statement of the problem, briefly, the definition, diagnosis, and incidence of osteoporosis are reviewed, and the consequences of the pathology significantly related to the older adult population are explored. Further, an overview of osteoclast differentiation and the cellular-level effects of Near Infrared Light Emitting Diode (NIR-LED) treatment (i.e., oxidative stress and mitochondrial function) on osteoclast cells (bone-resorbing cells) are presented to familiarize the reader with this study's intervention.

The purpose of this dissertation was to pilot test a new protocol designed to answer the broad research question: Does Near-Infrared Light Emitting Diode (NIR-LED) treatment affect Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) induced osteoclastogenesis in a cell culture model? Osteoporosis is defined as “a disease characterized by low bone mass and structural deterioration of bone tissue, leading to bone fragility and an increased susceptibility to fractures, especially of the hip, spine, and wrist” (World Health Organization scientific Group, [WHOSG], 2003, p. 4) and is the most common bone disorder (National Institutes of Health Consensus Development Panel on Osteoporosis [NIH-CDPO] 2001) Specifically, osteoporosis has been shown to increase the risk for fracture resulting from a fall, especially of the hip (Rizzoli, 2010; Schwartz, Nevitt, Brown, & Kelsey, 2005). The incidence of non-vertebral osteoporotic

fractures has been associated with increased chronological age, increased number of fall risk factors, and reduced bone density and strength (Crepaldi & Maggi, 2005; Cummings et al., 1995). The World Health Organization (WHO) reported in 2003 that osteoporosis-induced fractures burden society due to the great health care costs for treatment and rehabilitation. Reported in 2005, the yearly fracture rate for those individuals in the United States 50 years and old was greater than 20 million, with an estimated cost of \$17 billion which 72% of the expenditures were spent to treat hip fracture (Burge et al., 2007). In 2008, estimates of cost to treat osteoporosis and fractures were reported as \$22 billion (Blume & Curtis, 2011). These experts have estimated that, between 2005-2025, the incidence and economic burden to treat osteoporotic related fractures will increase 50% annually. Due to the current societal demographic growth and rising healthcare costs, it will become imperative to identify individuals at risk for falls and screen for bone loss. The development of additional interventions meant to slow the progression of osteoporosis may contribute to decreasing the burden of osteoporosis-induced fractures in the aging population.

One unique multidisciplinary approach to study skeletal health in the aging population involves teaming with researchers investigating skeletal health in a microgravity environment. Microgravity during space flight simulates complications of muscle (Trappe et al., 2001), and bone loss (Rodionova & Oganov, 2001) often experienced by older adults from prolonged bed rest during hospitalization (Trappe, 2009) which may result in self-limiting behaviors due to the fear of falling (Fletcher & Hirdes, 2004; Van Doorn et al., 2003),

A model for simulating conditions of micro-gravity uses a human bedrest model (Clement & Slenzka, 2006) Research in musculoskeletal health that develops and validates countermeasures taken during long duration space flights involves interventions and procedures designed to mitigate health and performance hazards present in a space environment (Clement, Bukley, & Paloski, 2007). Some researchers investigating bone loss in microgravity environments believe that there is the potential for translational research that could impact the pathology of bone loss in the aging population (Rittweger, 2007). Therefore, continued collaborative interdisciplinary research with the National Aeronautics and Space Administration (NASA) may assist researchers in using new technologies to mitigate bone loss for the older adult population to prevent fracture (Rittweger, 2007).

### **Statement of the Problem**

The following section presents the essence and rationale for investigating osteoporosis. Briefly, an overview of osteoporosis is provided relative to its incidence within the aging population and its contribution to fall induced fractures. The proposed use of NIR-LED as a possible future treatment for the management of osteoporosis, a brief review of bone physiology and pathology, diagnosis as it relates to falls, fractures and frailty and finishes with an overview of the cellular mechanisms of NIR-LED are discussed.

### **Aging Population**

It is projected that by the year 2030, the United States population of older adults will double to 71.5 million 8.9 million of these, will be over the age of 85 (U.S. Department of Health and Human Services Administration on Aging [USDHHS-AOA], 2006) and represent the fastest-growing sector of the U.S. population. Similar

projections apply globally with a world population of individuals over 60 years reaching approximately two billion by the year 2050 (United Nations, 2010). In fact, the number of Americans 65 and older has tripled between 1900-2005 (Friedland & Summer, 2005). According to the Alliance for Aging Research (1999), 6,000 Americans turn 65 each day. Further, it is projected that over the next 19 years, 10,000 Americans will turn 65 each day (Cohn & Taylor, 2010). Over time, this sub-set of the American population overtime will become vulnerable for disability, morbidity, and mortality (Ostir, Ottenbacher, & Markides, 2004).

### **Incidence of Osteoporosis**

In the US, osteoporosis affects approximately 10 million people over the age of 50 years and, of those, 8 million are women. In addition, 34 million Americans are at risk for developing osteoporosis due to low bone mass (osteopenia) (Harvey, Dennison, & Cooper, 2013; A. C. Looker, Melton, Harris, Borrud, & Shepherd, 2010). Due to ethnic differences in bone and mineral metabolism (Norris, Micklesfield, & Pettifor, 2013), osteoporosis is present more often in Caucasian than Hispanic and African American women. Few data exist that report ethnic patterns in men; however trends are similar to women. Bone loss is present more often in Caucasian than Hispanic and African American women.

There has been only a modest decline in the rate of osteoporosis over the past years, despite the development and use of medications and weight-bearing exercise (A. C. Looker et al., 2010). Based upon this fact, current interventions and treatments of osteoporosis have not been effective enough to impact and decrease the prevalence of

osteoporosis. Therefore, research investigating new interventions and treatments needs to be considered.

### **Osteoporotic-Fall Induced Fractures.**

Due to the bone loss and strength, experts have associated the presence of osteoporosis with fall-induced fractures (Schwartz et al., 2005; WHOSG, 2003). Falls are the leading cause of death by unintentional injury for individual 65 (Avdic, Pecar, & Mujic-Skikic, 2004; Centers for Disease Control and Prevention National Center for Injury Prevention and Control [CDCP-NCIPC], 2003; 2005; 2010a)

As individuals age they become susceptible to falling. Falls have been an independent indicator for functional decline and are identified as the causal event for 40% of nursing home admission (Bischoff-Ferrari, 2009; Tinetti & Williams, 1997). Further, a little over a half a million (1.4% of community dwelling older adults) experience a low-impact fall resulting in fracture annually (Morrison, Fan, Sen, & Weisenfluh, 2013). Hip fractures are the most serious osteoporosis-associated fracture resulting in permanent disability (Harvey et al., 2013; WHOSG, 2003; Ziden, Wenestam, & Hansson-Scherman, 2008). In fact, the mortality rate is estimated to be 20%-35% within the first year after suffering a hip fracture (Goldacre, Griffith, Gill, & Mackintosh, 2002; Goldacre, Roberts, & Yeates, 2002).

Further, a hip fracture may be considered the “triggering event” contributing to functional decline, possibly transitioning the individual into a frail state of health (Morley, 2002; Runge & Hunter, 2006; Suh & Lyles, 2003). Half of the people who are ambulatory prior to hip fracture cannot walk independently after the fall (Harvey et al., 2013) and suffer from fear, anxiety and depression, dramatically impacting their quality

of life (Ziden et al., 2008). However, it is unclear if a fall is an antecedent to frailty, or conversely, whether frailty is an antecedent to falls (J.E. Morley, 2002). Therefore, it is important to explore possible interventions to alter the development of osteoporosis with the intent to preserve or rebuild the bone architecture to withstand the impact of a fall, thus avoiding fracture.

### **Proposed use of NIR-LED**

One area to investigate when exploring potential new interventions could be cellular-level mechanisms involved with the pathology of osteoporosis. The intervention employed in this study—near infrared light—already has a large body of research supporting the idea that near infrared light has advantageous effects at the cellular level (Brondon, Stadler, & Lanzafame, 2005; Karu, Piatibrat, & Kalendo, 1987; Karu, 1988; 1999; 2003; Lubart, Wollman, Friedmann, Rochkind, & Laulicht, 1992; Tim et al., 2014; Whelan et al., 2008). However, the literature offers only a limited amount of research studying the effects of near infrared light on bone.

The initial literature search yielded six NIR-LED *in vitro* studies (cell culture model) (Pinheiro et al., 2002; Pinheiro & Gerbi, 2006; Renno, McDonnell, Parizotto, & Laakso, 2007; Stein, Benayahu, Maltz, & Oron, 2005; Yamada, 1991) that investigated *osteoblast* cells (bone-forming cells) and four *in vivo* studies (animal model) (Blaya, Guimaraes, Pozza, Weber, & de Oliveira, 2008; Pinheiro et al., 2008; Pinheiro et al., 2009; Torres, dos Santos, Monteiro, Amorim, & Pinheiro, 2008). Predominately, these investigations explored bone remodeling which would more specifically measure osteoclast and osteoblast interaction. Because we found one *in vitro* study that investigated *osteoclast* cells (bone-resorbing cells) (Aihara et al., 2006), the decision was

made to investigate the effects of NIR-LED on osteoclastogenesis due to this gap in the literature.

## **Background**

Presented below is a brief overview to introduce the literature's main tenets that provided foundational evidence for identifying gaps in the literature. This presentation will allow the reader to understand the dissertation's structure and introduce the main content related to the following subjects: bone physiology, diagnosis of osteoporosis, followed the discussion of osteoporosis as it relates to the incidence of frailty, falls, and fractures in the older adult population. This section will conclude with a more in-depth explanation for the study's intervention, NIR-LED treatment.

### **Bone Physiology**

Bone formation is central to growth and development during childhood and adolescence (Bonjour, Theintz, Law, Slosman, & Rizzoli, 1994). Peak bone mass is complete by early adulthood ((WHOSG, 2003). After the age of 20, bone resorption in males becomes a central process that demineralizes bone at about 4% per decade. Females maintain bone mineral content until menopause, then experience declines of 15% per decade (NIH-CDPO, 2001); WHOSG, World Health Organization Scientific Group (2003).

There are four types of bone cells that contribute to the formation and maintenance of bone: osteoprogenitor cells, osteocytes, osteoclasts, and osteoblasts (Porth, 2009). Osteoprogenitor cells are undifferentiated cells that are the source of all bone cells except osteoclasts which originate from monocyte/macrophages within the hemopoietic lineage. Osteocytes are mature bone cells that maintain the bony matrix. In

addition, they may respond to bone tissue strain and recruit osteoclasts to areas of bone requiring bone repair/remodeling (Lanyon, 1993). There are interconnected passageways called canaliculi that are found throughout the calcified matrix and contain extracellular fluid (Hadjidakis & Androulakis, 2006). These passageways provide a communication network between the neighboring osteocytes and blood system for the exchange of nutrients and metabolites because diffusion does not occur in calcified bone (Porth, 2009).

Osteoclasts and osteoblasts are two types of cells within mineralized bone that contribute to mineral homeostasis (Lemaire, Tobin, Greller, Cho, & Suva, 2004). They are responsible for building and maintaining bone structure. Osteoclasts are bone-resorbing cells that originate from monocyte/macrophages within the hemopoietic lineage, and their activity is regulated by osteoblasts, bone-forming cells, and numerous hormones and other growth factors: macrophage colony-stimulating factor (M-CSF), interleukin-1 (IL-1), and tumor necrosis factor (TNF) (Hock et al., 2001). Osteoblasts are differentiated mesenchymal stem cells, responsible for laying down the new bone matrix that becomes mineralized, thus replacing old bone previously reabsorbed by osteoclasts (Hadjidakis & Androulakis, 2006; Hock et al., 2001).

There are two pathways that induce osteoclastogenesis. On the osteoblast stromal cell membrane, Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) receptor, binds to the osteoclast precursor cell (macrophage) RANK receptor. When this occurs, RANK signaling activates the transcription factor Kappa-B to differentiate into osteoclasts (Kumar, 2010). The other pathway occurs with the osteoblast secretion of M-CSF and the osteoclast precursor cell (macrophage) M-CSF receptor. When joined,

tyrosine kinase activity is stimulated, also important to osteoclast formation. This signaling occurs in tandem with the RANKL/RANK pathway. Further bone marrow stromal/osteoblast cells secrete osteoprotegerin (OPG), a decoy receptor for RANKL, preventing RANKL from binding with the osteoclast precursor cell's RANK receptor, preventing bone absorption and attenuating osteoclast differentiation (Kumar, 2010).

### **Diagnosis of Osteoporosis**

Osteoporosis arises from the out-of-balance osteoclast/osteoblast activity in remodeling bone (Manolagas & Jilka, 1995). In healthy remodeling tissue of the adult, osteoblasts lay down a new matrix that becomes mineralized, thus replacing old bone previously reabsorbed by osteoclasts (Hock et al., 2001). This occurs in both spongy (cancellous) tissue and cortical (dense) bone, which together comprise intact bone; the relative contribution of cancellous and cortical tissues to a given bone varies.

The diagnosis of osteoporosis is made by identification of reduced bone density of vertebral and/or femur cortical and cancellous bone. Using dual energy x-ray absorptiometry (DEXA) scanning, a bone mineral density (BMD) that exceeds 2.5 standard deviations (SD) below the mean BMD found in 30 year old women confirms a diagnosis of osteoporosis. Osteopenia is identified by 1 to 2.5 SD below that same mean.

From 2005-2006, for older adult women in the U.S., the incidence of osteopenia and osteoporosis was 49% and 10%, respectively (A. C. Looker et al., 2010). Among older adult men, 36% had osteopenia, and 2% had osteoporosis (A. C. Looker et al., 2010) estimating that 5.3 million older men and women had osteoporosis at the femur neck and an additional 34.5 million had osteopenia. In data gathered by NHANES in 2005-2006, femur neck osteoporosis prevalence was lower by three percentage units in

men and by seven percentage units among women compared to the 1988-1994 data (Looker et al., 2010). Decreased prevalence in the 2005-2006 data may be attributed to differences in DEXA technology even though the same manufacture scanner was utilized. The 1988-1994 study, a first-generation pencil-beam scanner was used while the 2005-2006 study a third-generation scanner was employed. The authors attempted to adjust the data to control for the scanner change, but the first generation scanner had few published studies with direct comparison to the third generation scanner, and reported mixed results of significant differences. Further, correlations for the decline in femoral osteoporosis were not strongly associated with changes in body mass index or the use of osteoporosis medication. When combining the incidence of osteoporosis and osteopenia from the 2005-2006 data compared to the 1988-1994 data, more older adults had low femur neck BMD in 2005-2006, possibly due to the increased aged population (Looker et al., 2010). Regardless of the decline in prevalence, the estimates of how many older adults experience these conditions remain high.

### **Falls, Fractures, and Frailty**

Because falls are the leading cause of death by unintentional injury for individuals 65 and older (Avdic (Avdic et al., 2004), Pecar, & Mujic-Skikic, 2004; Centers for Disease Control and Prevention (Centers for Disease Control and Prevention National Center For Injury Prevention and Control [CDCP-NCIPC], 2005; 2010a; 2010b; Centers for Disease Control National Center for Injury Prevention and Control Office of Statistics and Programming, 2003) mortality rate has also increased over the past decade (Stevens, 2006). Bone loss (osteoporosis) has been associated with falls and fractures (Sinaki et al., 2002; Suh & Lyles, 2003; WHOSG, 2003) and a little over a half a million low impact

falls result in osteoporotic fractures (Morrison et al., 2013). In 1999, in the European Union, the Osteoporosis Foundation reported that every 30 seconds an individual has a fracture resulting from osteoporosis (World Health Organization, 1999).

Vertebrae are particularly rich in cancellous tissue (spongy), and the hip and wrist are rich with cortical bone (dense) (Wynghaarden, Smith, & Bennett, 1992). Vertebral compression fractures are the most common osteoporotic bone injury often experienced by women 45-60 years of age occurring in the early stages of the bone loss disease process. They occur asymptotically while performing normal activities of daily living such as lifting, and occur three times more often than hip fractures (Harvey et al., 2013).

As the bone loss disease process progresses, cortical composite weakening occurs and the prevalence of wrist and hip fractures become apparent (Wynghaarden et al., 1992). A wrist fracture most commonly occurs when a women falls sideways or backwards and uses her arm to break the fall, thus breaking their wrist (Melton & Cooper, 2001). A fall induced hip fracture is the most serious osteoporosis-associated incidental fracture resulting in permanent disability (Harvey et al., 2013; WHOSG, 2003). Hip fractures most commonly occur at the femoral neck resulting from landing directly on the hip during a fall (falling sideways) rather than falling forward (NIH-CDPO, 2001). As early as 1990, the WHO declared that hip fracture may become a worldwide epidemic by the middle of the 21<sup>st</sup> century, increasing from an incidence of 1.7 million in 1990 to a projected 6.3 million by 2050 (C. Cooper, Campion, & Melton, 1992). Therefore, impaired bone strength predisposes older adults to fracture when they fall and may lead to the development of frailty (Runge & Hunter, 2006; Suh & Lyles, 2003). This transition

may be acute if it stems from a “triggering event,” such as a hip fracture (Hamerman, 1999).

Frailty is often described as a decline in the functioning of the neuromuscular system, abnormal aging, vulnerability to adverse health outcomes, and loss of physiological reserve to handle minor stresses (Abate et al., 2007; Hamerman, 1999; Kinney, 2004; Morley, 2002) and is most frequently identified in older adults over 85 years of age (Suzman, Willis, & Manton, 1992). The current prevalence of frailty in persons aged 65 and older ranges from 4.0% to 17% (Collard, Boter, Schoevers, & Oude Voshaar, 2012).

As the number of older adults increases, fall-risk will increase, as well as the incidence of fall induced osteoporotic fractures. There is an extensive body of literature concerning the study of bone loss and interventions to prevent bone loss over that past 15 years; however the incidence of osteoporosis remains relatively unchanged. Thus, investigating innovative technologies that may improve bone cell function and overall skeletal health in this population remains critical to preserve an older adult’s quality of life is important.

### **Near-Infrared Light Emitting Diodes**

Presented are a brief overview of the use of Near-Infrared Light Emitting Diodes (NIR-LED) and rational for the proposed method of experimentation in a cell culture model. A short review of associated tenets of aging affecting bone health, oxidative stress, mitochondrial dysfunction and increased apoptosis is presented broadly and then related to the aging skeleton. This section will end with the research questions

formulated to begin a program of research investigating a potential intervention for the treatment and management of osteoporosis.

The spectrum of electromagnetic radiation (ER) includes light and ranges from radio waves to gamma rays. Light has amplitude (brightness), and wavelength defines the color and angle of wave vibration. According to quantum theory, ER contains particles called photons, packets of energy that move at the speed of light (Chung et al., 2012). Typically a laser output is a narrow beam of light often named a “pencil beam” (Chung et al., 2012). The peak irradiance is greatest at the center of the beam and dissipates outward.

Light Emitting Diodes (LED) are semiconductors of light and emit low-intensity red light in ultraviolet and infrared wavelengths (Chung et al., 2012). The mechanism that occurs within the diode releases energy in the form of photons (electroluminescence and color of light). An LED is less than  $1\text{mm}^2$  in diameter, thus, LED devices are made with numerous LED lights. The optical window of low level laser light treatment (LLLT) is in the red and near-infrared wavelengths (600–1070 nm). Wavelengths that effectively treat superficial tissues range between 600 and 700 nm and deeper tissues require longer wavelength ranges between 780–950 nm. Wavelengths that are longer range, that is, between 700–770 nm, have little biological effect (Chung et al., 2012). Initial research used a Helium Neon (HeNe) laser at a wavelength of 632.8-nm. Most recently semi-conductor diode lasers such as gallium arsenide lasers (GaAs) are used. It is unknown if there really is a difference between lasers and LEDs.

In search of innovative technologies for interventions that may improve bone cell function and skeletal health, particularly those that would inform the understanding of

osteoclast differentiation (bone-resorbing cells), the pilot use of Near Infrared Light-Emitting Diodes (NIR-LEDs) photobiomodulation was proposed as a possible future intervention to treat bone loss and/or preserve bone health. Although the focus of this study was to determine the effects of photobiomodulation upon osteoclastogenesis, it is important to understand the cellular effects noted in the NIR-LED literature for future research questions that may be appropriate to determine cellular effects in osteoclasts.

The theory of biological aging related to human aging has been associated with cellular oxidative stress (OS) and mitochondrial dysfunction (Melov, 2000), and these may induce bone loss (Srinivasan & Avadhani, 2007). Thirty years ago, somatic genetic errors, mutations of the nuclear genome, were postulated as a basis for aging (Hayflick, 1985b; Shock, 1985) by negatively altering cell metabolism, resulting in free radical oxidative stress (ROS) (Hayflick, 1985a). Research about mitochondrial DNA (mtDNA) indicates that mutations of the mitochondrial genome are vulnerable to oxidative damage (Linnane, Marzuki, Ozawa, & Tanaka, 1989) and may cause degenerative diseases and aging.

Cell mitochondria, which are producers of cell energy, regulators of apoptosis—a naturally occurring process of programmed cell death—and cell function, are vulnerable to OS and have an essential role in accelerating cell death (Dirks & Leeuwenburgh, 2005; Joza et al., 2005). Oxidative stress can result from the generation of excess free radicals (Beckman & Ames, 1998) which causes an imbalance with cellular antioxidants that, in turn, damages cellular components and function. Specifically, free radicals are highly unstable and reactive with other molecules, and can instigate oxidative cell destruction. The oxygen molecule has an atypical placement of electrons within electron orbitals and

oxygen prefers to accept one electron at a time during chemical reactions, rather than two electrons. Accepting two electrons at a time would keep the oxygen molecule in a stable state. When electrons are accepted one at a time, the oxygen molecule becomes unstable; and superoxide ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ) are generated. These reactive oxygen species undergo further biochemical cellular reactions, and results the production of extremely reactive molecules such as hydroxyl radicals ( $OH^-$ ) (Beckman & Ames, 1998) affecting mitochondrial function, and cellular energy production.

The aging skeleton is associated histologically by bone wall thinning due to a decreased bone matrix depositing (Almeida & O'Brien, 2013). This is thought to be due to an insufficient number of functioning osteoblasts available for mineral deposit related to decreased bone marrow mesenchymal stem cells (MSC) (precursor osteoblast and adipocyte cells), poor differentiation of osteoblast progenitor cells or more progenitor cells utilized for adipocyte formation rather than osteoblastogenesis, as well as apoptosis (cell death) (Almeida & O'Brien, 2013; Muruganandan, Roman, & Sinal, 2009). The loss of the ability for MCS cells to regenerate due to aging leads to a diminished compensation to stress and increase reactive oxidative species. The use of NIR-LED may decrease oxidative stress and improve mitochondrial function by affecting COX-IV, a specific segment of the cell's energy production system, thus enhancing cell function (Karu, 2008).

The use of NIR-LED (630-1000nm) photobiomodulation—an FDA-approved treatment—has been found to be effective in improving wound healing (Whelan et al., 2003), bone regeneration (Pinheiro et al., 2009), mitochondrial function (J. T. Eells et al., 2004; Wong-Riley, Bai, Buchmann, & Whelan, 2001), attenuating cellular oxidative

stress (Desmet et al., 2006; Karu et al., 1987), decreasing inflammation and pain, aiding in recovery of ischemic cardiac injury (Oron, 2006; Oron et al., 2001), and attenuating retinal/optic nerve degeneration (Eells et al., 2007; Eells et al., 2004; Eells et al., 2003; Liang et al., 2006). Reducing oxidative stress delays the activation of the apoptotic signaling pathways, thus preventing bone loss and mitochondrial dysfunction (Banfi, Iorio, & Corsi, 2008). Mitochondrial dysfunction has been reported in several slow-dividing tissue types such as central nervous system, cardiac, skeletal muscle, and liver tissues (Nagley et al., 1992; Varanasi, Francis, Berger, Papiha, & Datta, 1999). Investigators have suggested that mitochondrial dysfunction and sarcoplasmic reticulum stress may stimulate apoptosis and alter the mechanical signaling in bone (Booth & Criswell, 1997; Hock et al., 2001).

Photobiomodulation, also referred to in the literature as low-level laser therapy, cold-laser therapy, or laser biostimulation, was introduced in the 1980's. Laser light-treatment uses light in the infrared to near-infrared region of the absorption spectrum (630-1000nm), as this range is known to affect numerous cell functions that are dependent on the energy production in the mitochondria (Desmet et al., 2006; Karu et al., 1987). (Karu (1988) ) postulated that the mechanism of the effect of a low-power laser is at the cellular level due to changes in components of the electron transport chain system within the mitochondria.. The electron transport system of the cell is directly related to the production of Adenosine triphosphate (ATP), the primary energy compound for the cell.

The role of the electron transport chain oxidation reaction pathway (redox) transfers electrons and signals synthesis of apoptotic proteins activating the caspase-3

pathway (Hock et al., 2001; Karu, 1999). This cascade is responsible for signaling apoptosis (programmed cell death). It occurs when the signal leaves the mitochondria, fueling cytoplasmic transcription factors, which upregulates cytoprotective and antioxidant genes that are thought to prevent free radical damage from oxidative stress. This increased transcription preserves cell survival (Eells et al., 2007; Karu, 2003; Squier, 2001). The mechanism of action may be through release of nitrous oxide or initiating superoxide signaling. Further, transcription factor nuclear factor B (NF- $\kappa$ -B), regulated by changes in cellular redox state, is activated with low level light treatment producing protective and stimulatory gene products (Huang, Sharma, Carroll, & Hamblin, 2011). Light treatment may improve oxidative metabolism and mitochondrial function, and preserve cell life. It still remains unknown if the activation of macrophage nuclear factor B (NF- $\kappa$ -B) is helpful in attenuating osteoclastogenesis.

In order to study light treatment, researcher must understand how to calculate the correct irradiation parameters. To define LLLT according to the “medicine” (irradiation parameters) and “dose” (irradiation time) standards, delivery is a key to understanding and developing interventions using light treatment. Energy Density  $\text{J}/\text{cm}^2$  often reflect the dose of treatment, however, energy has two components, power and time. Energy (J) = Power (W) x Time (s). There is reciprocity between the variables meaning that if the power was doubled and the time is cut in half the same energy is delivered but a different biological effect would be seen (Huang et al., 2009). It is best to describe the parameters separate, irradiation parameters and dose. This study used a continuous wave NIR-LED with a wavelength of 670nm, power intensity of  $60\text{mw}/\text{cm}^2$  delivering and energy density of  $4.5 \text{ J}/\text{cm}^2$  and  $45\text{J}/\text{cm}^2$  in 80 and 800 seconds, respectively.

## Methods

### Cell Culture Model

A cell culture model was chosen because a proposal to test any kind of intervention in a vulnerable population of older adults gives rise to many ethical considerations. For example, directly extracting bone cells in a human model by a researcher would be an invasive and painful procedure (WHOSG, 2003). Interventions that alter the cellular function of bone cells require a careful progression of research.

Thus, to gain an understanding of the efficacy and safety of a technology that is known to alter cellular functioning, an appropriate place to begin would be in a cell culture model because an *in vitro* method is economical compared to animal and human models, easily implemented for experimental replication to interpret data for protocol development determining dose response curves, and is exempt from Institutional Review Board submission. This research was undertaken using a cell culture model with RAW 264.7 animal cell line derived from mouse leukemia monocyte macrophages. These cells are precursor osteoblastic cells (macrophages). This particular cell line requires the induction of Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) in order to promote osteoclast formation. The induction method occurs when osteoblast stromal cell membrane RANKL receptors, bind to the osteoclast precursor cell (macrophage) RANK receptors. When this occurs, RANK signaling activates the transcription nuclear factor Kappa-B to differentiate into osteoclasts (Kumar, 2010).

Two manufactures of RANKL (Sigma<sup>TM</sup> & PeproTech<sup>TM</sup>) were tested to determine the best ligand to induce osteoclastogenesis because of poor osteoclast cell counts in preliminary experiments. In order to determine if cellular response occurred

during RANKL suspension, a vehicle control of Bovine Serum Albumin (BSA) was tested and was found to be negative and the stability of the RANKL was preserved.

Based upon results of robust cell culture experiments, progressing the research into an animal may be advantageous to begin using primary cells obtained directly from experimental animals and, ultimately, if the intervention were deemed safe and beneficial at the cellular level—a human model may be appropriate to further bridge research from bench to bedside using non-invasive measurements of bone-mineral density. In other words, if the outcomes from the cell culture model warrant positive effects, the research could progress to experimentation in animals. In an animal model, the researcher is able to use skeletal remains, thus enabling direct maceration of a whole bone in order to flush and extract bone cells which are closer to human cells for study (Rittweger, 2007). If the experimental treatment is deemed efficacious and safe in animals, then it may be undertaken in human model.

In order to understand maximal doses for RANKL and energy density dose response curves were conducted in the experiments. Biphase response has occurred in low level light treatment (LLLT) research (Huang et al., 2009) however, a biphasic dose response curve for RANKL is lacking in the literature. The “Arndt-Schultz Law”, the model to describe dose dependence, states a weak stimuli will induce slight activity, while a stronger stimuli will induce activity higher, but once a peak is reached (minimal threshold) an even stronger stimuli will suppress the activity until it has a negative response (Huang et al, 2009). Therefore, the biphasic curve, described as insufficient energy, is applied there will be no response, if more is provided then the minimal threshold is crossed, but if too much energy is given, the response will disappear. Huang

and colleagues (2009) report that energy density ( $\text{J}/\text{cm}^2$ ) is used to describe the light treatment dose, however, the energy density has two components, power and time. They recommend describing the light treatment as two variables, the medicine (irradiation) and the time (dose).

### **Purpose**

Our primary goal in this study was to determine the effects of NIR-LED treatment on osteoclast cell differentiation (osteoclastogenesis) in cell-cultured RAW264.7 cells using the WARP 75™ NIR-LED light. Our specific research questions and hypotheses were as follows.

**Question 1.** Is there a difference in osteoclast cell count between single light-treated Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL)-induced cell cultures as compared to non-light-treated controls? **Hypothesis.** Single light-treatment of RANKL-induced cell cultures will impair osteoclastogenesis.

**Question 2.** Is there a difference in osteoclast cell count between light-treated RANKL-induced cell cultures treated at different energy densities of light-treatment ( $2.25$ ,  $4.5$  and  $45 \text{ J}/\text{cm}^2$ ) compared to non-light-treated controls? **Hypothesis.** Light-treatment of RANKL-induced cell cultures at a higher energy density ( $45 \text{ J}/\text{cm}^2$ ) will stimulate less osteoclastogenesis compared to lower energy densities ( $2.25$  and  $4.5 \text{ J}/\text{cm}^2$ ).

**Question 3.** Is there a difference in osteoclast cell count between single light-treated RANKL-induced cell cultures compared to multiple light-treated RANKL-induced cell cultures? **Hypothesis.** Multiple light-treatments of RANKL-induced cell cultures will stimulate osteoclastogenesis less compared to single light-treated RANKL-

induced cell cultures.

**Experimental Control Research Question- Dose Response Curve:** Is there an expected biphasic dose dependent response curve in RANKL induced (0ng/ml, 2.5ng/ml, 10ng/ml, 25ng/ml and 50ng/ml) cell cultures? **Hypothesis:** Lower dose RANKL-induced cell cultures will have lower osteoclast cell counts than higher dosed RANKL cell cultures until the minimum dose threshold is reached then osteoclast cell counts then will decrease inversely.

### **Implications**

Conducting research brings a strong commitment and responsibility to disseminate results to impact public policy, nursing practice, and research. Nursing historically is rich with political activism. From the beginning of nursing's evolution pioneers such as Florence Nightingale and Lillian Wald began over a century ago to improve deplorable hospital conditions and public health for all (Backer, 1993; Nightingale, 1989). Nursing in the past has also been dominated by the political influences of medicine. Due to subsequent professional conflicts, advocacy for vulnerable populations at times was challenging (Mechanic & Reinhard, 2002). As nursing has become more autonomous and has experienced professional growth, progress is made working collaboratively with medicine and other bench scientists through multidisciplinary professional organizations, advanced practice nursing, clinical practice settings and public policy to bridge new partnerships.

### **Nursing Practice and Research**

Nurses are interested in investigating new interventions to prevent or treat osteoporosis because of its primary commitment to the patient, as nurses advocate and

protect the health and safety of patients, as well as improve the quality of care (American Nurses Association, 2001). Nurses recognize evidence based practice (EBP) as the gold standard for providing safe and compassionate care (Brown, Wickline, Ecoff, & Glaser, 2009). Evidenced based practice is recognized by the American Nurses Credentialing Center's Magnet programme as key component for quality care (McClure & Hinshaw, 2002). Nurses many times are the most prevalent and consistent health care provider that older adults encounter throughout their health care service delivery. Nursing has the opportunity to identify individuals at risk and implement timely interventions to prevent disease or implement the most beneficial appropriate intervention for the patient. This type of interventional research is the first step in moving bench science to the bedside.

Per the National Institutes of Health, translational research can be defined as a two stage process: the application of innovative laboratory research into preclinical studies to design human clinical trials and translation with implementation of best practices into the community objectives to achieve cost-effectiveness in prevention and treatment (National Institutes of Health, 2007). Research is transferred from the laboratory's bench (basic research) to the patient's bedside (clinical research) then clinical study and trial findings transferred into the community and practice settings to improve health outcomes (Rubio et al., 2010).

### **Public Policy**

As stated previously, the older adult population is aging in great numbers every day. In fact, fracture totals in 2005 were estimated to be 20 million with a cost to treat \$17 billion, and estimated by 2025, fracture rate and costs will increase 50% (Burge et al., 2007). This program of research has one main public policy implication to discover

interventions to treat and manage osteoporosis in an effort to preserve, re-build and strengthen bone architecture. This in turn may ultimately decrease the incidence of fracture rate and impact cost containment to treat osteoporotic-induced fractures. Once significant benefit is established, the medical device must be approved by the Federal Drug Association (FDA). Choosing the use of an FDA approved device avoids the arduous process of establishing efficacy and safety. NIR-LED light used in this study is an FDA approved device invented by Quantum Devices Incorporated (Barneveld, WI) and was developed as a NASA technology entitled High Emissivity Aluminiferous Lighting Substrate <sup>TM</sup> (HEALS), to grow plants for long-term space flight. Receiving FDA approval, they successfully developed a commercial device; the Warfighter's Accelerated Recovery by Photobiomodulation (WARP 10), a portable Near Infrared Light Emitting Diode (NIR-LED), used in combat to provide first aid for minor injuries and pain and received FDA approval. In 2000, they received a Space Technology Hall Of Fame Award for their *Innovation of Light Emitting Diodes for Medical Applications* (National Astronautical Space Agency, 2008; Quantum Devices Incorporated, 2004).

Nursing and NASA technology have common goals, the care of astronauts and patients. Since 1976, 1,300 space related technologies have been applied to industry, daily life, and patient care (Plush & O'Rangers, 2004). Such examples include: cellular phones and personal data assistants (PDAs), CT scanners/MRI, as well as the ventricular assist device, a miniature heart pump based upon a NASA fuel pump which was developed by Michael DeBakjey, MD (Plush & O'Rangers, 2004). Further, the translation of technology has been made in this current study, as the NIR-LED was developed for NASA to grow plants during space flight (National Astronautical Space

Agency, 2008). Given time and funding, this proposed new intervention to either prevent or treat osteoporosis has promise.

### **Future Chapters**

The subsequent chapters of this dissertation are comprised of three manuscripts on the topics of osteoporosis and current interventions as well as a summary chapter. Chapter Two, the review of the literature, is divided into two sections; thus, two review manuscripts were written according to submission guidelines for *MEDSURG NURSING: Official Journal of the Academy of Medical-Surgical Nurses*. Manuscript One, *New Insights-Understanding the Etiology and Consequences of Osteoporosis*, provides an overview of the definition, significance, incidence and pathophysiology for osteoporosis. This is followed by a discussion regarding outcomes of this pathology, osteoporotic induced fractures and frailty. Manuscript two *Nursing Implications for Osteoporosis Management*, reviews current nursing implications for the generalist nurse and recommendations for nursing process, as well as pertinent osteoporosis medical management to inform the nurse's knowledge base.

The experimental bench research protocol development at the NASA Ames Bone and Signaling Laboratory that was consequently used for the experiments in the final manuscript is described in chapter III. The specific protocols are found in Appendix A-G, tables Appendix H and letters of permission Appendix I. Further, the preliminary data that were collected during the piloting of the experimental protocols are presented in this chapter.

In Chapter IV, the third manuscript, experiment pilot data, considered the dissertation study as described. This manuscript was presented at the 62<sup>nd</sup> International

Astronautical Congress: African Astronaissance, Cape Town, South Africa, on October 3-7, 2011. Lastly in, the relationship between manuscripts is reviewed. Findings are discussed, conclusions and limitations highlighted, and directions for future research offered.

## **CHAPTER TWO: REVIEW OF THE LITERATURE**

This chapter is divided into two sections comprised of two manuscripts prepared for submission to *MEDSURG NURSING: Official Journal of the Academy of Medical-Surgical Nurses*. These manuscripts comprise a two-part series. The first manuscript, entitled *New Insights-Understanding the Etiology and Consequences of Osteoporosis* and the second, entitled *Nursing Implications: Osteoporosis Management* set the stage for the dissertation reported in Chapter Four. The first manuscript provides the context by clarifying definitions, highlighting the prevalence, reviewing normal physiology and pathology of bone, factors to consider for making the diagnosis of osteoporosis, and osteoporosis health outcomes. Manuscript two is a review of current nursing implications for the generalist nurse with recommendations for nursing interventions, new insights in osteoporosis management.

### **Manuscript One**

#### **New Insights-Understanding the Etiology and Consequences of Osteoporosis**

##### **Abstract**

Osteoporosis is a growing worldwide public health problem given the increasing numbers of individuals over the age of 65, affecting 75 million people in Europe, Japan and the United States. Osteoporosis, described as compromised bone strength, is a silent epidemic occurring virtually unnoticed, associated with staggering consequences of osteoporotic induced fractures, specifically, hip fracture. In the near future, due to primary care provider shortages, generalist clinicians will be responsible to provide the majority of health care to those at risk for this condition. It is important that generalist nurses become familiar with the basic underpinnings associated with this disease process.

The purpose of this review is to provide the generalist nurse with the most current definition, an overview of bone physiology and pathology, including its causes, and summarizes the negative health outcomes, including the societal financial burden. Further, new insights in osteoporosis management for evaluation of bone architecture and strength, new medications, and new therapies; Near-Infrared Light Emitting Diodes (NIR-LED) treatment and whole body vibration (WBV) therapy are introduced.

### **Introduction**

Osteoporosis, the most common bone disorder (National Institutes of Health Consensus Development Panel on Osteoporosis [NIH-CDPO], 2001) is quickly becoming a worldwide public health problem (International Osteoporosis Foundation, 2013). Given the increasing numbers of individuals over the age of 65 and that it affects 200 million people (Burge et al., 2007; Colón-Emeric, 2013). Older adults with low bone mineral densities (BMD) are at risk for fracture when they fall. Therefore, the risk of osteoporotic-induced fractures, specifically those of the hip, increases as persons age (Rizzoli, 2010). Hip fractures experienced by the older adult population are one of the most serious sentinel events that are associated with overall morbidity and mortality, and are associated with high treatment costs to the health care system. It is projected that by the year 2030, the US older adult population will double to 71.5 million, 8.9 million of whom will be over the age of 85 (U.S. Department of Health and Human Services Administration on Aging, 2006), thus being the fastest-growing sector of the U.S. population. Similar projections apply globally with a world population of individuals over 60 years reaching approximately two billion by the year 2050 (United Nations, 2010). Over time, this older adult population will become vulnerable for disability,

morbidity, and mortality due to aging (Ostir, Ottenbacher, & Markides, 2004). The focus of this manuscript is on individuals over aged 50 years.

Osteoporosis-induced fractures burden society by over-use of available medical resources (Burge et al., 2007). In 2008, estimated US costs to treat osteoporosis and fractures was reported as \$22 billion (Blume & Curtis, 2011). Within a year of fracture, one third of cases are admitted to long-term care; of these, 20% are found to be frail (Orsini et al., 2005). In 2005, the cost associated with two million fractures was estimated to be \$17 billion, of which 72% of the expenditures were spent to treat hip fractures. Costs are projected to increase approximately by 50% (Blume & Curtis, 2011; Burge et al., 2007). However, these figures do not take into account indirect costs such as lost wages or the decrease productivity of caregivers or the post-fracture medical management expenditures (NIH-CDPO, 2001). Kilgore et al. (2009) reported the average cost to treat a wrist fracture was \$7788 and an open hip fracture \$31,310 (Iowa Foundation for Medical Care, 2008). Given current societal demographic growth and rising healthcare cost it will become imperative to identify individuals at risk for falls, screen for bone loss and implement appropriate tailored interventions to improve bone loss and prevent falls. This in turn will ultimately impact morbidity and mortality outcomes and conserve medical resources. In order to achieve these objectives, nurses must build a foundation of knowledge stemming from the basic physiological and pathological tenants associated with bone loss.

Osteoporosis is a silent epidemic because it occurs without symptoms or pain. While considerable literature exists on this topic (European Foundation for Osteoporosis and Bone Disease & National Osteoporosis Foundation, USA [EFOBD & NOFUSA],

1997; National Guideline Clearinghouse [NGC], 2006 [Revised 2010]; North American Menopause Society [NAMS], 2010; World Health Organization Scientific Group [WHOSG], 2003), synthesizing the literature can be time consuming. Therefore, the purpose of this critical review of the literature is to provide the generalist nurse with the most current definition, an overview of bone physiology and pathology, causes, and health outcomes. Fall-induced fractures, especially hip fractures, are highlighted given their association with osteoporosis, in terms of their health, social, and financial toll. Lastly future proposed cellular level physiological research utilizing near-infrared light emitting diode treatment (NIR-LED) that may impact reversing, preventing, or slowing bone loss is discussed. Nursing implications are discussed in another manuscript.

### **Method**

An extensive search of the literature published between 2000 – 2013 was conducted focusing on osteoporosis, its etiology, and societal and health outcomes. Databases searched included PubMed, CINAHL, Academic Search Complete/EBSCO, Medline, National Guideline Clearinghouse, Google Scholar, Primo Central/Ex-Libris, as well as references from articles and books. Moreover, a Google web search was employed to gather full text copies of standards and guidelines. Seminal articles prior to 1999 were subsequently authenticated from the extrapolated literature. Expert identified literature also was reviewed. Of note, the *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, eighth edition* which is distributed to the entire membership of The American Society for Bone and Mineral Research, was utilized to as the source of leading expert bone health researcher opinions (Rosen, 2013).

The following key words were used: bone, in combination with physiology, pathology, remodeling, architecture, apoptosis, osteoblasts, osteoclasts, bone turnover, NIR-LED, low level light laser therapy, and whole body vibration treatment. The term osteoporosis was searched in combination with epidemiology, definition, guidelines, position statement, primary and secondary causes, risk factors, cost analysis, fall induced fractures, hip fracture/risk, falls and frailty and was limited to English. However, the main focus of the search was on the age group of 50+ years.

The search, when feasible, was limited to meta-analyses (2), systematic reviews (4), clinical cohort studies (18), epidemiological studies (11), professional guidelines (1), position statements (6), government reports (8), theory (3), reviews of literature (29), bench research (10), articles from peer-reviewed journals (75), professional texts (14), and one qualitative study. Whenever possible, each article was critically evaluated for quality and reliability using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist as a guideline to evaluate academic peer-reviewed articles (Moher, Liberati, Tetzlaff, Altman, & The Prisma Group, 2009).

### **Findings**

Findings are summarized in the following order: definition, incidence, and significance for osteoporosis followed by an overview regarding bone physiology and pathology associated with bone demineralization, identifying the primary and secondary cause of osteoporosis, negative health outcomes, and societal financial burden. Lastly, new alternative osteoporosis identifying diagnostics, medications and bioengineering treatment such as NIR-LED treatment and whole body vibration (WBV) therapy are discussed.

## **Definition and Diagnosis for Osteoporosis**

The definition of osteoporosis, evolving over time through a series of professional position statements, is often described as compromised bone strength (EFOBD & NOF-USA, 1997; NIH-CDPO, 2001). It is defined conceptually as a skeletal disease characterized by low bone mass and structural deterioration of bone tissue, leading to bone fragility and an increased susceptibility to fracture, especially of the hip, spine, and wrist (EFOBD & NOFUSA, 1997; NIH-CDPO, 2001).

The World Health Organization (WHO) defines osteoporosis by diagnostic assessment of bone mineral density (BMD) measurements that categorize the degree of bone loss (WHOSG, 2003). The diagnosis of osteoporosis is identified by reduced bone density of vertebral and/or femur cortical and cancellous bone. Using dual energy x-ray absorptiometry (DEXA) scanning, reported as grams of mineral per area volume, a bone mineral density (BMD) that exceeds 2.5 standard deviations (SD) below the mean BMD found in 30 year old women, diagnoses osteoporosis (EFOBD & NIHUSA, 1997; Kanis, 1994; NIH-CDPO, 2001; WHOSG, 2003). (see Table 1)

## **Incidence**

In the US, osteoporosis affects approximately 10 million people over the age of 50 years of those, 8 million are women. In addition, 34 million Americans are at risk for developing osteoporosis due to low bone mass (osteopenia) (Harvey, Dennison, & Cooper, 2013; A. C. Looker, Melton, Harris, Borrud, & Shepherd, 2010). The incidence of osteoporosis is more common in women than men due to postmenopausal hormonal changes and a longer life expectancy (NIH-CDPO, 2001; WHOSG, 2003).

Due to ethnic differences in bone and mineral metabolism (Norris, Micklesfield, & Pettifor, 2013), osteoporosis is present more often in Caucasian than Hispanic and

African American women. Few data exist that report ethnic patterns in men; however trends are similar to women. When comparing incidence rates of osteoporosis and osteopenia between 2005-2008, 9% of non-institutionalized 50 year old Americans had osteoporosis of either the femur neck or lumbar spine and approximately 50% had osteopenia (Looker, Borrud, Dawson-Hughes, Shepherd, & Wright, 2012).

### **Bone Anatomy**

The generalist clinician should have an appreciation of bone anatomy and physiology so that he or she can practice knowledgeably and understand how interventions to prevent and treat osteoporosis affect these physiologic pathways. Bone is best described according to its function, architecture, and tissue type. Bone remodeling occurs throughout the lifespan and has a specific systematic process in reabsorbing and replacing bone.

**Function.** Anatomically, the function of bone supports the body while standing erect, protects vital organs, and assists ambulation and movement through muscular system linkages. Physiologically, bone is responsible for the manufacture of blood cells in the bone marrow and serves as a reservoir for phosphorus, magnesium, and 99% of the body's calcium. Further, the bone's ability to maintain metabolic function is achieved by mineral homeostasis through acid-base balance (Boskey & Robey, 2013; Hadjidakis & Androulakis, 2006; Sherwood, 2013).

There are two parts to the skeletal system: the axial skeleton (the cranium, thorax and vertebral column), and appendicular skeleton (upper and lower extremities) (Porth, 2009). Every bone has an extracellular matrix (ECM) which is comprised of two main components: mineral composite and collagen, as well as water, proteins, and lipids

(Boskey & Robey, 2013). The mineral composite is made of hydroxylapatite, which are mainly calcium phosphate salts, providing strength via crystallization around the collagen fibers in the ECM. Collagen type I is the basic building block of the bone matrix and contributes to bone strength (Sherwood, 2013).

**Bone Architecture.** There are two types of bone matrix: cancellous (spongy) bone and cortical (compact) bone. Both types of bone matrix are found in all bone; however, the relative contribution of cancellous and cortical tissues to a given bone varies (Porth, 2009). Cancellous bone, found within the bone's inner core, comprises 20% of the skeleton, and is often referred to as trabecular bone because trabeculae form a lattice pattern (Adler, 2000; Hadjidakis & Androulakis, 2006). The lattice is lined with bone marrow and osteogenic cells and is fairly light, but able to withstand tensile strength for weight bearing (Porth, 2009; Seeman, 2008), 2008). Cancellous bone is described as being elastic compared to cortical bone (Hadjidakis & Androulakis, 2006). Vertebrae are particularly rich with cancellous or trabecular bone. Cortical bone comprises approximately 80% of the skeleton. It is a rigid, densely calcified, and compact matrix that forms the bone's outer shell, and it is the predominate type of tubular bones found in the upper and lower extremities, hip, and wrist. Structurally, cortical bone is more resistant to bending and torque (Hadjidakis & Androulakis, 2006; Porth, 2009).

Two important considerations in evaluating the bone quality are bone structure and strength. Bone structure determines the energy load that bone can withstand, and the energy load in turn affects the bone structure through adaptation by modeling and remodeling bone tissue in response to the energy load (Seeman, 2008). Currently, bone quality remains indefinable by current dual energy x-ray absorptiometry bone mineral

testing (DEXA BMD) testing. Because spine and hip Bone Mineral Density (BMD) can estimate approximately 70% of bone strength (Suh & Lyles, 2003), newer technologies for measurement of bone quality may be achieved through areal BMD measurement using computed tomography (Genant et al., 2008; MacNeil, Boyd, MacNeil, & Boyd, 2008). BMD, however, does little to help the clinician evaluate bone architecture. It is important to understand that an increase in BMD does not always render improved bone quality, a result that may affect true bone strength considerably. Recently, trabecular bone scores (TBS) are being researched as an added measurement and may become a standard measurement with traditional DEXA testing (TBSiNsight, 2014).

Bone architecture determines the energy load that a bone can withstand, and the energy load in turn affects the bone architecture through adaptation. Remodeling bone tissue responds to the energy load exerted upon that bone structure (Seeman, 2008). The greater the energy load, the greater the rate of bone deposition or density and excess load beyond bone tensile strength results in fracture (Sherwood, 2013). Therefore, weight bearing exercise is imperative for bone architecture. Moreover, in order for one to stand erect against gravity, the bones collectively must function like levers; they must be stiff and able to resist buckling when absorbing energy, such as a spring (Seeman, 2008). Bone acting as a “spring” must change shape without cracking, must shorten or widen according to the compression that the energy load expends, must lengthen or narrow in tension, and remain light enough to allow for rapid motion (Seeman, 2008). If bone is too stiff or too flexible, however, bone fragility may occur

**Bone Tissue and Cells.** There are two types of bone tissue: woven and lamellar. Both types can be either mineralized or unmineralized (osteoid). Woven bone is not

strong and is rapidly deposited as a temporary structure during fracture healing. Lamellar bone is matured bone that forms slowly and is highly organized.

There are four types of bone cells that contribute to the formation and maintenance of bone: osteoprogenitor cells, osteocytes, osteoclasts, and osteoblasts (Porth, 2009). Osteoprogenitor cells are undifferentiated cells that are the source of all bone cells except osteoclasts which originate from monocyte/macrophages within the hemopoietic lineage. Osteocytes are mature bone cells that maintain the bony matrix. In addition, they may respond to bone tissue strain and recruit osteoclasts to areas of bone requiring bone repair/remodeling (Lanyon, 1993). There are interconnected passageways, canaliculi, found throughout the calcified matrix that contain extracellular fluid (Hadjidakis & Androulakis, 2006) that provide a communication network between the neighboring osteocytes and blood system for the exchange of nutrients and metabolites because diffusion does not occur in calcified bone (Porth, 2009).

Osteoclasts and osteoblasts are two types of cells within mineralized bone that contribute to mineral homeostasis (Lemaire, Tobin, Greller, Cho, & Suva, 2004). They are responsible for building and maintaining bone structure. Osteoclasts are bone-resorbing cells that originate from monocyte/macrophages within the hemopoietic lineage, and their activity is regulated by osteoblasts, bone-forming cells, and numerous hormones and other growth factors, macrophage colony-stimulating factor (M-CSF), interleukin-1 (IL-1), and tumor necrosis factor (TNF) (Hock et al., 2001). Osteoblasts are differentiated mesenchymal stem cells, responsible for laying down the new bone matrix that becomes mineralized, thus replacing old bone previously reabsorbed by osteoclasts (Hadjidakis & Androulakis, 2006; Hock et al., 2001).

There are two pathways that induce osteoclastogenesis. On the osteoblast stromal cell membrane, Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) receptor, binds to the osteoclast precursor cell (macrophage) RANK receptor. When this occurs, RANK signaling activates the transcription factor Kappa-B to differentiate into osteoclasts (Kumar, 2010). The other pathway occurs with the osteoblast secretion of M-CSF and the osteoclast precursor cell (macrophage) M-CSF receptor. When joined tyrosine kinase activity is stimulated, also important to osteoclast formation. This signaling occurs in tandem with the RANKL/RANK pathway. Further bone marrow stromal/osteoblast cells secrete osteoprotegerin (OPG), a decoy receptor for RANKL preventing RANKL from binding with the osteoclast precursor cell's RANK receptor. Thus, preventing bone absorption and attenuating osteoclast differentiation (Kumar, 2010).

### **Bone Physiology**

**Normal Bone Remodeling.** Bone is a connective and living tissue that undergoes continuous remodeling. Approximately every 10 years, a healthy human adult skeleton is completely replaced (Lippuner, 2012; Sherwood, 2013) and every year, 5-10% of bone is replaced in specific bones (WHOSG, 2003). Normal bone remodeling is a coupling process between osteoclasts and osteoblasts, between bone gain and loss, where bone absorption and bone formation is balanced (homeostasis) (NAMS, Bruzzaniti & Baron, 2006; 2010). As the bone adapts to mechanical load and strain, old bone is replaced with new tissue through the coordination between osteoblasts and osteoclasts (Hadjidakis & Androulakis, 2006). Bone remodeling begins with bone resorption in both cortical and cancellous bone by the osteoclasts over a period of a few days.

Osteoprotegerin (OPG) production and is vital to the regulation of osteoclastogenesis, menopausal transition and post-menopause, the RANKL secretion by osteoblasts is greater than the OPG generation due to loss of estrogen (Tella, Gallagher, Tella, & Gallagher, 2013).

Bone remodeling begins with bone resorption in both cortical and cancellous bone by osteoclasts over a period of 30-40 days (Eriksen, 2010). Osteoclasts attach to the mineralized bone surface and resorb bone via secretion of hydrogen ions and cathepsin K (Raisz, 1999), creating irregular, scalloped cavities named Howship lacunae in trabecular bone or cylindrical Haversian canals in cortical bone. Osteoprogenitor cells found in the periosteum and endosteum will differentiate into cuboidal-shaped osteoblasts. Osteoblasts lay down a new organic extracellular matrix in two stages; ossification, formation of unmineralized bone (osteoid), and calcification that becomes mineralized with calcium and phosphate and fills the resorption lacunae over approximately 150 days (Eriksen, 2010; Raisz, 1999).

Bone structure reflects the quality of the bone tissue (EFOBD & NOFUSA, 1997). Microarchitectural bone changes may be independently associated with bone weakness, but not associated with a change in BMD (Harvey et al., 2013). An extracellular matrix (ECM) of bone is comprised of two main components: mineral composite and collagen, as well as water, proteins, and lipids (Gehron-Robey & Boskey, 2008). The mineral composite is made of hydroxylapatite, mainly calcium phosphate salts, which provide strength via crystallization around the collagen fibers in the ECM (Sherwood, 2004). Collagen Type I is the basic building block of the bone matrix and contributes to bone strength.

## **Pathogenesis of Osteoporosis**

There are primary and secondary causes for the pathogenesis of osteoporosis. When secondary causes for bone loss cannot be determined, the patient is diagnosed with primary osteoporosis (Ardawi, Al-Kadi, Rouzi, & Qari, 2011). Primary causes of bone loss are due primarily to menopause and aging (senile osteoporosis) which overlap clinically, as well as the failure to achieve optimal peak bone mass (PBM) and strength initially during adolescence (NIH Osteoporosis and Related Bone Diseases (NIH Osteoporosis and Related Bone Diseases ~ National Resource Center, 2009; Raisz, 2008). Achieving peak bone mass (PBM) and bone strength is critical to an individual's overall lifespan skeletal health and is a determinant of the future adult skeleton's strength (Raisz, 2008; WHOSG, 2003). Optimal PBM may not be obtained due to genetic mutations, lack of adequate dietary calcium and vitamin D intake, as well as lifestyle choices such as physical activity. Secondary causes occur idiopathically in younger men and premenopausal women who present with osteoporotic fragility fractures attributed to iatrogenic factors and disease pathogenesis (Hudec & Camacho, 2013).

Osteoporosis arises from the out-of-balance osteoclast/osteoblast activity in remodeling bone (Shih, 2012). Bone loss has been described as a two-stage process in women. The first stage is due to menopausal estrogen loss usually around 51 years and the second, that occurs post-menopausally, is a slow continuous loss of bone due to aging rather than the loss of estrogen prior to menopausal transition. The first to two years of perimenopause may account for the greatest amount of lost bone; trabecular (37%) and cortical (6%) loss found before age 50 (Armas & Recker, 2012; Riggs et al., 2008). It is estimated that menopausal transition spans 5-7 years. Approximately, 12% of total bone mass is lost during this time which is equal to one T-score (-1 SD) of measured bone

density by DEXA and corresponds to enough bone loss as diagnosed osteopenia (Armas & Recker, 2012; Recker, Lappe, Davies, & Heaney, 2000). Bone loss occurs because osteoclastogenesis becomes proportionally greater than osteoblastogenesis (Shih, 2012). The beginning of the second stage varies individually in post-menopausal women and is a slow continuous loss of bone due to aging rather than the loss of estrogen. During this later stage cortical and trabecular bone loss occurs at an equal rate until the end of the life span. Unlike women, men begin this slow continuous loss of bone due to aging during middle age (Khosla & Riggs, 2005).

Independent of aging or estrogen deficiency, secondary osteoporosis may be due to a group of heterogeneous causes leading to bone loss. Secondary causes of bone loss occur in approximately two thirds of men, over half of premenopausal women and 30% of postmenopausal women (Recker, 2011). One main cause is iatrogenic effects of medications that negatively alter the bone remodeling homeostasis. However, not all drugs have negative effect; some medications have been associated with increase bone density (see Table 2). Specific attention has centered on proton-pump inhibitor use associated with decreased BMD and possible increased hip fracture/fracture risk. Calcium absorption is thought to be impaired with gastrointestinal PH changes (Gray et al., 2010; Targownik, Lix, Leung, & Leslie, 2010). It has also been suggested that some medications may, in fact, have an off label therapeutic benefit to skeletal health such as the combined use of  $\beta$ -blockers and thiazides (Wiens, Etmnan, Gill, & Takkouche, 2006), and lipid lowering agents (statins) (Uzzan, Cohen, Nicolas, Cucherat, & Perret, 2007), but investigators cannot recommend preventive treatment without further research. Further, there are several specific disease states of the endocrine, hematological, gastro-

intestinal systems that have their own epidemiologic characteristics and pathogenesis that contribute to bone loss (Diem et al., 2007; Haney et al., 2007; Osteogenesis Imperfecta Foundation, 2010; Yang, Lewis, Epstein, & Metz, 2006) (see Table 3).

### **Outcomes of Osteoporosis**

Two main osteoporosis outcomes are highlighted: frailty and fall induced osteoporotic fractures, specifically hip fracture. Both of these result in a societal and financial burden to treat. Researchers have identified frailty as a precursor to falls and falls as a precursor to frailty (Runge & Hunter, 2006). Falls and fractures accelerate frailty in already frail older adults or acutely initiate transitioning into frailty due to a “triggering event,” such as a hip fracture.

In a series of studies, the association between frailty and falls is well documented in the literature (Ensrud et al., 2007; Runge & Hunter, 2006) in both men (Ensrud et al., 2009) and women (Ensrud et al., 2008) and there is considerable research that associates osteoporosis with fall-induced fractures (Morrison, Fan, Sen, & Weisenfluh, 2013; Schwartz, Nevitt, Brown, & Kelsey, 2005). Therefore, individuals who may or may not be frail, who experience a fall, and who have low bone mass, are more likely to experience an osteoporotic induced fracture, which may accelerate or initiate frailty.

Frailty, described as a decline in the functioning of the neuromuscular system and the loss of physiological reserve to handle minor stresses, increases susceptibility to adverse health outcomes and progression of disease and comorbidities (Abate et al., 2007; Fried et al., 2001; Morley et al., 2013). Frailty is seen most frequently in older adults over 80 years of age. An individual is identified as being frail when at least three of the following factors are present: unintentional weight loss (10 pounds or more in a

year), self-reported exhaustion, weakness as measured by grip strength, observable slowed walking speed and self-reported low physical activity (Fried et al., 2001; Mahoney, Glysch, Guilfoyle, Hale, & Katcher, 2005; Morley et al., 2013). The current prevalence of frailty in persons aged 65 and older ranges from 4.0% to 17% (Collard, Boter, Schoevers, & C, 2012).

As individuals age they become susceptible to falling. Falls have been an independent indicator for functional decline and are identified as the causal event for 40% of nursing home admissions (Bischoff-Ferrari, 2009; 2013; Tinetti & Williams, 1997). Approximately 1.4% of community dwelling older adults experience a low-impact fall resulting in fracture annually (Morrison et al., 2013). Currently, falls remain the leading nonfatal injury treated in the emergency room (Centers for Disease Control and Prevention National Center for Injury Prevention and Control [CDCP-NCIPC], 2010) and the leading cause of death by unintentional injury for individuals 65 and older (Avdic, Pecar, & Mujic-Skikic, 2004; CDC-NCIPC, 2003; 2005). Consequently, over the past decade, the mortality rate has also increased (Stevens, 2006). In older adults with low bone mass, who are in a pre-frail state, a condition between frail and nonfrail, falling may initiate functional decline initiating the progression into a frail state; and they are more likely to experience another significant consequence of osteoporosis, fall-induced fractures.

The lifetime fracture risk for white women over 50 years is 50% (Armas & Recker, 2012) and for osteoporotic adults  $60 \geq$  years who experience a fracture, 24% of women and 20% of men will re-fracture within five years; and within one year 26% of women and 37% men will die, three times greater risk than the general population

(Colón-Emeric, 2013; Kannegaard, van der Mark, Eiken, & Abrahamsen, 2010). Further, a little over a half a million low impact falls result in osteoporotic fractures (Morrison et al., 2013). Fall induced hip fracture is the most serious osteoporosis-associated incidental fracture resulting in permanent disability (Harvey et al., 2013; WHOSG, 2003).

The three most common osteoporotic fall-induced fractures are vertebral compression, wrist, and hip. Wrist and vertebral spine fractures are most often experienced by women 45-60 years of age, and are thought to be attributed to age-related changes of neuromuscular reflexes. A wrist fracture most commonly occurs when a woman falls sideways or backwards and uses her arm to break the fall, thus breaking their wrist (Melton & Cooper, 2001). Vertebral fractures usually occur asymptotically while performing normal activities of daily living such as lifting, and occur three times more often than hip fractures (Harvey et al., 2013). It is important to understand that vertebral fractures are painful and affect patient morbidity, while hip fractures affect patient mortality. Therefore, older adults living with back pain tend to self-limit mobility which results in a functional decline, thus increasing risk for hip fracture and frailty.

Hip fractures most commonly occur at the femoral neck resulting from landing directly on the hip during a fall (falling sideways) rather than falling forward (NIH-CDPO, 2001). As early as 1999, the WHO declared that hip fracture may become a worldwide epidemic by the middle of the 21<sup>st</sup> century, increasing from an incidence of 1.7 million in 1990 to a projected 6.3 million by 2050 (Cooper, Campion, & Melton, 1992). Further, estimates for the number of hip fracture and costs could possibly double or triple by 2040 (US Department of Health and Human Services, 2004). The majority of hip fractures are known to occur in Europe and North America, but over the next 50

years, it is projected that 75 % of the world's total hip fractures will occur in developing countries. Reported in a systematic review, there is a global 10-fold variation in the global 10 year hip fracture and fracture probability rates and more study is required to initiate global strategies to affect rates of hip fracture (Kanis, Oden, et al., 2012).

Most importantly, of the individuals who experience a fall and consequent hip fracture, many do not return to their original functional level nor resume original social activities after the fall. Only one-third of older adults regain their pre-fracture functional level (NIH-CDPO, 2001) and another third will be admitted to long-term care (NIH-CDPO, 2001). Half of the people who are ambulatory prior to a hip fracture cannot walk independently after the fall (Harvey et al., 2013), and suffer from fear, anxiety and depression, which dramatically impacts their quality of life (Ziden, Wenestam, & Hansson-Scherman, 2008).

In summary, osteoporosis is a growing world-wide problem requiring international collaboration to investigate and develop strategies to predict and stratify risk for falls and fractures, as well as address issues concerning access to appropriate therapy and education. This disease process is silent and usually not diagnosed until a fracture has occurred resulting in staggering consequences for the quality of life of many older adults. The population of older adults is expected to increase exponentially, resulting in rising health care costs and society burden to treat, as well as a growing population of frail older adults. Therefore, researchers are actively working together to develop new technologies for disease diagnostics, osteoporosis medications based on new findings in bone biology, as well as new treatment options.

## **New Treatment Insights**

Generalist nurses with osteoporosis pathophysiological knowledge can appreciate the new advances for the management of osteoporosis. Alternative diagnostics, medications, and bioengineering treatment options are in various stages of discovery. Newer technologies for measurement for bone quality may be achieved using areal BMD testing, measuring bone for size, thickness, and volumetric mineral density using volume quantitative computed tomography (vQCT) and micro-computed tomography ( $\mu$ CT) scanning and are undergoing substantiation for specificity and reliability (Genant et al., 2008; MacNeil et al., 2008). They potentially may provide valuable BMD measurements in monitoring bone response to medical management.

Trabecular Bone Score (TBS), a measurement of bone microarchitecture not captured in BMD testing, is a metric calculated from a two-dimensional lumbar-spine DEXA scan image. This measurement is a newly recommended metric to further evaluate postmenopausal women for bone loss and strength (Silva et al., 2014). A lower score indicates weaker bone. In women who experienced fragility fractures, lower TBS scores were found, but many of these women were not identified according to DEXA T-score as osteoporotic or osteopenic. Possibly TBS will become a standard measurement for bone strength, added to clinical practice guidelines, and be used as a predictor for fracture in women not found to be osteopenic.

Currently, bisphosphonates, which are osteoclast antiresorptives, are the first line medication used to manage osteoporosis (see Table 4). Based upon advances in understanding mechanisms in parathyroid (PTH) bone stimulation pathways, a PTH bone anabolic, Teriparatide (Forteo®), is now considered first line treatment for severe

osteoporosis (Kanis, Reginster, et al., 2012; Rizzoli et al., 2011). Another drug in use, Denosumab (Prolia ® or XGEVA®), a Receptor Activator of Nuclear Factor Ligand (RANKL) -inhibitor has been effective in inhibiting osteoclast formation, thus decreasing the incidence of vertebral, non-vertebral and hip fractures (Cummings et al., 2009). Current experimental drugs, JTT-305, known as Calcilytics, stimulate PTH secretion *in vivo* [animal study model] (Avdic et al., 2004) and human dose response studies are promising (Cabal et al., 2013). Further, a molecular pathway, Wnt/ $\beta$ -catenin, responsible for osteoblastic differentiation and recruitment has been discovered. The drug, AMG 785, consisting of antibodies to antagonize this pathway resulted in significant increases in lumbar spine BMD (Kanis, Reginster, et al., 2012; Padhi, Jang, Stouch, Fang, & Posvar, 2011).

Some exciting developments in treatment involve using bioengineering equipment such as near-infrared light emitting diode (NIR-LED) treatment and vibration therapy. Low level light treatment has been found to be effective in many clinical settings, mainly improving wound healing and cell regeneration and function, and decreasing inflammation/pain (Desmet et al., 2006; Eells et al., 2004; Pinheiro & Gerbi, 2006; Pinheiro et al., 2008; Pinheiro et al., 2009; Whelan et al., 2003; Wong-Riley, Bai, Buchmann, & Whelan, 2001). The hypothesis behind light treatment is that light absorption spectrum in the far-red to near-infrared range (630-1000nm) and low energy densities  $4\text{J}/\text{cm}^2$  positively affects numerous cell functions (Desmet et al., 2006). Traditionally, lasers have been used at higher wavelengths and energy densities to cut, coagulate, and ablate by intense heat tissue, thus destroying the tissue. At low intensity, such as 670 nm, infrared wavelengths are thought to have cellular benefit rather than

destructive properties. When the light is applied to the tissue, a photochemical conversion of energy occurs, absorbed by non-specialized cellular photo acceptors that enhances the production of energy, adenosine tri-phosphate (ATP), and ultimately reduces cellular oxidative stress, known to negatively affect cell function associated with aging (Eells et al., 2004; Karu, 1988). Light treatment increases osteoblastogenesis (Stein, Benayahu, Maltz, & Oron, 2005; Yamada, 1991) and osteoclastogenesis (Aihara, Yamaguchi, & Kasai, 2006) in a cell culture model and in animal models. It is suggested that light treatment promotes osteogenesis increasing amounts of well-organized bone trabeculae and vertical bone regeneration (Blaya, Guimaraes, Pozza, Weber, & de Oliveira, 2008; Pinheiro et al., 2009; Torres, dos Santos, Monteiro, Amorim, & Pinheiro, 2008). Based upon this research, low level light treatment may be useful to treat the osteoporotic population by applying the light to spine and hip. However, no current studies exist.

Whole Body Vibration (WBV) has been reported to have potential benefits to prevent and treat osteoporosis (Chan, Uzer, & Rubin, 2013). However this technology, if not administered in the correct intensity, has risk to damage the musculoskeletal system. Bone is sensitive to mechanical signaling, which is beneficial to build and maintain bone mass; however parameters for duration, frequency or intensity remains unclear (Chan et al., 2013). Studies have investigated bone response in postmenopausal women with low BMD reporting significant increases in bone mineral density with 6 months of treatment (Verschueren et al., 2004), but others report no changes in BMD in 11 weeks of treatment (Gomez-Cabello et al., 2014). As a result, researchers need to develop WBV safety protocol studies to determine the parameters for standard treatment, but it appears that

low intensity vibration of one gravitational force (1g), earth's gravitational force, is safe and may be effective to treat osteoporosis (Chan et al., 2013).

### **Conclusion**

The incidence of osteoporosis remains high within a population expected to reach 70 million by 2020, with costs to treat projected to double or triple by 2040 in the US alone. Moreover, the worldwide incidence of osteoporosis is expected to reach 200 million people by 2050, contributing to a significant societal burden worldwide. Given these numbers, the possibility that a nurse will interact with someone with osteoporosis is quite real. Having a solid understanding of normal and pathologic bone anatomy and physiology will be critical in order to provide evidence-informed care.

Although, as discussed, there are various promising advances in osteoporosis management and professional organizations such as the International Osteoporosis Foundation are readily evaluating literature and updating practice guidelines. This organization has an extensive worldwide presence with 12 working groups in bone loss health. The annual report is comprehensive (International Osteoporosis Foundation, 2013), but how does this information reach the generalist nurses, the healthcare professionals who have the potential for the most frequent contact with patients? How nurses can best care for persons with osteoporosis is the focus of another manuscript.

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Table 1

*WHO Diagnostic Criteria for the Defining Osteoporosis*

Definition	Bone Mineral Density	T-Score
Normal	BMD within 1 SD below the mean BMD of 30 year old women	$\geq -1$
Low Bone Mass (Osteopenia)	BMD 1-2.5 SD below the mean BMD of 30 year old women	Between -1 and -2.5
Osteoporosis	BMD $\leq -2.5$ below the mean BMD of 30 year old women	$\leq -2.5$
Severe Osteoporosis	$\leq -2.5$ below the mean BMD of 30 year old women that has $\geq 1$ fractures	$\leq -2.5$ with fragility fracture

Sources: Kanis & Adami, 1994; EFOBD & NOFUSA, 1997; WHOSG, 2003

Table 2

*Affecting Medications in Bones*

Initiates Bone Loss	Prevents Bone Loss
Glucocorticoids	$\beta$ -blockers
Anti-Hormonal	Thiazides,
Aromatase Inhibitors	Statins
Thiazolidinediones	
Acid Suppressants	
Anti-Epileptics	
Selective Serotonin Receptor Uptake Inhibitors	
Long-Term Heparin use	

Sources: Gray et al., 2010; Targownik et al., 2010; Uzzna et al., 2006

Table 3  
*Secondary Causes Associated with Bone Loss*

Endocrine	Hematological	Rhematological	GI Diseases	Other
Hyperthyroidism	Bone Marrow Mastocytosis	Rheumatoid Arthritis	Cohn's Disease	Osteogenesis Imperfecta
Primary Hyperparathyroidism			Celiac Disease	Chronic Renal Failure
Vitamin D Deficiency				Solid Organ Transplant
Hypogonadism				Chronic Obstructive Pulmonary Disease
Cushing's Syndrome				
Idiopathic Hypercalciuria				
Paget's Disease				
Diabetes Mellitus				

*Sources: Compston, 2008; Diem et al, 2007; Haney et al., 2007; Osteogenesis Imperfecta Foundation, 2010 and Yang et al., 2006*

## **Manuscript Two**

### **Nursing Implications for Osteoporosis Management**

#### **Abstract**

Osteoporosis is a worldwide silent epidemic affecting the quality of life of 75 million people 65 years and older. Osteoporotic induced fractures, an associated negative health outcome, may contribute to initiating or accelerating the downward spiral of frailty. The costs associated with bone loss are expected to increase to \$85 billion in 2020 posing a significant societal financial burden. Given the dearth of geriatric clinicians, generalist clinicians will be responsible for rendering the majority of health care to populations at risk for this condition. Currently, osteoporosis and osteopenia are significantly underdiagnosed and not usually assessed until after a fracture occurs. Among the healthcare team, nurses have frequent patient contact, patient trust, and opportunity to improve and implement evidence based practice. It is critical that the care they provide is drawn from the most current evidence. In a prior manuscript, the etiology of osteoporosis was reported in preparation for this review. The purpose of this review is to provide the generalist nurse with knowledge to appropriately assess and identify osteoporosis, implement tailored interventions, provide disease monitoring and discuss nursing implications. Ultimately, nurses, active members of the multidisciplinary team, are positioned to assist patients in understanding bone loss.

#### **Introduction**

Nurses have the most frequent patient contact, are trusted among many patient populations (Dinç, Gastmans, Dinç, & Gastmans, 2013), embrace the implementation and improvement of evidence based practice (Lee et al., 2013), and are an integral part of the

multidisciplinary health care team (Wilkes et al., 2014). Estimates of cost to treat osteoporotic-induced fractures are expected to at least double by 2040 (US Department of Health and Human Services [USDHHS], 2004), placing a great societal financial burden to care for the anticipated 70 million older adults in 2030 (U.S. Department of Health and Human Services Administration on Aging, 2006). Nurses have an opportunity to guide women and men to improved bone health and reduce or reverse bone loss, thus preventing fractures and mortality and improving the quality life of older adults through thoughtful risk and clinical assessment, attentively monitoring and implementing tailored interventions (Teng, Curtis, & Saag, 2009). In this manuscript, the literature is reviewed to provide the generalist nurse with knowledge to implement the nursing process to appropriately assess and identify osteoporosis, implement tailored interventions, and provide disease monitoring.

Given the rapidly expanding osteoporosis literature, it is nearly impossible for nurses to remain abreast of all new recommendations for patient treatment. Therefore, the purpose of this article is to provide a brief overview of the problem, and identify the current practice recommendations for risk assessment, screening, diagnosis, treatment options, prevention, and disease monitoring for the management of osteoporosis and provide implications for nursing practice and policy. Information is presented in the following order: bone loss and fall risk assessment, health history, screening, physical exam, diagnostic tests, and tailored interventions, prevention and nursing implications. It will become imperative to identify individuals at risk for falls, screen for bone loss and implement appropriate tailored interventions to improve bone loss and prevent falls, in order to effectively impact the combined outcomes of morbidity and mortality, and

conserve medical resources. These nursing practice recommendations are aimed toward adults 50 years and older.

### **Problem**

Nurses must recognize that osteoporosis is a silent killer and not a customary disease associated as a general characteristic of aging. Nonetheless, it is well known that bone loss can be currently identified, prevented and managed (National Osteoporosis Foundation [NOF], 2010 [revised 2013]). Osteoporosis, the most common skeletal disease seen in older adults, is characterized by low bone mineral density (BMD) with microarchitectural bone deterioration that compromises bone strength, thus, increasing risk for bone fragility and fracture (National Institutes of Health Consensus Development Panel on Osteoporosis [NIH-CDPO], 2001; NOF, 2010 [revised 2013]; Nuti et al., 2009). Primary causes of osteoporosis are due primarily to menopause and aging (senile osteoporosis) and the failure to achieve optimal peak bone mass (PBM) and strength during adolescence. Secondary causes occur idiopathically in younger men and premenopausal women who present with osteoporotic fragility fractures attributed to iatrogenic factors and disease pathogenesis (Bonjour, Theintz, Law, Slosman, & Rizzoli, 1994; National Institutes of Health Osteoporosis and Related Bone Diseases ~ National Resource Center, 2009; Raisz, 2008).

In the US, 10 million people over the age of 50 years are affected by osteoporosis; of these, 8 million are women and an additional 34 million Americans have low bone mass (osteopenia) (A. C. Looker, Melton, Harris, Borrud, & Shepherd, 2010). Osteoporosis is more common in women than men due to postmenopausal hormonal changes and longer life span and more prevalent in Caucasian women and men than

Hispanics and African-Americans (Looker, Borrud, Dawson-Hughes, Shepard, & Wright, 2012).

Not all individuals at risk for bone loss are identified and treated. Giangregorio, Papaioannou, Cranney, Zytaruk, and Adachi (2006) found that only 30% of patients were diagnosed with osteoporosis prior to a fracture. In the National Osteoporosis Risk Assessment Study (NORA) of women 50 years and older, almost half of the 200,160 subjects had low bone mineral density (BMD), osteopenia, and 7% met the criteria for osteoporosis and were not receiving treatment. Further, 11% had a history of an osteoporotic related fracture retrospectively beginning at age 45 (Siris et al., 2001).

For white women over 50 years, the lifetime fracture risk is 50% (Armas & Recker, 2012) and every year in the US, there are over a half a million low impact falls that result in an osteoporotic fracture (Morrison, Fan, Sen, & Weisenfluh, 2013). Currently, falls are the leading cause of death by unintentional injury for individuals 65 and older (Centers for Disease Control and Prevention National Center for Injury Prevention and Control [CDCP-NCIPC], 2003; 2005; 2010) . The estimated lifetime risk for experiencing an osteoporotic fracture (vertebral, wrist, and hip) is 40%, similar to the lifetime risk for coronary heart disease ((World Health Organization Scientific Group [WHOSG], 2003). With an aging population, the incidence of falls will increase, as will fracture risk.

Hip fracture is the most serious incidental fracture associated with morbidity and mortality in older adults (Harvey, Dennison, & Cooper, 2013; Johnell & Kanis, 2006; Ziden, Wenestam, & Hansson-Scherman, 2008), and the most costly fracture to treat (Kilgore et al., 2009). In 2008, estimated US costs to treat osteoporosis and fractures was

reported as \$22 billion (Blume & Curtis, 2011). Hip fractures usually result in hospitalization, with mortality rates within the first year reported in men (37.1%) and women (26.4%) (Kannegaard, van der Mark, Eiken, & Abrahamsen, 2010). In fact, 50% of those ambulatory prior to fracturing their hip, cannot walk independently (Harvey et al., 2013), suffer from fear, anxiety, and depression which dramatically impacts their quality of life (Eastwood et al., 2002; NOF, 2010 [revised 2013]; NIH-CDPO, 2000; Ziden et al., 2008).

### **Method**

An extensive search of the literature focusing on osteoporosis, its etiology, societal and health outcomes published between 2000 – 2013 was conducted. Databases searched included PubMed, CINAHL, Academic Search Complete/EBSCO, Medline, National Guideline Clearinghouse, Google Scholar, Primo Central/Ex-Libris, as well as references from articles and books. In order to gather full text copies and guidelines, a Google web search was conducted. Articles published prior to 1999 were considered seminal and were authenticated from the extrapolated literature. Further, expert opinion literature was reviewed and of note, the *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, eighth edition*, published by The American Society for Bone and Mineral Research, was utilized to obtain leading expert bone health researcher's opinions (Rosen, 2013). The term osteoporosis was searched in combination with epidemiology, definition, clinical practice guidelines, diagnosis and treatment, risk factors and assessment, cost analysis, nursing interventions, exercise, calcium, vitamin D, bisphosphonates, and estrogen. The search was limited to English, focused on the age

group of 50+ years, and utilized US practice guidelines addressing osteoporosis management.

Critical appraisal of the literature followed the recommendations of nursing researchers Polit and Beck (2008) evaluating for existence of research questions, hypotheses, study design, methods of sampling and data collection, and analysis. Systematic reviews were evaluated using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) method (Moher, Liberati, Tetzlaff, Altman, & The Prisma Group, 2009). The search, when feasible, was limited to National Guideline Clearing House (2), professional guidelines (5), professional position statements (11), meta-analyses (1), systematic reviews (6), clinical trials (5), clinical cohort studies (21), epidemiological studies (6), reviews of literature (15), articles from peer-reviewed journals (57), professional texts (7), and government reports (19).

### **Findings**

Over the past 20 years, clinical practice guidelines have evolved into sophisticated tools designed to guide clinical decision making, optimize efficiency and patient care by facilitating the translation of research to the bedside and serves as an excellent nursing resource. These guidelines are statements that reflect a systematic review of evidence that examines the scientific quality of relevant literature assessing the risks and benefits for specific types of clinical care (Institute of Medicine of the National Academies, 2011). Their purpose is not to dictate patient care, but to provide healthcare professions with quality information to make informed clinical decisions which may be tailored to each distinctive patient.

## **Osteoporosis Management**

The main goals in osteoporosis management are to accurately identify patients at increased risk for fracture and educate patients at every opportunity regarding disease process, prevention, and treatment options (McCloskey, 2009). The nurse must be aware of the appropriate time in the lifespan to screen individuals for bone loss through health history, risk assessment, physical exam, tailored interventions and yearly follow up.

Menopausal transition has become the focus in bone health research, suggesting screening women prior to the age of 65. It is thought that the greatest amount of bone loss occurs one year prior to the final menstrual period and the first two years after the final menstrual period (Lo, Burnett-Bowie, & Finkelstein, 2011). The exact point in time is difficult to determine, however, because definitions of menopausal status lack clarity and vary individually. Updated clinical practice guidelines address the need to consider screening perimenopausal women and men age 50-69 years with one osteoporosis risk factor (Lim, Hoeksema, & Sherin, 2009; Michigan Quality Improvement Consortium [MQIC], 2014; North American Menopause Society [NAMS], 2010). Also, some researchers believe that risk should be assessed in adolescence (Schettler & Gustafson, 2004). A synthesis of five clinical practice guidelines can be found in Table 1.

A disconnect between evidence-based practice and real-world clinical practice in diagnosing and preventing osteoporosis despite the great advancement in identifying and treating osteoporosis (Teng et al., 2009) presented in the literature, however, improvement is needed in transferring these interventions into clinical settings (NOF, 2010 [revised 2013]; Rojas-Fernandez, Lapane, MacKnight, & Howard, 2002; Warriner et al., 2009). Actually, identification of osteoporosis was reported to be greater in older

adults, yet, treatment occurred more often in younger individuals (Giangregorio et al., 2006).

Giangregorio, Papaioannou, Cranney, Zytaruk, and Adachi (2006) in their systematic review of 35 studies that investigated osteoporosis treatment after experiencing a fragility fracture reported a little less than half were diagnosed with osteoporosis prior to fracture; and of those identified with osteoporosis, one third did not take calcium/vitamin D supplements or osteoporotic medications, the most common diet and medication interventions. Also reported, (Siris et al., 2001) of the National Osteoporosis Risk Assessment Study of women 50 years and older, 7% of the subjects, not previously diagnosed, met the criteria for osteoporosis and were not receiving treatment and 11% had a history of an osteoporotic related fracture retrospectively beginning at age 45.

Once an individual has been screened and is identified at risk or with low BMD through risk assessment and/or BMD testing, the individual requires a detailed history and physical, diagnostic tests as to evaluate further for fracture risk and rule out other secondary causes for low BMD. Through this work up, interventions can be implemented to modify risk factors, and initiate pharmacological treatment if appropriate to treat osteoporosis (NAMS, 2010). Therefore, enhancing the nurse's knowledge about appropriate risk assessment, interventions and vigilant monitoring has potential to improve patient outcomes.

**Health History.** It is important to not consider a possible diagnosis of osteoporosis based solely upon a measurement of low bone mass. Nurses should begin with a detailed health history. The history should include obtaining information about

five major risk factors: calcium intake through diet history, alcohol and cigarette use, menarche history, medication review for drugs attributed to bone loss such as glucocorticoids, and sun exposure review (NOF, 2010 [revised 2013]; WHOSG, 2003).

**Screening.** The National Osteoporosis Foundation (NOF) recommends that all post-menopausal women and men 50 years and older receive a comprehensive bone loss assessment employing a detailed history and physical, Bone Mineral Density (BMD) testing and vertebral imaging, if needed, and a calculation of the World Health Organization (WHO) 10-year Fracture Risk Assessment Tool (FRAX<sup>®</sup>) probability score (Lim et al., 2009), as well as fall risk assessment (NOF, 2010 [revised 2013]; NAMS, 2010). The University of Michigan practice guidelines recommend assessing for risk factors to perform the FRAX<sup>®</sup> in all women at risk, then if needed, measure BMD if the 10-year hip fracture risk score  $\geq 9.3\%$  (equivalent risk of a 65 year old women) (MQIC, 2012 [revised 2014]).

Many nurses are aware that the screening measurement of BMD alone is a strong predictor for fracture; however, it has low sensitivity because the majority of fractures occur in the diagnostic osteopenic range (McCloskey, 2009). The combination of BMD scores and clinical risk factors increases the discrimination value of predicting fracture risk (Kanis et al., 2007). One tool that includes this combination is the FRAX<sup>®</sup>. This tool, typically used in men and women 65 years and older, has also been adopted internationally as a valid and sensitive for use in men and women age 40-50. It is available in a desktop application and more information regarding the development and international utilization and endorsement of the FRAX can be found in Table 2. It is

administered efficiently, requiring minimal training and is especially useful in communities where BMD testing is scarce (NOF, 2010 [revised 2013]).

The FRAX<sup>®</sup> tool identifies the most pertinent independent risk factors for fracture and bone loss: prior fragility fracture, parental hip fracture history, tobacco use, history or use of long-term oral glucocorticoids, alcohol consumption  $\geq$  to 3 units, and a diagnosis of Rheumatoid Arthritis. These factors are then calculated into an algorithm with the individual's body mass index (McCloskey, 2009), as BMI is a significant risk factor for bone loss. Currently, the tool is available in several languages through the website or downloaded at <http://www.shef.ac.uk/FRAX> (World Health Organization, 2012).

In addition to using the FRAX, a multifactorial fall assessment is essential since the majority of osteoporotic-induced fractures occur due to falls. Assessment should include an evaluation of orthostatic hypotension, muscle strength, gait and balance with use of adaptive aids, functional status, and home environment. Further, an assessment for comorbidities known to increase risk such as a history of falls (strongest predictor), depression, syncope, arrhythmia, poor vision, and fear of falling should be included (Panel on Prevention of Falls in Older Persons & British Geriatrics, 2011). Lastly, performing a comprehensive pharmacology review to identify medications known to increase the risk of falling such as benzodiazepines, psychotropics, narcotic analgesics, and anticonvulsants is recommended (NOF, 2010 [revised 2013]; Panel on Prevention of Falls in Older Persons & British Geriatrics, 2011).

**Physical Exam.** Height loss of 1.5 inches or greater indicates the strong possibility for vertebral fracture and requires BMD testing and vertebral imaging (NOF,

2010 [revised 2013]; NAMS, 2010). Chronic and acute back pain with percussive tenderness over the thoracic spine 11-12 and first lumbar, the most common sites for vertebral fracture, should be assessed for the presence of acute vertebral fractures ((NAMS, 2010; Siminoski, Warshawski, Jen, & Lee, 2006). In addition, weight should be monitored for changes in body mass index as this will affect interpretation of future BMD values and weight loss over time has been associated with bone loss (NAMS, 2010). Although kyphosis, height loss, and back pain can occur without the presence of osteoporosis, such conditions call for further evaluation and consideration of the presence of osteoporosis, thus being accurate in obtaining height measurements become critical. In addition, entering data into the electronic medical record can enhance sharing within a healthcare system. Such a strategy would alert nurses to be vigilant when future hospital admission occur.

**Diagnostic Tests.** It is important that nurses are familiar with the most common tests. A primary care provider would then order BMD testing, as well as routine laboratory tests (see Table 3) (Ardawi, Al-Kadi, Rouzi, & Qari, 2011; Dawson-Hughes, Harris, Ceglia, & Palermo, 2014; NOF, 2010 [revised 2013]; NAMS, 2010; WHOSG, 2003) These laboratory values are useful to rule out secondary causes of bone loss such as anemia, multiple myeloma, GI malabsorption, vitamin D deficiency, hyperparathyroidism, renal failure, and hypothyroidism.

**Tailored Interventions.** Nurses have the ability to initiate interventions to prevent falls, provide patient education to engage health promotion, evaluate medication interactions, improve osteoporosis medication compliance and provide yearly bone loss monitoring. Interventions are aimed to impact modifiable bone loss risk factors.

However, some risk factors such as not reaching optimal peak bone mass by the end of adolescence, aging, sun exposure, genetics, and heredity are non-modifiable. Others can be modified by nutrition, lifestyle choices, body mass index, falls, history of prior fracture, low bone density, muscle strength, and frailty (NIH-CDPO, 2001; Suh & Lyles, 2003; WHOSG, 2003).

Fall prevention is one of the most important interventions to implement.

Multicomponent interventions, such as muscle strengthening, balance and weight-bearing exercises, referrals to multiple resources such as occupational and physical therapy, home care and primary care, and vitamin D supplementation are most effective (Chang et al., 2004). Specific examples are provided in Table 4. Nurses have the opportunity through patient education to inform women about bone health early in menopause transition. Patient education could be implemented using the resources from the NOF (National Osteoporosis Foundation: Bone Source, 2013). These materials are created to provide osteoporosis facts, prevention, fitness programs, as well medication information. Further, electronic medical record programs incorporate patient education topics into the outpatient visit summary, taken home by the patient. This may occur in the outpatient setting or during an inpatient admission.

The most common interventions in the prevention and maintenance of BMD are overall lifestyle changes to diet, exercise routines, and lifestyle change. Dietary modifications target sufficient calcium/vitamin D and protein intake, in addition to a weight and non-weight bearing exercise programs, smoking cessation, and moderate alcohol consumption (Carter, Kannus, & Khan, 2001; Kannus, Uusi-Rasi, Palvanen, & Parkkari, 2005; NAMS, 2010). Further, nurses have the ability to assess patient's needs

and refer for physical therapy and nutritional evaluation and gerontological clinical nurse specialist consultation.

Age-related changes of the gastrointestinal tract, such as malabsorption and changes in gastric PH can impair calcium and vitamin D absorption (Perez-Lopez et al., 2012) and some patients require supplementation. Bone stores 99% of the body's calcium and if intake of calcium is inadequate, calcium is leached from the bones to maintain serum calcium homeostasis. Vitamin D is essential for calcium absorption, as well as cardiac and skeletal muscle function. The best sources for calcium and vitamin D absorption are found in a patient's diet (see Table 5). Adult calcium intake is 500-700mg a day (NOF, 2010 [revised 2013]); therefore, it is important to evaluate the patient's diet history for calcium intake. General supplementation recommendations are provided in Table 5.

Weight bearing and strengthening exercises are instrumental in preventing falls, but also have a known physiological response that improves and preserves bone health ((National Guideline Clearinghouse [NGC], 2010 [revised 2012]; NOF, 2010 [revised 2013]). Implementing these fall prevention weight bearing interventions will achieve this positive bone remodeling result (NAMS, 2010; Howe et al., 2011). Postmenopausal women who exercised lost 0.85% and 1.03% less bone at the spine and hip, respectively, and those who used combinations of weight and strengthening exercise regimes averaged 3.2% less bone loss than women who did not exercise (Howe et al., 2011).

The two most important lifestyle changes the nurse can recommend to patients are to quit smoking and to consume alcohol moderately. Smokers lose bone more rapidly, reach menopause two years earlier, and have higher rates of fracture than non-smokers

(NAMS, 2010). Low to moderate alcohol consumption [one drink per day or >3 drinks per week] has been reported to have protective of bone health effects (Sommer et al., 2013). However, women who consume three or more drinks a day are associated with low BMD, falls and may indicate alcoholism (NOF, 2010 [revised 2013]).

Since there are multiple medications to treat osteoporosis, knowledgeable nurses are critical to guide the administration and compliance to medication therapy. There are several medications to inhibit bone resorption and stimulate bone formation (Kleerekoper, 2008) (see Table 6). Post-menopausal women who have experienced a hip or vertebral fracture have a T-score  $\leq -2.5$ , and are osteopenic with a FRAX score of  $\geq 20\%$  or Hip FRAX score of  $\geq 3\%$  should be pharmacologically treated for osteoporosis (NGCH, 2010 [revised 2012]; NOF, 2010 [revised 2013]; NAMS, 2010).

Hormone replacement therapy (estrogen-progesterone) and Selective Estrogen Receptor Modulators (SERMs) are useful to preserve bone and prevent fracture in the beginning of the post-menopausal period; however, patients need to be informed regarding the risk factors of developing breast cancer (Chlebowski et al., 2003), deep vein thrombosis, and stroke (NAMS, 2010). Bisphosphonates are the first-line treatment to inhibiting bone loss by slowing the osteoclast activity and are reported to decrease vertebral fractures 40-70% and hip 15-35% (NAMS, 2010). Bisphosphonates are metabolized through the renal system; therefore, renal function must be evaluated prior to initiating therapy and nurses must assist monitoring serum creatinine levels for signs of renal failure (see Table 6). The most common side effect is gastrointestinal upset, because the medication must be taken on an empty stomach sitting upright for 30-60 minutes. Further, suppressing bone turnover with long term bisphosphonate therapy may

attribute to bone brittleness, however this has been reported in small case studies and still remains controversial (Nevaser, Lane, Lenart, Edobor-Osula, & Lorich, 2008; NAMS, 2010). Newer medications such as parathyroid hormone and Denosumab (Prolia or Xgeva), a Receptor Activator of Nuclear Factor kappa-B (RANK) Ligand (RANKL) Inhibitor, are used in high risk patients for fracture due to breast or prostate cancer (Food and Drug Administration, 2002, 2013a, 2013b, 2013c, 2013d). (see Table 6)

Yearly height and weight measurement, along with repeating BMD testing, are recommended every 2-5 years to monitor an individual's response to pharmacologic treatment and 1-2 years if not treated (Lim et al., 2009; NAMS, 2010; ). Also, a primary care provider may wish to order bone turnover markers (BTMs) measured in 3-6 months after initiating medication management. These are reported to be useful in monitoring response to treatment or evaluating treatment adherence (NOF, 2010 [revised 2013]). However, clinicians must use caution with interpretation of results because of the lack of test assay standardization, diet influences, and variation in day-to-day values (Lim et al., 2009; Miller et al., 1999; NGC, 2010 [revised 2011]; NAMS, 2010). Lastly, fall risk assessment should be addressed yearly.

### **Nursing Implications**

Implications for practice and policy can be drawn from the literature, even as new original research questions are proposed to determine cellular bone loss causality and potential points in the lifespan to intervene and preserve bone health. A nurse driven comprehensive lifespan bone health program may be beneficial to preserve and treat bone loss. Bone remodeling occurs throughout the lifespan and has a specific systematic process in reabsorbing and replacing bone. It is important to understand how crucial to

optimize peak bone mass early in the lifespan because individuals whom have low peak bone mass are at higher risk for fracture and osteoporosis. Once peak bone mass is reached, it must then be preserved throughout the lifespan (Bonjour et al., 1997; Kannus et al., 2005; Mahon et al., 2010; Weaver & Heaney, 2008). Therefore, interventions should be implemented in the following areas of practice: beginning with maternal-child health, adolescent, and proceeding to perimenopause, menopause and postmenopause.

### **Practice**

Nurses have the ability to impact osteoporosis screening, management, and medication adherence through patient education in both inpatient and outpatient settings. Bone loss is a multifaceted healthcare concern. Ultimately, research is needed to create comprehensive intervention programs that are collectively implemented throughout a health care system.

For example, (Majumdar et al., 2007) designed a Canadian nurse case management intervention study. They divided patients into two groups, usual care, and nurse case management. Usual care received osteoporosis, fall prevention, and dietary patient education materials and were encouraged to discuss these materials with their caregivers and/or primary care provider. The intervention group was provided one-on-one counseling with a nurse case manager. Here they discussed the risks and benefits of bisphosphonate therapy, arranged BMD testing, and if needed obtained prescriptions from their primary care provider. This program was implemented throughout three hospital health care systems and was found to be cost-effective, with a cost of \$50 per patient and requiring approximately 70 minutes of case management. Osteoporosis

treatment was significantly improved in the nurse case manager group over the standard care group (67% vs. 26%) (Majumdar et al., 2007).

Further, Australian researchers created a patient education nurse driven program that provided post-fracture information. One group received a patient education letter and the other group received in addition to the letter free BMD testing (Bliuc, Eisman, & Center, 2006). A significant increase in osteoporosis workup was found in those who received a letter and free BMD, but no effect in treatment rates (6%) was seen. Further, 23% of patients contacted their physician, and of these only 25% were recommended treatment. Comparing these two studies results, one-to-one patient education and case management is a key to improving osteoporosis management outcomes.

These above mentioned intervention programs can be replicated in the outpatient setting. Anticipating future shortages of primary care providers, nurses will be responsible to care for those at risk for bone loss. The best clinical setting for generalist nurses to impact the negative outcomes for osteoporosis are in outpatient care settings because approximately 994 million patients are seen annually (Hsiao, Cherry, Beatty, & Rechtsteiner, 2010). Most concerning is the lack of registered nurses working in this setting.

Over the past five years, 2008-2013, only (9%) and (7%) of registered nurses (RNs) were employed in the outpatient clinics compared to (62%) and (60%) of medical assistants, respectively (Bureau of Labor Statistics, 2008a, 2008b, 2013a, 2013b). Of concern is the report from the American Academy of Ambulatory Care (2012) that RNs are performing below their scope of practice while unlicensed assistive personnel are performing above their scope of practice. Since 2008, the US has undergone a major

change in health care delivery, mainly the enactment of the Healthcare Affordability Act, resulting in shifting services from the inpatient to outpatient settings. In 2013, 58% of RNs worked in inpatient settings; therefore, it is evident that there is a significant difference in the access to registered nurses for the majority of the patient population (Bureau of Labor Statistics, 2013a).

Due to perceived cost savings, hospital systems choose to employ more unlicensed personal than registered nurses, thus providing “nursing care” through numerous types of licensed and unlicensed nursing staff. Role confusion exists because of lacking standardization or quality control which may impact negatively patient safety. Use of RNs in outpatient settings has demonstrated improvement in quality patient outcomes and safety, patient satisfaction, reducing adverse events and hospital/emergency room visits (American Academy of Ambulatory Care, 2012; Haas, 2008). Critical to improving osteoporosis outcomes is recognition that educated RNs are needed not only to provide bone health risk assessment and patient education in the post-menopausal population, but across the lifespan.

With this said, nurses in outpatient clinic settings, can develop a comprehensive bone health educational programs that provides intervention at the four points of the lifespan: maternal/infant health, pediatrics, peri-menopause/menopause (women’s health) and post-menopause (gerontology). Each point in the life span has implications for nursing practice. Much is known about post-menopausal bone loss; however, researchers are beginning to study other points in the lifespan. This is evident by the addition of approximately 30 more chapters added to the *Primer on the Metabolic Bone*

*Diseases and Disorders of Mineral Metabolism, eighth edition*, providing new advances in basic bone biology and clinical osteoporosis management (Rosen, 2013).

**Maternal/Infant.** No discussion is complete without recognizing the importance of early bone health assessment. The tenets of fetal bone development are beneficial to later in life bone health. The body of literature concerning maternal/infant calcium and vitamin D status is developing. It is known that calcium demands are greater during pregnancy and lactation, instrumental to fetal growth, and commonly prescribed if a women's intake is < 500 mg/day, but the use of vitamin D supplementation remains vague. However, there is concern that the physiological demands of pregnancy for calcium and vitamin D are not being met by the use of prenatal vitamins and require assessment during prenatal visits (Hacker, Fung, & King, 2012).

Vitamin D deficiency and insufficiency is prevalent in western and non-western pregnant women. It is associated with impairing fetal bone development, low birth weight, and poor childhood growth (Cooper, Javaid, Westlake, Harvey, & Dennison, 2005; De-Regil, Palacios, Ansary, Kulier, & Pena-Rosas, 2012; Mahon et al., 2010). However, due to limited quality studies, recommendations to use vitamin D supplementation remains questionable as there is inconsistent results regarding efficacy and safety for use during pregnancy (De-Regil et al., 2012), this topic continues to be actively researched today as a worthwhile intervention.

Vitamin D deficiency, common in the newborn population, is found more often in African American newborns. There is concern regarding the vitamin D contents of breast milk, which is dependent on the mother's vitamin D status, and infant formulas. Both provide an adequate intake of essential vitamins and minerals but not necessarily enough

vitamin D (Weaver & Heaney, 2008). Of infants supplemented with 400IU of vitamin D daily, an increase in mean vitamin D measurement was found but not to optimal newborn levels (Hanson, Armas, Lyden, & Anderson-Berry, 2011). Further research is required to provide clear recommendations for supplementing vitamin D in pregnancy and newborns.

**Pediatrics.** Pediatric researchers regard bone loss as a “*pediatric disease with geriatric consequences*” (Randi-Schoenfeld, Ng, Henderson, & WU; 2010 p. 104 ) and believe evaluation should begin in adolescence (Schettler & Gustafson, 2004). High calcium intake in prepubertal girls has been associated with increased Peak Bone Mass (PBM) accrual, and adolescence milk consumption positively affects postmenopausal BMD. Because 90% of PBM is attained by age 18 (NIH Osteoporosis and Related Bone Diseases ~ National Resource Center, 2009) preventive medicine (Randi Schoenfeld, Ng, Henderson, & Wu, 2010) and physical therapist researchers (Magee, Stuberg, & Schmutte, 2008) have engaged to develop adolescent educational interventions. An internet based educational program significantly improved knowledge, self-efficacy, and calcium consumption, but was not as effective to impact life-style change (Randi Schoenfeld et al., 2010).

Nurses can assess calcium and vitamin D intake, provide nutritional guidelines, present adolescent health patient education, and monitor status throughout adolescence. Further, evaluation for specific adolescent risk factors associated with impairing PBM; anorexia nervosa, exercise-induced amenorrhea, use of oral contraceptives, poor adolescent physical activity, and cigarette smoking should be added to the electronic medical record (Anderson, Chad, & Spink, 2005; Krall & Dawson-Hughes, 1993; Martin

et al., 2004; Randi-Schoenfeld et al., 2010; Schettler & Gustafson, 2004; WHOSG, 2003).

**Perimenopause.** This is one of the most significant gaps in the literature: identifying menopausal transition, specifically perimenopause. It is alarming that only one preventive guideline recommends screening all patients with potential risk, therefore peri-menopausal (NGC, 2010 [revised 2012]). However, others screen peri-menopausal women only if they present with risk factors, most likely a fracture (NGC, 2010 [revised 2012]; NOF, 2010 [revised 2013]; NAMS, 2010).

Bone loss is reported to be stable during pre-menopause, but accelerates significantly in late perimenopause (Finkelstein et al., 2008). In the 5-7 year span of perimenopause and menopause, approximately, 12% of total bone mass is lost; which is equal to one T-score (-1 SD) of measured bone density by DEXA, and corresponds to a diagnosis of osteopenia (Armas & Recker, 2012; Recker, Lappe, Davies, & Heaney, 2000). Most bone loss studies and interventions have focused on post-menopausal women; however, menopausal status definitions lack clarity (Lo et al., 2011). Therefore, menopausal transition has come into the forefront of the bone health research and may be useful to identify when to screen women for bone loss.

The stage of menopausal transition is not evaluated regularly but is defined as premenopausal; regular menstrual cycles between 26-32 days, peri-menopausal; cycles longer than 35 days, irregularity > 5 days between cycles or hot flashes > 5 days per cycle, and postmenopausal; > one year between cycles. In a nine year prospective study, trabecular bone loss in midlife women averaged 3.2% per year, a 28.8 % total loss of bone with the greatest percentage of loss occurred between years 2-6, accelerating in late

peri-menopause (Seifert-Klauss et al., 2012). Researchers in search of bio-markers for perimenopause found accelerated bone loss was associated with increased gonadotropin levels, follicle stimulating hormone, luteinizing hormone and  $17\beta$ -estradiol, and may be helpful to determine a women's menopausal stage (Seifert-Klauss et al., 2012) (see Table 7). The above definitions and values were successfully supported by study data to determine pre and post menopause (Seifert-Klauss et al., 2012), but the use of clinical bone markers, osteocalcin, bone-specific alkaline phosphatase, and c-terminal telopeptide cross-linked collagen type I, were not able to operationally define the perimenopausal state. However, during menopausal transition, bone markers were the greatest. These menopausal transition indicators could be incorporated into a women's yearly gynecological exam assessed by nursing. The question remains whether bone health screening occurs too late in the life-span.

**Post-Menopause.** The majority of post-menopausal women more than likely will have experienced some degree of bone loss (Reid, 2013). However, it still remains a significantly under diagnosed condition as osteopenia presents asymptotically (Teng et al., 2009; USDHHS, 2004). Because the greatest amount of bone lost early in menopausal transition is trabecular bone, bone loss is not discovered until a patient presents with back pain as a result of a vertebral fracture (Rachner, Khosla, & Hofbauer, 2012).

Nursing is an active member of the multidisciplinary team positioned to assist patients in understanding bone loss. Through risk and clinical assessment, attentive monitoring, will assist to improve osteoporosis health outcomes by early identification

and implementing timely interventions, thus in turn precludes fractures and mortality, improving the quality life of older adults and averting the transition into frailty.

### **Policy**

Policy implications are twofold. First, in order to be effective, hospital systems must employ more registered nurses in their clinic systems to provide assessment and second, to provide research data to support screening perimenopausal women beginning at age 45. Exploring the literature, specifically, employment of outpatient registered nurses was positively associated with cost-savings and patient outcomes. Specific articles written to reflect policy implications are lacking; found were the political agenda and position statements formulated by the American Academy of Ambulatory Care Nursing website (American Academy of Ambulatory Care, 2014).

Perimenopause has been a focus in the literature since the mid-1990s. However, the prevention and screening for osteopenia or evaluating perimenopausal women for bone loss is lacking. The phases of menopausal transition are becoming more understood, but lack of research supporting early screening of women without risk factors is void in the literature. Therefore, it is uncertain with limited statistical evidence that practice guidelines will change in the near future, but active programs of research exist.

The University of Michigan successfully has investigated follicle-stimulating hormone (FSH) levels in 629 women, aged 24-44 for 14 years, and identified four menopausal transitions with eight chronological aging periods occurring between ages 26-60 years (Sowers et al., 2008). Further they found, spine and femoral neck bone loss accelerated at FSH stage 3, defined as FSH levels of 34 mIU/ml corresponding 2-3 years before their final menstrual period (FMP). Bone loss continued into FSH stage 4 and

they concluded that accelerated bone loss was associated with 2 years prior and 2 years after their FMP (Sowers et al., 2010).

In 2012, the University of Michigan Management and Prevention of Osteoporosis practice guidelines recommended the inclusion that all men and women patients having clinical risk factors for bone loss to calculate a FRAX<sup>®</sup> score. Based on this research, perimenopausal women would be included (MQIC, 2012 [revised 2014]) and then if needed, measure BMD if the 10-year hip fracture risk score  $\geq 9.3\%$  (equivalent risk of a 65 year old women) (Lash, Van Harrison, McCort, Nicholson, & Velez, 2013). Implementing this tool is non-invasive and quickly administered and has the potential to be included routinely during yearly visits and potentially identify those not outwardly at risk with beginning stages of bone loss.

### **Conclusion**

Nurses are active members of the multidisciplinary team positioned to assist patients in understanding bone loss. Osteoporosis and osteopenia are significantly underdiagnosed and usually are not assessed until after a fracture occurs. Osteoporosis research over the past two decades has been extensive. However, only a modest impact has been shown in decreasing fracture rates; thus, the societal economic impact to treat remains high and will only increase as the population ages. Nurses have an opportunity through thoughtful risk and clinical assessment and attentive monitoring, to improve osteoporosis health outcomes. Through early identification of bone loss, implementing timely interventions that improve or prevent bone loss will in turn preclude fractures and mortality, and ultimately improve the quality life of older adults and averting the transition into frailty.

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## Tables

Table 1  
*Osteoporosis Management Practice Guideline Synthesis*

	WHO (2003)	ACPM (2009)	NAMS (2010)	MQIC (2012[revised 2014])	NOF (2013)
<b>Risk Assessment</b>	<b>Screen</b>	No recommendation	♂/♀ ≥ 50	♀ ≥ 65	All at Risk
	<b>Use FRAX</b>	-	+	+ Consider Peri-M	+ PM
	<b>Fall Risk</b>	+	-	+	+
<b>Clinical Evaluation</b>	<b>DEXA Preferred</b>	+	+	+	+
	<b>Age</b>	None Stated	All ♀ ≥ 65 ♂ ≥ 70	All ♀ ≥ 65 ♂ ≥ 70	White ♀ ≥ 65 ♀/♂ FRAX score > 9.3% ♀ ≥ 65 ♂ ≥ 70
	<b>Order DEXA</b>				
	<b>With Risk Factor</b>	Any of below	♀/♂ 50-69	♀ ≥ 50	♂ & ♀ 50-69
	Fx/ Fragility Fx	+	+	♀ ≥ 50	♂ & ♀ after 50
	2 <sup>nd</sup> Causes	+	+	+ PM	+
	Peri-menopause	Premature	Premature	Consider	No-consider with risk
	Steroid use	6 months	> 3 months	+	3 months
	X-ray Osteopenia	Consider w/risk	+	-	-
	Low BMI	+	+	+	+
	Maternal hx of hip fx	+	-	Parental	Parental
	Kyphosis-Height Loss	+	-	+	+
	Rheumatoid Arthritis	-	+	+	+
	Alcohol use > 2 day	-	+	+	+
	Smoker	+	+	+	+
	<b>Adolescent Screen</b>	-	-	-	No
	<b>Vertebral Imaging</b>	-	-	+	Only Assess Height yearly
	<b>R/O Secondary Causes</b>	+	-	+	+
	<b>Labs</b>				
	CBC, CMP, TSH	+ cortisol	-	+	-
	Serum 25(OH) D	-	-	+	PTH, Urine: cortisol, histamine, protein electrophoresis
	Biomarker	+	Limited utility	+	+
				?	Predict Fx Risk
<b>Diagnosis</b>	<b>WHO Definition</b>	+	+	+	FRAX >20%
<b>Interventions</b>	<b>Age to Implement</b>	-	-	-	All
	<b>Advise Diet</b>	+	+	+	PM & ♂ 50 ≥
	Calcium: mg/day, Age	Elderly	1200	1500	1,200
			♀/♂ ≥ 50	♀ ≥ 50	♂ & ♀
	Children-Adolescence	No	-	-	-
	Vitamin D: IU/ daily, Age	400-800	800-1,000	800-1,000	800-1,000, PRN in
		Elderly	♀/♂ ≥ 50	♂ & ♀	♂ & ♀ ≥ 50

	WHO (2003)	ACPM (2009)	NAMS (2010)	MQIC (2012)	NOF (2013)
<b>Exercise</b>					
Weight Bearing	+	+	+	+	+
Muscle Strengthening	+	-	+	+	+
Balance Training- PT/OT					+
Adolescence	+	-	-	-	+
<b>Fall Prevention</b>	+	-	+	+	+
<b>Lifestyle</b>					
Patient Education	+	+	?	?	+
Smoking/Alcohol Cessation	+	+	+	+	+
<b>Medication</b>					
<b>Treat</b>					
w/ Hip or Vertebral Fx	-	-	+ PM	+	+
T-Score ≤ -2.5	+	+	+ PM	+	+ Includes Lumbar
Osteopenia PM	Consider	-	+	Consider	♀ & ♂ ≥50
Osteopenia and FRAX score ≥ 20% OR Hip-≥ 3%	-	-	+	+	+
Steroid Use & T-Score ≤ -1.0	-	-	-	+	-
<b>Duration</b>	-	unknown	-	-	3 – 5 years
<b>Medication Preference</b>	Counsel HRT Consider SERM Bisphosphonates	*Bisphosphonates *Estrogen *Raloxifene & Teriparatide	Bisphosphonates SERM Raloxifene in younger PM ET/EPT early PM Teriparatide PM high risk for fx Calcitonin NOT first- line treatment	Use Generic Steroids: Use Bisphosphonates Men: Alendronate	Clinician Choice
<b>Monitoring</b>					
<b>Repeat DEXA</b>	-	2 years	2-5 years/untreated 1-2 years/treated Clinical Changes	If normal Insufficient evidence when to repeat	2-5 years
<b>Height/BMI</b>	+	-	Yearly	Yearly	+
<b>Fall Risk</b>	-	-	Yearly	-	+
<b>Biomarkers</b>	-	Use to monitor Response/Adherence	No	-	3-6 months, Monitor Response /Adherence

WHO = World Health Organization, ACPM = American College of Preventive Medicine, NAMS = North American Menopause Society, MQIC = Michigan Quality Improvement Consortium, NOF = National Osteoporosis Society

FRAX = Fracture Risk Assessment Tool, ♂ = Female, ♀ = Male, Peri-M = Perimenopausal, PM = Post-Menopausal, FX= fracture, HX = History

CBC = Complete Blood Count, CMP = Complete Metabolic Panel, TSH = Thyroid Stimulating Hormone, PTH = Parathyroid Hormone

PRN = as needed, PT/OT = Physical – Occupational Therapy, BMI = Body Mass Index, DEXA = dual energy x-ray absorptiometry, SERM = Selective estrogen receptor modulators, ET/EPT = Estrogen/Estrogen-Progesterone Therapy, HRT = Hormone Replacement Therapy

+ = guideline recommends, - = absent in guideline

Table 2  
*Osteoporosis Management Resources*

Organization	Link	Resources
Who Fracture Risk Assessment Tool	<a href="http://www.shef.ac.uk/FRAX/">www.shef.ac.uk/FRAX/</a>	Desktop application iPhone© application Web Version 3.8
International Osteoporosis Foundation	<a href="http://www.iofbonehealth.org">www.iofbonehealth.org</a>	Management
National Osteoporosis Foundation	<a href="http://www.nof.org">www.nof.org</a>	Practice Guidelines
Japan Osteoporosis Foundation	<a href="http://www.jpof.or.jp">www.jpof.or.jp</a>	Data & Publications Patient Education Materials
The European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis	<a href="http://www.exceo.org">www.exceo.org</a>	Publications Reports
National Guideline Clearing House	<a href="http://www.guideline.gov/search/search.aspx?term=osteoporosis">www.guideline.gov search/search.aspx?term= osteoporosis</a>	162 Reported Osteoporosis Practice Guidelines

Table 3  
*Baseline Laboratory Tests for Osteoporosis*

Baseline Labs	Disorder	Reference Range
CBC	Low HCT/HG: Anemia, Sickle Cell, Multiple Myeloma, Alcohol Abuse	HCT 35-52 %
		HG 12-18 g/dL
		PLT 150-450 10 <sup>3</sup> /mm <sup>3</sup>
		WBC 4-11 10 <sup>3</sup> /mm <sup>3</sup>
Serum Chemistries	Hypercalcemia: Malignancy or Hyperparathyroidism	CA 8.6-10.2 mg/dL
		Hypocalcemia: absorption of calcium from bone, GI Malabsorption
	Low Magnesium: alters calcium absorption and metabolism	MG 1.6-2.6 mEq/dL
	Low Creatinine: associated with increased hyperparathyroidism	CRT 0.6-1.3 mg/dL
	Elevated Creatinine: Multiple Myeloma, Renal failure	
TSH	Elevated TSH: Hypothyroidism	TSH 0.3-3.0 mIU/L
Liver Function Tests	Elevated ALT/AST: Alcohol Abuse, Liver Disease	ALT 10-40 IU/L
		AST 10-34 IU/L
Serum 25-Hydroxyvitamin D	Low values indicates Vitamin D Deficiency	25(OH)D 30ng/mL

*Sources: Clinical Laboratory Reference, 2013; Jacobs-Kosmin, 2013; NOF, 2010[revised 2013]*

*CBC = Complete Blood Count, HCT=Hematocrit, HG=Hemoglobin, PLT= Platelets, WBC= White Blood Count, BMP= Basic Metabolic Panel, CA=Calcium, MG=Magnesium, CRT=Creatinine, TSH- Thyroid-Simulation Hormone ALT-Alanine Aminotransferase, AST=Aspartate Aminotransferase, 25(OH)D=Serum 25-Hydroxyvitamin D*

Table 4  
Fall Prevention

Intervention	Component
Muscle Strengthening	<p>Core &amp; Lower Extremities: In home program</p> <ul style="list-style-type: none"> <li>• Ankle cuff weights 3 times a week: 2-5 lbs., 2 sets of 10 repetitions 2 times a day for 10-20 min. (<i>Gardner et al., 2001</i>)</li> <li>• LiFE in-home exercise program; set of integrated balance and strength train exercises integrated into activities of daily living (<i>Clemson et al., 2012</i>)</li> </ul>
Balance	<ul style="list-style-type: none"> <li>• Walking; tandem; on toes-heels, backwards, sideways</li> <li>• Turning Around, Stair Climbing, Stepping &amp; Picking Up Objects, Sit to Stand</li> </ul> <p>(<i>Campbell et al., 1997; Clemson et al., 2012; Gardner et al., 2001</i>)</p>
Weight Bearing Exercise	<ul style="list-style-type: none"> <li>• TaiChi (<i>Bischoff-Ferrari, 2013</i>)</li> <li>• Dancing (<i>Howe, Rochester, Neil, Skelton, &amp; Ballinger, 2011</i>)</li> <li>• Yoga (<i>Tiedemann et al., 2013</i>)</li> <li>• Walking-Jogging; 30 min- three times a week Increase 5 min. per week until total of 30 min (<i>Gardner et al., 2001; Bischoff-Ferrari, 2013</i>) (<i>Heike A. Bischoff-Ferrari, 2013</i>) (<i>Heike A. Bischoff-Ferrari, 2013</i>) (<i>Heike A. Bischoff-Ferrari, 2013</i>) (<i>Heike A. Bischoff-Ferrari, 2013</i>)</li> </ul>
Referrals	<p>Optometry; cataract evaluation (<i>Bischoff-Ferrari, 2013</i>)</p> <p>Primary Care</p> <p>Neuropathy-Arthritis Evaluation</p> <p>Medication review; Beers List (<i>AGS 2012 Beers Criteria Update Expert Panel, 2012</i>)</p> <p>Community Fall Prevention Programs</p> <p>Home Health: <i>NOF, 2010[revised 2013]</i></p> <p>Home Assessment; remove obstacles, throw rugs, install bathroom assistive devices</p> <p>Footwear Evaluation; use athletic shoes (<i>Menz et al., 2006</i>)</p> <p>Functional Assessment; include fear of falling evaluation</p> <p>Homemaker Services</p> <p>PT- OT; assistive device evaluation,</p> <p>Lifeline Monitoring</p>
Diet	<p>Vitamin D Supplementation-800-1000 IU daily (<i>Bischoff-Ferrari, 2013</i>)</p>

Table 5  
*Bone Loss Prevention*

Intervention	Component
Diet Modification	Diet History: <ul style="list-style-type: none"> <li>• Calcium rich foods: milk, cheese, yogurt, fortified juices</li> <li>• Vitamin D rich foods: fish (salmon-tuna), fish liver oils, fortified milk and cereals</li> <li>• Moderate sun exposure</li> </ul> Supplementation: <ul style="list-style-type: none"> <li>• Institutes of Medicine                Men aged 50-70- 1000 mg of calcium daily                Women &gt; 51 &amp; Men &gt; 7- 1,200 mg calcium daily</li> <li>• Collective Guideline Recommendations                Women &amp; men age 50 and older: 1200-1500mg of calcium daily, 800-1,00 IU Vitamin D daily</li> </ul>
Weight Bearing Exercise	Walking-Jogging; 30 min- three times a week
Medication Management	Administration and Side Effect Profile See Table 5
Lifestyle Modification	Smoking cessation Moderate alcohol consumption Healthy basal metabolic index
Monitoring	Yearly follow up: height-weight, BMD 2-5 years, Fall Risk

*Sources: Lim et al., 2009; NOF, 2010 [revised 2013]; NAMS, 2010; Office of Dietary Supplements: National Institutes of Health, 2013, 2012; National Research Council, 2011*

*(National Research Council, 2011; Office of Dietary Supplements: National Institutes of Health, 2013; Office of Dietary Supplements: National Institutes of Health, 2012)(National Research Council, 2011; Office of Dietary Supplements: National Institutes of Health, 2013; Office of Dietary Supplements: National Institutes of Health, 2012)(National Research Council, 2011; Office of Dietary Supplements: National Institutes of Health, 2013; Office of Dietary Supplements: National Institutes of Health, 2012)(National Research Council, 2011; Office of Dietary Supplements: National Institutes of Health, 2013; Office of Dietary Supplements: National Institutes of Health, 2012)*

Table 6  
*Osteoporosis Medications*

Medication	Administration/Monitoring	Common Side Effects
Hormone Replacement Selective estrogen receptor modulators EVISTA	Prescribed Post-Menopausal	Blood Clot Breast Cancer Stroke
Bisphosphonates: Alendronate (Fosamax) Risedronate (Actonel)	Take on empty stomach with 8oz. of water sitting upright for 30 min.	GI Upset
Boniva	Oral: Take on empty stomach with 8oz. of water sitting upright for 60 min IV: administer over 15-30 sec every 3 months	GI Upset Renal Failure: monitor creatinine levels
Zoledronic Acid (Reclast)	IV: administer over 15 min every 1-2 years Pre-treat with acetaminophen	Arthralgia Headache Myalgia or fever
Parathyroid: Teriparatide (Forteo)	Use in high risk for fracture and secondary bone loss Administer subcutaneously daily Do not stop abruptly- rapid bone loss ensues Do not use in patients with: Skeletal radiation treatment Bone metastases Hyperkalemia History of skeletal cancers	Leg cramps Nausea Dizziness Risk of osteosarcoma
Receptor Activator of Nuclear Factor kappa-B (RANK) Ligand (RANKL) Inhibitor: Denosumab (Prolia/Xgeva)	Use high risk for fracture with bone loss due to breast or prostate cancer: Bone metastases from solid tumors or giant cell tumor of bone Administered subcutaneously every six months Monitor for hypocalcaemia, cellulitis, rash, Osteonecrosis of the Jaw (ONJ) and atypical femur fractures Do not stop abruptly-rapid bone loss ensues	Fatigue Hypophosphatemia Nausea Arthralgia Headache Back pain Extremity pain

Sources: Chlebowski et al., 2003; FDA, 2002, 2013a, 2013b, 2013c, 2013d, 2013e; Kleerekoper, 2008

Table 7  
*Standard Menopausal Status Laboratory Values*

Laboratory Test	Pre-Menopause	Post-Menopause
FSH	1.5 – 8.5 mIU/ml	30-100 mIU/ml
LH	1 – 15 mIU/ml	20-60 mIU/ml
Estradiol	30-120 pg/ml FP 100-210 pg/ml LF	10-35 pg/ml

*FSH=Follicular Stimulating Hormone, LH=Luteinizing Hormone, FP=Follicular Phase, LF=Luteal Phase*

## **CHAPTER THREE: METHODOLOGY**

This chapter is divided into two sections: the experimental protocol and procedures are presented in the first section, and the series of four experiments performed during protocol development are presented in the second section. The actual written protocols and procedures may be found in Appendices A-G.

### **Introduction**

Bone loss in astronauts, a subject of scientific and practical interest to NASA, (Willey, Lloyd, Nelson, & Bateman, 2011) occurs as a consequence of microgravity environments; bone loss in older adults, a major focus of this investigator, occurs as a consequence of aging and/or pathological disease processes. This shared interest in bone loss led to a collaborative relationship between this investigator and the NASA Ames Research Center Bone and Signaling Laboratory, located in Mountain View, California.

Bone loss is thought to occur due to an imbalance in osteoblast and osteoclast activity. A search of the literature for an appropriate experimental protocol revealed that, although there were a few protocols for investigating the effects of Near Infrared Light Emitting Diode (NIR-LED) in osteoblast cell culture models (Renno et al., 2007; Yamada, 1991), there were no existing protocols to examine the effects of NIR-LED on Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL)-induced osteoclastogenesis (osteoclast proliferation and differentiation) in cell culture. Therefore, the first step in this study was to develop a protocol that would allow the investigation of the effects NIR-LED photobiomodulation affecting osteoclast proliferation and differentiation in a cell culture model. The purpose of this chapter is to describe the development of a new experimental protocol conducted at the NASA Ames Bone and

Signaling Laboratory. A custom designed protocol resulted in an experimental design that would allow the investigation of NIR-LED treatment in a cell culture model as an intervention possibly to prevent and/or treat bone loss.

Before undertaking the creation of an experimental protocol, a review of the literature was conducted to identify any experimental protocols similar to this study's hypothesis. The literature search yielded five similar experimental protocols and procedures investigating the use of NIR-LED treatments affecting osteoclastogenesis (Aihara, Yamaguchi, & Kasai, 2006), osteoblastogenesis (Renno et al., 2007; Yamada, 1991) and other different cell-type culture models (Wong-Riley et al., 2001; Wong-Riley et al., 2005). This protocol was developed by first creating a template that combined aspects of an existing experimental protocol investigating osteoclastogenesis at NASA Ames Bone and Signaling Laboratory. The existing experimental protocol was entitled "collection medium protocol" (see Appendix A) and was created by a post-doctoral fellow for another experimental project that investigated the effects of heavy ion irradiation on osteoclastogenesis in cancellous tissues under the conditions of musculoskeletal disuse (Yumoto et al., 2009). This osteoclast cell culture protocol was found to be successful in yielding consistent proliferation and differentiation of osteoclast cells therefore; it was used as a template protocol model.

## **Section One**

### **Protocol Description**

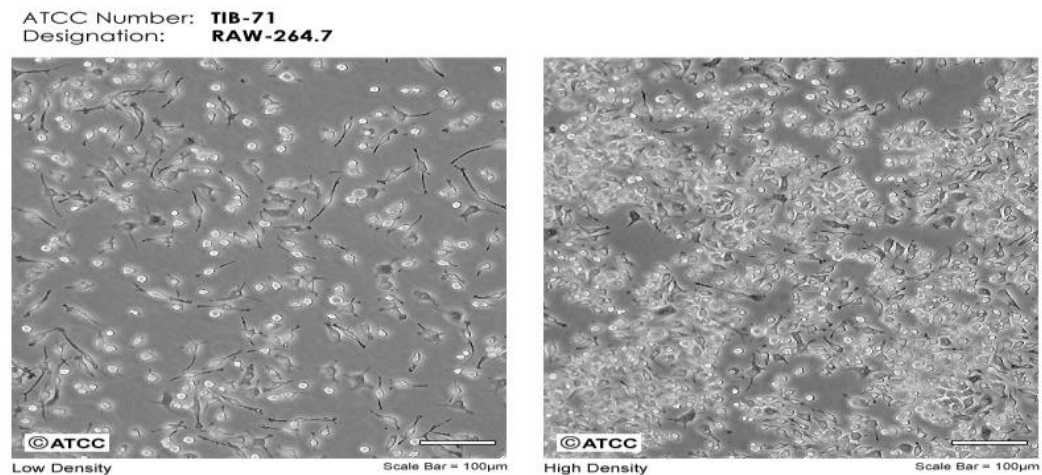
Each experiment took 7 days to complete and was comprised of the following steps: Step 1) preparation of the RAW 264.7 cell line derived from mouse leukemia monocyte macrophages and culture medium for cell propagation (see Appendix B); Step



## Step 1: Preparation of the Cell Line and Culture Medium

**Cell line:** The RAW 264.7 cell line is derived from mouse leukemia monocyte macrophages obtained from American Type Culture Collection (ATCC). This cell line was developed by Raschke and colleagues in 1978 and is kept in a cell repository for purchase through the ATCC for research (American Type Culture Collection [ATCC], 2009; Raschke, 2010; Raschke, Baird, Ralph, & Nakoinz, 1978) (See Figure 2). A microscopic cell appearance example is presented in Figure 2. The letter of permission can be found in Appendix J.

Figure. 2  
*RAW 264.7 Cell Appearance*



(American Type Culture Collection, 2009)

<http://www.atcc.org/~media/6FC6DC948CC64496A158674CEFEE7826.ashx>

Frozen RAW 264.7 cells were shipped directly from ATCC to the laboratory and were handled under bio-safety level 2, moderate risk-suitable for personnel with specific training in handling pathogenic agents (U.S. Department of Health and Human Services Public Health Service, 2007), and were stored at  $-80^{\circ}\text{C}$  in a polypropylene 3ml conical tube.

**Culture Medium:** In the late 1950's, Eagle (Sigma-Aldrich, 2010) developed one of the first cell culture growth mediums called Basal Medium Eagle (BME). Eagle then modified this medium with a higher concentration of essential nutrients which was named Modified Eagle's Medium (MEM). Dulbecco (JR Scientific Inc., 2007) made one of the many modifications to Eagle's BME, thus naming the culture medium Dulbecco's Modified Eagle's Medium that contains a four-fold concentration of amino acids and vitamins compared to BME to support growth of primary mouse and chicken cell cultures it is one of the most widely used cell culture growth mediums. Alpha Modification of Modified Eagle's Medium ( $\alpha$ -MEM) was developed to grow hybrid mouse and hamster cells (Atlanta Biologicals, 2010) and is further supplemented with additional amino acids and vitamins.

The ATCC recommends a base culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) (Raschke, 2010) stored at 2-8°C with the added components of 10% fetal bovine serum (FBS) (ATTC, 2009; Invitrogen, 2010a). However, the Ames Bone and Signaling Laboratory found that RAW 264.7 cell growth was greater when  $\alpha$ -MEM Invitrogen Catalog number: 12571071 (Invitrogen, 2010b) with the added components of 10% FBS (Yumoto et al., 2009) was used for experiments stored at 2-8°C.

Cell culture growth medium was prepared prior to cell line thawing (RAW 264.7 cells) and the manufactures procedure for heat inactivation was followed (HyClone, 2003). The culture medium used for RAW 264.7 cell propagation was  $\alpha$ -MEM, with 10% FBS. The FBS was derived from the blood of animals (bovine, equine, porcine). Fetal bovine serum must be heat inactivated and filtered prior to being added to the base

medium. The FBS was heat inactivated by heating in a 56°C water bath to destroy heat-labile complement proteins. The medium was ready for cell culture propagation and cell line thawing was initiated. Both procedures are found in Appendix B.

### **Step 2: Propagation of the RAW 264.7 Cell Line**

The goal was to propagate a healthy cell line for experimentation and there are two stages in this step. To begin, cells must be propagated over a week to observe for proper cell function and judge their viability for experimentation.

According to Cooper (2000), cell division is variable depending on cell type, but most eukaryotic cells in culture divide approximately every 24 hours. There are two basic parts of the cell cycle: mitosis (nuclear division) and interphase (DNA replication). During cell mitosis there is the separation of the daughter chromosomes, ending with cytokinesis (cell division). Since mitosis and cytokinesis only last for about one hour, the majority of the cell cycle is spent in interphase where cell growth and DNA replication occur to prepare for cell division in RAW 264.7 cells. By allowing newly plated cells to proliferate over several days, the researcher is able to observe the morphology (cell appearance) and behavior of the cell line (RAW 264.7) prior to experimentation. In order for successful propagation to occur, the cell line was maintained in an incubator (Thermo Scientific: CO<sup>2</sup> Series II water jacketed) at 37°C in 5% CO<sup>2</sup> for no longer than 30 days, and passaged (as described below) (Rice University, 2010) every three days with 10% FBS with  $\alpha$ -MEM according to the ATCC cell propagation guidelines to maintain optimal cellular metabolism and health (Raschke, 2010). The incubator provides an environment maintained at a consistent temperature. Once the cell line is determined to

be healthy by observed cell growth by increasing cell confluence (coming together), cells may be used for experimentation.

**RAW 264.7 cell passaging:** Passaging culture cells means to split or divide cells from mature cell culture plates. This procedure is done to maintain the viability and growth of cells within the cell culture line. When RAW 264.7 cells are left in culture for an extended period of time, usually greater than three days, they become confluent (run together) and grow in clumps. If the cells are not “split up” they will grow on top of one another and will eventually die due to the lack of surface area upon which to grow. Cell passage is the method by which cell clumps are dislodged via cell scraping and then sub-cultures are created. A certain percentage of cells are re-suspended with fresh medium in new cell culture dishes according to cell concentration ratios. (see Appendix. C)

### **Step 3. Cell Plating Procedure**

This step required two experimental days, Day 0 (Plate labeling & cell plating) and 1 (RANKL induction).

**Day 0: Plate labeling:** Each plate was labeled according to experimental condition: number of light treatments (1 or 4), energy density (varied by experiment: 4.5 or 45 J/cm<sup>2</sup>), RANKL dose (varied by experiment: 0, 25, or 50 ng/ml or 0, 2.5, 10, 25 or 50ng/ml). The following is a labeling example used for the experiments: control (no light treatment), single light treatment, and multiple light treatment groups, respectively.

1. *Control* [0-0-0], translates to [0] light treatment, energy density [0 J/cm<sup>2</sup>], and RANKL Dose [0 ng/ml].
2. *Single* [1-4.5-50], translates to [1] light treatment, energy density [4.5 J/cm<sup>2</sup>], and RANKL Dose [50 ng/ml].
3. *Multiple* [4-45-25], translates to [4] light treatments, energy density [45 J/cm<sup>2</sup>], and RANKL Dose [25 ng/ml].

Experimental conditions, RANKL dose, and energy density are described in subsequent sections in which the NIR-LED equipment and procedure are explicated. However, in the first four experiments due to the limitations of the WARP™ 10, the surface area of light illumination could optimally irradiate 2 wells at one time (well row B by columns 3-4). Therefore, multiple 24-well plates had to be used (see Figure 3 in List of Figures). In the final two experiments, the WARP™ 75 surface area of light illumination was great enough to irradiate a maximum of 15 wells at one time (well row A-B by columns 2-6).

The experimental condition labeling procedures for the final two experiments used a higher sample number than our first four experiments summarized in Appendix H, Table 1. Summarizing the steps involved to cell plate the experiment included: the number of wells for each cell culture plate was calculated. Second, medium was placed into the wells, and then from the propagated cell line culture, a cell suspension was calculated to determine the amount of cells to be deposited into each well. On Day 0 (first day of experiment) RAW 264.7 cells were plated at  $2 \times 10^4$  cells per well ( $4 \times 10^4$  cell/ml) in two center wells of a 24-well plate (well diameter: 1.77mm) for the first four experiments and 5 wells for the last two experiments for each experimental condition. The full procedure is found in Appendix D.

**Day 1- RANKL Induction Procedure:** The plates were cultured for 24 hours prior to RANKL induction. At the end of the 24 hour period, RANKL was added to each plate well at a dose of 0, 2.5, 10, 25 or 50ng/ml. This particular cell line requires the induction of Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) in order to promote osteoclast formation. The induction method is as follows: Osteoblast stromal

cell membrane RANKL receptor, binds to the osteoclast precursor cell (macrophage) RANK receptor. When this occurs, RANK signaling activates the transcription nuclear factor Kappa-B to differentiate into osteoclasts (Kumar, 2010).

In order to calculate the correct RANKL dose added to the cell culture, a serial dilution was used. A serial dilution is a stepwise dilution of a substance, in this case RANKL. At each step of dilution, the dilution factor changes as seen in Table 2, Appendix H.

The original stock concentration of RANKL from the manufacturer was 100 $\mu$ g/ml (PeproTech). Converted to ng this would be  $100 \times 10^3$  ng/ml. For experimental use, RANKL was aliquoted into smaller volumes (10 $\mu$ l) at a 1250ng/ml concentration (see Appendix E)

#### **Step 4: NIR-LED Treatment**

NIR-LED treatment was initiated on Day 2 for all experiments regardless of whether the experiment was testing single or multiple light-treatments. Experiments in which multiple light-treatments were the focus had repeated exposure on Days 3-5 for a total of 4 light treatments. A continuous wave NIR-LED WARP™10 (Quantum Devices, Barneville, WI) with a wavelength of 670nm, power intensity of 60mw/cm<sup>2</sup> and energy density of 5 J/cm<sup>2</sup> was used in the experiments because it was shown in previous studies to produce positive cellular effects (Desmet et al., 2006; Eells et al., 2007; Eells, 2004; Karu, 1988; Liang, Whelan, Eells, & Wong-Riley, 2008; Pinheiro et al., 2002; Whelan et al., 2003; Whelan et al., 2008; Wong-Riley et al., 2001; Wong-Riley et al., 2005). The dosage of light treatment was determined by applying the following formula: Power (mW/cm<sup>2</sup>) x Time (seconds) ÷ 1,000 = Energy Density (J/cm<sup>2</sup>) in order to deliver 4.5

J/cm<sup>2</sup> and 45 J/cm<sup>2</sup>. To ensure delivery of an accurate energy density of 4.5 J/cm<sup>2</sup> and 45 J/cm<sup>2</sup>, each light treatment was timed to 80 seconds to deliver 4.5 J/cm<sup>2</sup> and to 800 seconds to deliver 45 J/cm<sup>2</sup>.

Light treatment began on cell culture Day 2 (Stein et al., 2005; Yamada, 1991) under a sterile laminar hood with the dish lid in place. When multiple light treatments occurred, three more consecutive days of treatments for a total of four treatments were performed. The plates receiving multiple light treatments were treated according to the following protocol. On culture day two of the light treatment, plate 1 was treated first and then plate 2, 3, and 4 were treated. On culture day three of the light treatment, plate 2 was treated first, and then plates 3 and 4 were treated while plate 1 was placed at the end of the treatment order. On culture day four of the light treatment, plate 3 was treated first and then plates 4, 1, and 2. On culture day five of the light treatment, plate 4 was treated first and then plates 1, 2 and 3 were treated. This was to control for objectivity and amount of time the plates were out of the incubator.

#### **Step 5: TRAP Staining Day 7**

For purposes of these experiments, TRAP staining (SIGMA #387) was the method employed for manual osteoclast cell counting. Using a visual cell counting classification tool, those multinucleated cells that were stained with a light pink/purple were identified by light microscopy (inverted microscope, 10x magnification; Nikon Eclipse TS100) and counted. The procedure used for TRAP cell staining was obtained from SIGMA Acid Phosphatase Leukocyte Kit (prod #387) (see Appendix F). Cells were fixed with a formaldehyde solution to stop the cellular activity and prepares them for staining. The cells were stained with fast garnet solution which identifies the release of the enzyme,

TRAP. The cells that were both stained a light pink/purple and were multinucleated were assumed to be releasing TRAP and, therefore, were considered osteoclast cells.

#### **Step 6: Manual Cell Counting Classification Tool**

When macrophages differentiate into osteoclast cells, the enzyme, bound in the cell membrane, Tartrate-Resistant Acid Phosphatase (TRAP) is stained for cell counting. This enzymatic activity has been historically regarded as one of the reliable markers of osteoclast identification (Nakano, Toyosawa, & Takano, 2004). There are several methods to identify osteoclast cells, such as TRAP staining (Susa, Luong-Nguyen, Cappellen, Zamurovic, & Gamse, 2004), TRAP5b ELISA assay (Halleen et al., 2000), or computer imaging of histological slides (Reed, Conzemius, Robinson, & Brown, 2004).

However, for this study a visual cell counting classification tool was developed to guide in the cell counting process (see Appendix G). Plates were blinded. To be counted as an osteoclast, all cells were TRAP<sup>+</sup> stained with a pink/purple or dark purple bordering on black (if nuclei were visible). To determine the faintest acceptable pink, the stain color should match the RANKL control pink stain. The counted cells should be stained darker than the control faint pink. The cell was counted if there were three or more nuclei present. While clumps of TRAP<sup>+</sup> mono-nucleated cells were not counted, and cells connected by cell processes were counted as separate cells. Mean osteoclast well counts per well and standard deviations were calculated by taking the sum of multinucleated TRAP<sup>+</sup> osteoclast cells for each well and dividing by the well surface area, expressed as cells per cm<sup>2</sup>. To determine light treatment effects, differences between osteoclast cell counts in treatment plates and controls were calculated.

Six experiments were conducted. Presented below are the first four experiments conducted. These four experiments were used as an exercise by which to develop the experimental protocol and procedure to ensure the reliability of equipment and cell culture and procedure techniques. Results are reported with the subsequent change to the experimental protocol and procedure. The final two experiments are presented in Chapter IV, in the manuscript entitled, *Effects of Photobiomodulation in Osteoclast Formation in Vitro: a Pilot Study* and represent the culmination of the dissertation work.

The following are the equipment testing experiments for the WARP™ 10 and WARP™ 75 NIR-LED lights [e1386, e1389] which are followed by the four WARP™ 10 NIR-LED experiments (e1386, e1380, e1383, e1384, and e1385). Each experiment is identified by an experiment number (assigned and recorded by the NASA Bone and Signaling Laboratory). Problems that occurred and the resolution to correct the problem within the protocol are discussed for each experiment.

### **Equipment Testing Experiments**

In order to determine the functionality and possible experimental confounding effect of the WARP™ 10 and WARP™ 75, NIR-LED lights, two equipment tests were conducted.

**e1386: WARP™ 10 and e1389: WARP™ 75 Equipment Tests:** In laser medicine the most common use of lasers has been in surgery to cut, coagulate, or ablate tissue. The laser's mechanism of action in this instance is to destroy tissue and provide precise surgical incisions. This is achieved by heating the tissue at a high irradiation intensity and energy density. However, there are other uses for lasers.

The rationale for testing the equipment was to determine possible confounding variables that could affect the experiments. In the 1980's photobiomodulation—referred to in the literature as low-level laser therapy, cold-laser therapy, or laser biostimulation—was introduced. This laser “light treatment” uses light in the infrared to near-infrared region of the absorption spectrum (630-1000nm) which is known to affect numerous cell functions that are dependent on the energy production in the mitochondria (Desmet et al., 2006; Karu et al., 1987). At low light intensity, such as 670 nm, a photochemical conversion of energy absorbed by non-specialized photoacceptors on the mitochondria and generates heat (Karu, 1988; 1999; Letokhov, 1991). Raising the temperature of these molecules triggers biochemical reactions that may activate or inhibit cellular enzymes that turn cellular functions “on” and “off” (Karu, 1999). This may result in positive or negative events. If the heat generated exceeds the optimal cell line temperature for cell growth, in this case body temperature (37°C), it is referred to as “heat shock”. Heat shock at 41°C can stress the cells and result in the release of heat shock proteins, which may cause harm to the cell (Levin, Wickliffe, Leppla, & Moayeri, 2008). Several investigators reported that continuous wave light treatment generates minimal local heating of photoacceptors because the wavelength used is of low irradiation intensity (670nm) (Karu, 1992; Letokhov, 1991), thus, heat shock is avoided.

Because there were only a few studies testing local heating of photoacceptor molecules, this principle was investigated. In addition, there was concern regarding possible heat shock due to the use of the WARP™ 10 and WARP™ 75, because they were commercial continuous wave light sources that automatically shut off every 80 seconds requiring a manual restart. Therefore, the 80-second light treatment (4.5 J/cm<sup>2</sup>)

did not require manual restarts, but the 800 second light treatment ( $45\text{J}/\text{cm}^2$ ) required 10 manual restarts. This manual restarting of the light treatment causes a brief surge of local heating. This surge could be considered pulsing light treatment which has been shown in the literature to give a greater biological effect than continuous wave light treatment (Karu, 1992).

Light treatment temperature testing was performed with a thermistor in both the WARP™ 10 (e1386) and WARP™ 75 (e1389) as described in the “Light Treatment Procedure”. Cell culture plates were removed from the  $37^\circ\text{C}$  incubator and placed on a metal table. The temperature of the cell culture medium at the beginning of the experiment was  $30.7^\circ\text{C} - 32.3^\circ\text{C}$ . The temperature testing procedure at room temperature was repeated two or three times at 80 seconds and 800 seconds (the length of both light treatment groups in this experiment). During the light treatment exposure the temperature of the cell culture medium never rose above  $37^\circ\text{C}$ .

Treated with the WARP™ 10 for 80 seconds, the cell culture medium had an overall drop in temperature of  $0.9^\circ\text{C} - 1.4^\circ\text{C}$ , while that treated for 800 seconds had an overall drop in cell culture medium temperature of  $5.6^\circ\text{C} - 7.6^\circ\text{C}$ . The cell culture medium treated with the WARP™ 75 had an overall drop in temperature of  $1.8^\circ\text{C}$  for both the 80- and 800-second light treatment. During the WARP™ 75 multiple light treatments (which required 10 manual restarts), temperature of the cell culture medium initially dropped by  $0.9^\circ\text{C} - 2.3^\circ\text{C}$ , but then at approximately 265 seconds (4 minutes, 25 seconds) into the treatment, the temperature increased overall by increments of  $1.6^\circ\text{C} - 1.8^\circ\text{C}$ . It was concluded that heat shock had most likely not occurred because temperature change did not reach  $41^\circ\text{C}$ .

## Section Two

### Four Protocol Development Experiments

#### NIR-LED Experiments

Experiments e1380 and e1383 tested multiple light treatments and single light treatments. Experiments e1384 and e1385 tested only single light treatment. All experiments tested energy densities of 4.5 & 45 J/cm<sup>2</sup>. Lastly, RANKL dose response was tested at 0, 25, & 50 ng/ml in experiments e1380, e1383, e1384, e1385. (see Appendix H Table 3)

#### Experiment # 1: e1380

In this experiment, six complications were encountered. Each complication is discussed with its subsequent decision made in changing the protocol or procedure. This experiment multiple light treatments and single light treatments at 4.5 J/cm<sup>2</sup> and 45 J/cm<sup>2</sup> at RANKL doses of 0, 25, and 50ng/ml were tested. A lack of osteoclast cell differentiation was noted in this first experiment. The following are the encountered six complications.

1. A lack of osteoclast cell differentiation may have occurred due to premature RANKL induction. In accordance with the “collection medium protocol” (see Appendix A) RANKL induction occurred 6 hours after cell plating. Possibly, the RAW 264.7 cells did not have enough time to adhere to the well plate and proliferate sufficiently for osteoclast cell differentiation. **Correction:** Therefore, other NIR-LED cell culture model experiments found in the literature (Renno et al., 2007; Wong-Riley et al., 2001; Yamada, 1991) were consulted and the decision was made to induct RANKL 24 hours after cell plating to allow for better cell/plate adhesion. This decision was reached because it was

thought that when the medium was changed, many free floating cells were extracted leaving too few cells to propagate and adhere.

2. A lack of osteoclast cell differentiation may have occurred due to miscalculating the cell density when preparing a cell suspension used to plate cells at cell density of  $4 \times 10^4$  cells per/ml. An error was made in loading the hemocytometer: there were too few cells counted in the cell suspension. **Correction.** In subsequent experiments, the investigator consulted with the lab technician to avoid errors such as checking mathematical calculations and observing the investigator's pipetting technique because many cell wells were void of cells mid-well due to investigator aggressive pipetting, resulting in loss of reliable data collection. The investigator practiced pipetting to prevent mid-well washing, hemocytometer calculation, and propagating cells RAW cells. The importance of consistent cell plating with correct cell suspension is imperative because if too many or few cells are plated, cell wells may encounter cell confluence (making cell counting difficult) or non-cell adhesion.

3. A lack of osteoclast cell differentiation may have occurred due to leaving all of the cell culture plates out of the incubator during all of the light treatments. **Correction.** In future experiments each cell culture plate was kept outside the incubator only for the length of its light treatment, approximately 80 to 800 seconds. A decrease in cell well medium temperature may affect cell differentiation adding a confounding variable because the control did not encounter the same experimental conditions.

4. A lack of osteoclast cell differentiation may have occurred due to pre-mature NIR-LED shut-off and limitations of the WARP™ 10 unit. Because the WARP™ 10 is a commercial product for the general public, it is battery powered and automatically turns

off every 80sec to avoid overheating because of repeated consecutive use. In addition, over the span of the treatment time the light intensity may have diminished due to it being battery operated affecting the dosing of NIR-LED photobiomodulation and altering reliable data collection. **Correction.** These limitations had to be accepted during these preliminary experiments and were controlled by manually restarting the WARP™ 10 by switching to rechargeable batteries which were cooler and may have delayed overheating, by changing to fresh batteries more frequently, and by allowing the light to cool five minutes between the 45 J/cm<sup>2</sup> treatments. This was done to avoid heating the cell culture medium.

5. A lack of osteoclast cell differentiation may have occurred due to the investigator error of cell plating only two control wells. It was determined that two control wells were too few because one well was lost due to contamination, leaving only one control well. **Correction.** An extra set of control wells per experimental condition (n = 5). In the final experiments reported in chapter four, two sets of controls were plated (n = 10) to prevent the loss of controls. If an experiment's controls do not produce reliable cell counts or are flawed, the experiment data cannot be interpreted. If the first control was flawed, then the second control was used for data analysis.

6. Lastly, a lack of osteoclast cell differentiation may have occurred due to possible RANKL derogation. In order to determine if the potential cause for poor osteoclastogenesis occurring in experiment e1390, it was thought that possibly the PeproTech RANKL may have derogated due to freezing/thaw cycles or perhaps the carrier protein, Bovine Serum Albumin (BSA), added to the RANKL delusion was responsible for the lack of osteoclastogenesis. This also may have occurred due to using a

manufacturer of RANKL (PeproTech) that differed from the manufacturer of the “culture medium protocol” (Sigma) (see Appendix A). Therefore, experiment e1382 was conducted by the lab technician who compared osteoclastogenesis using PeproTech RANKL and Sigma RANKL and BSA vehicle control. **Correction.** Based on experimental results, PeproTech RANKL was chosen for subsequent experiments because it yielded three times more osteoclasts with doubled RANKL, larger cell size, and dark-pink and/or purple TRAP+ stained cells with bright distinctive nuclei compared to Sigma RANKL. Further, BSA did not affect cell growth or morphology. Therefore, RANKL could be reconstituted for future experiment use in sterile pyron-free water with 0.1% BSA carrier protein which will remain stable for three months stored at -20°C. Consequently, RANKL used in e1390 was derogated and any unused RANKL that was prepared for osteoclast induction was discarded after use and not refrozen. If the cytokine added to cell cultures is derogated, a poor biological signal results and data analysis may be flawed due to derogated cytokine versus a freshly suspended cytokine.

**Conclusion.** Because of the above complications the data collected in this experiment were not reliable and were not analyzed. However, alterations were made to the experimental protocol in experiment #2 (e1383). Overall, the investigator acquired cell culture knowledge in technique, control of samples, cytokine preparation, cell plating, and suspension. The most important protocol change was the increase in number of control cell wells because losing control data nullifies an entire experiment. New information and learning was evident by understanding how manufactures of RANKL may differ, inexperienced cell culture skills can alter data collection reliability, as well as simple miscalculation errors.

**Experiment # 2: e1383**

With these protocol changes mentioned above, the biphasic dose response curve for both RANKL dose and energy density were repeated in our next experiment to illustrate the expected dose response to photomodulation and RANKL dose. If insufficient energy or RANKL dose was delivered, there would be no response because the dose minimum threshold was not met. However, if too much energy or RANKL dose, minimum threshold was crossed then biostimulation disappears and only bioinhibition occurs (Huang, 2009). A new treatment variable was added, multiple light treatment, the literature reflected a gap in this type of NIR-LED light treatment (Huang, 2009). This experiment tested multiple light treatments and single light treatments at 4.5 J/cm<sup>2</sup> and 45 J/cm<sup>2</sup> at RANKL doses of 0, 25, and 50ng/ml.

1. It was noted that the expected normal RANKL dose response during osteoclastogenesis was reversed between RANKL doses 25 ng/ml and 50 ng/ml in one of the control plates. A normal dose response during osteoclastogenesis would be a one-fold increase from the 25ng/ml to the 50 ng/ml doses. **Correction.** Because there was a correct dose response in the treatment plates, it was unclear where an error had been made. Possibly the RANKL dose was inversely inducted. The backup control was used for data analysis. No changes were made to the protocol.

2. Investigator error was noted in miscalculating the cell density when preparing a cell suspension for plating cells, even though this was checked by the lab technician. An error may have been made when loading the hemocytometer, again resulting in too few cells for the cell suspension. However, there was success in osteoclastogenesis throughout all experimental and control plates, so it was thought that enough cells

adhered to the cell plate. The lab technician questioned the cell plating density because there was confusion in the lab notes from the “conditioned medium protocol”. The “conditioned medium protocol” stated that the cell plating density was  $4 \times 10^4$  cells/ml (see Appendix A). Usually, cell plating density is reported as the number of cells per well not the number of cells per milliliter. The lab notebook stated the cell plating density to be  $2 \times 10^4$  cells/well. This may result in too few cultured RAW cells and therefore few osteoclasts may differentiate with RANKL. **Correction.** To check if the cell plating density was correct, the lab technician calculated that  $4 \times 10^4$  cells/ml equals  $2 \times 10^4$  cells/well. The protocol continued to use  $4 \times 10^4$  cells/ml. Due to this continued problem of calculating the cell density and cell plating, the lab technician calculated and performed the cell density and cell plated experiment e1384 as to correct problems with intra-rater reliability.

**Conclusion.** The effects of single light treatments at energy densities of  $4.5 \text{ J/cm}^2$  and  $45 \text{ J/cm}^2$  at RANKL doses of 0, 25 and 50ng/ml are presented in Figures 4 and 5. Comparing single NIR treatment at  $4.5$  and  $45 \text{ J/cm}^2$  and RANKL dose 25ng/ml there was less of a biostimulatory effect by 54% (see Figure 4) and 32% respectively (see Figure 5). To a lesser extent, osteoclastogenesis was inhibited in RANKL dose 50ng/ml at  $4.5 \text{ J/cm}^2$  and  $45 \text{ J/cm}^2$ , 37% and 10% respectively compared to controls. The dose response of RANKL may have reached minimal threshold at 25ng/ml and require testing a lower RANKL dose. However, light treatment was not biostimulatory at both energy densities. If a normal biphasic energy density dose response was evident, there should be evidence of stimulatory effect. Therefore, a lower energy density variable ( $2.25 \text{ J/cm}^2$ ) was added to the experiment #4 e1385 protocol. It is premature to determine if these energy

densities are biostimulating or bioinhibiting, as it is possible the energy density is too intense for the RAW RANKL induced osteoclast *in vitro* cell type compared to other light treatment cell culture studies using similar variables. Further knowledge was gained in regards to protocol development based upon dose response curves.

Figure 4

*e1383 WARP™ 10: Single NIR treatment 4.5 J/cm<sup>2</sup> RANKL dose 25 & 50ng/ml*

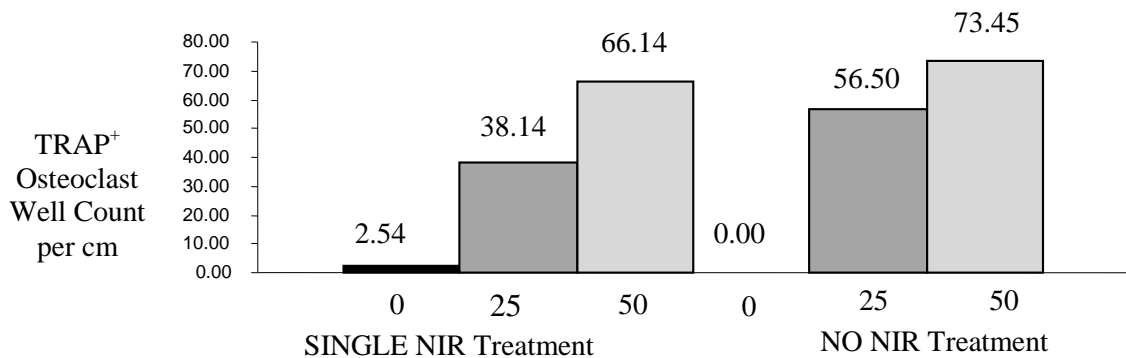
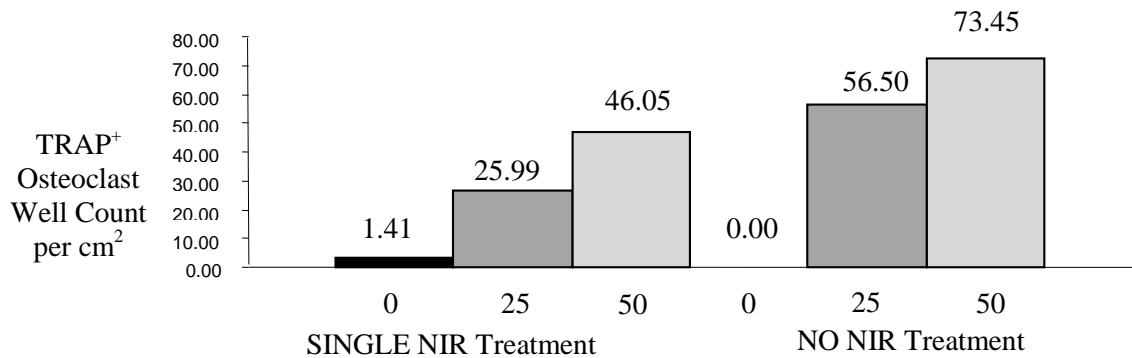


Figure 5.

*e1383 WARP™ 10: Single NIR treatment 45 J/cm<sup>2</sup> RANKL dose 25 & 50ng/ml*



A summary of the effects of multiple light treatments at energy densities of 4.5 J/cm<sup>2</sup> and 45 J/cm<sup>2</sup> at RANKL doses of 0, 25 and 50ng/ml are presented in Figures 6 and 7. Multiple light treatments at 4.5 & 45 J/cm<sup>2</sup> and RANKL dose 25ng/ml inhibited osteoclastogenesis by 70% (see Figure 6) and 44%, respectively compared to controls

(see Figure 7). No effect was seen in RANKL dose 50ng/ml at 4.5 J/cm<sup>2</sup> and 45 J/cm<sup>2</sup> or in single vs. multiple light treatment groups. The same rationale was applied to these results as above to investigate variables that may produce a stimulatory response. Therefore, the protocol was simplified to test single light treatment at 4.5 J/cm<sup>2</sup> and 45 J/cm<sup>2</sup> with RANKL doses 0, 25, and 50ng/ml in order to gain a better understanding of the inhibition or biostimulatory effect.

Figure 6

*e1383 WARP™ 10 Multiple light treatment 4.5 J/cm<sup>2</sup> RANKL dose 25 & 50ng/ml*

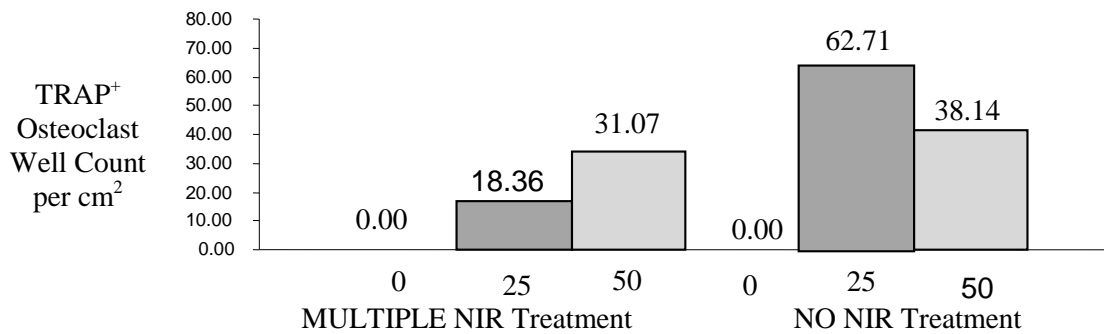
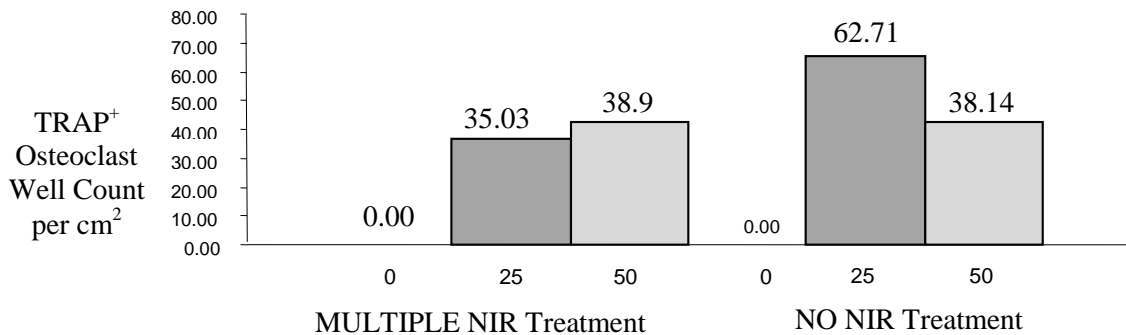


Figure 7

*e1383 WARP™ 10 Multiple light treatment 45 J/cm<sup>2</sup> RANKL dose 25 & 50ng/ml*

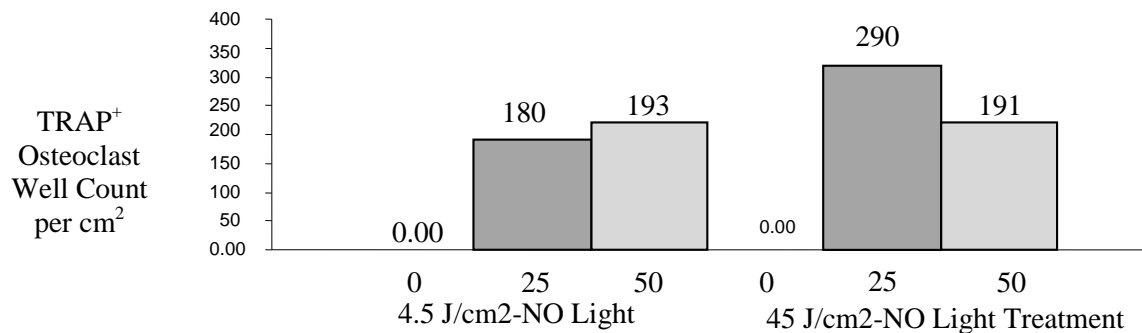


### Experiment #3 e1384

Two complications problems were encountered in this experiment which tested only single light treatments at  $4.5 \text{ J/cm}^2$  and  $45 \text{ J/cm}^2$  at RANKL doses of 0, 25, and 50ng/ml.

1. Increased cell well confluence, cell floaters, and large cell clumps were noted in all control plates starting on cell culture day 1. It was noted that the expected normal RANKL dose response during osteoclastogenesis was reversed between RANKL doses 25ng/ml and 50 ng/ml in the control plates (see Figure 8). The experiment was continued because all other cell wells in treatment plates were progressing normally. However, when TRAP staining was performed fewer TRAP<sup>+</sup> cells were noted in the control wells and it was determined that the control plates were flawed. **Correction.** The data from the controls were was not to be used in the data analysis; however the results from this experiment could be used to determine NIR-LED effects between single-treatment  $4.5 \text{ J/cm}^2$  and  $45 \text{ J/cm}^2$  light treatment groups. In reflection, it would have been more cost effective to have stopped the experiment and restart the protocol. The loss of controls nullified the majority of the experimental data.

Figure 8  
e1384 WARP™ 10 Dose Response Control Plates  $4.5 \text{ J/cm}^2$  &  $45 \text{ J/cm}^2$  RANKL dose 25 & 50ng/ml

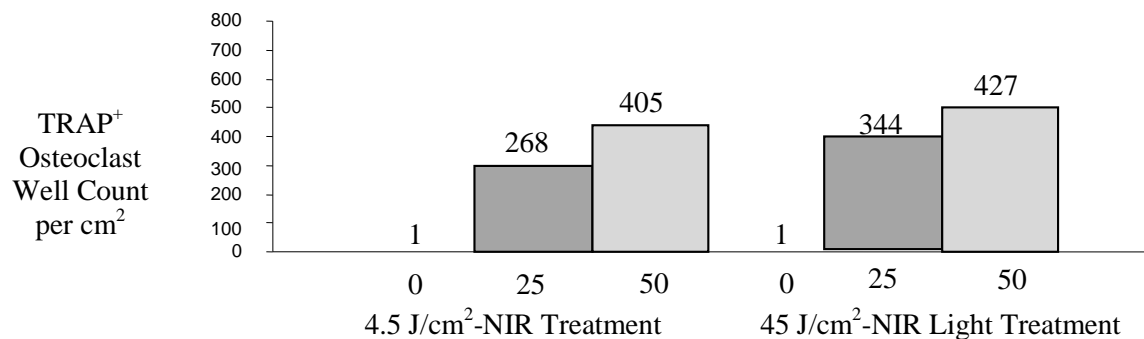


**Problem 2:** A protocol error was made on Cell Culture Day 0 which is the day that RANKL is added to the medium. Usually the cell culture medium is changed only on Day 3; however the investigator mistakenly changed the medium and then added RANKL on Day 0. There was a concern that some cells were discarded with the medium, leaving too few cells to adhere to the cell plate. **Resolution:** In order to control for this change in the protocol to determine the reliability of the data collected from e1384 NIR #3 another experiment e1385 NIR #4 was conducted to determine if changing medium on cell culture Day 0 vs. Day 3 would effect osteoclastogenesis.

**Conclusion:** Presented in Figure 9 is the comparison of the effects of single light treatments at energy densities of  $4.5 \text{ J/cm}^2$  and  $45 \text{ J/cm}^2$  at RANKL doses of 0, 25 and  $50 \text{ ng/ml}$ . Comparing Single Light treatments at an energy density of  $4.5 \text{ J/cm}^2$  vs.  $45 \text{ J/cm}^2$  at RANKL dose  $25 \text{ ng/ml}$  and  $50 \text{ ng/ml}$ ,  $4.5 \text{ J/cm}^2$  inhibited osteoclastogenesis by 29% and 5%, respectively. It appears that the  $4.5 \text{ J/cm}^2$  treatment dose stimulated osteoclastogenesis less than the  $45 \text{ J/cm}^2$  light treatment dose. The RANKL dose appears to produce a stepwise progression, therefore the RANKL dose should be replicated with possibly additional low dose RANKL added. It is unclear if the energy density response is significant, and the experiment would benefit from testing at a lower energy density.

Figure 9

*e1384 WARP™ 10 Single Light Treatment  $4.5 \text{ J/cm}^2$  vs.  $45 \text{ J/cm}^2$  RANKL dose 25 &  $50 \text{ ng/ml}$*



### **Experiment #4 e1385**

This experiment was performed using the final protocol developed from the previous experiments. The purpose of this experiment was to determine if changing the medium on cell culture day 0 instead of cell culture day 3 affected osteoclastogenesis in e1384. This experiment tested only single light treatments at  $4.5 \text{ J/cm}^2$  and  $45 \text{ J/cm}^2$  at RANKL doses of 0, 25, and 50ng/ml. There were no problems associated with this experiment and it was determined that changing the medium did not affect osteoclastogenesis.

### **Conclusion**

RANKL induced osteoclast formation by RAW cells occurred as expected in all experiments, however in e1383 and e1384 a typical dose-dependent response (0, 25, 50 ng/ml) was not observed in controls but in treatment groups. In all experiments using light treatment without the induction of RANKL at these doses and regimes, no effect was measured on osteoclast formation. A single NIR light treatment at energy density  $4.5 \text{ J/cm}^2$  may inhibit RANKL (25ng/ml)-induced osteoclast formation seen in e1383. However, controls were in inverse dose response. Multiple light treatments may be more effective in inhibiting osteoclastogenesis than single light treatment alone as seen in e1390. Based upon these summarized data, protocol modifications were made accordingly and effects were noted for future comparison to pilot data. The final protocol timeline reflects the experimental design divided by cell culture Days 0-7. (see Figure 1)

In some of the experimental control groups, there was a progressive biological dose response in osteoclast cell count that correlated to increasing doses of RANKL. Low dose RANKL-induced cell cultures had lower osteoclast cell counts compared to

RANKL-induced cell cultures with progressively increasing RANKL doses. After a successful experimental protocol was developed, the next step was to use this protocol in a pilot study to obtain reproducible data validating the experimental methodology. A pilot study was planned with the use of a more reliably functioning light source (WARP™ 75) (as determined by e1389) to allow for greater control to deliver light treatments with low variability of energy density and light intensity. Added lower doses of RANKL dose 2.5 and 10ng/ml and lower energy density dose 2.25 J/cm<sup>2</sup> was added and multiple vs. single light treatments will be retested.

Presented in the next chapter are the results of the final two experiments that were used for data analysis in the Chapter IV manuscript, *Effects of Photobiomodulation in Osteoclast Formation in Vitro: a Pilot Study*.

## CHAPTER FOUR: DATA ANALYSIS

The following chapter is a manuscript reporting the findings from the final two experiments e1837 and e1390 and represents the culmination of this dissertation work. This manuscript was presented at the 62<sup>nd</sup> International Astronautical Congress: African Astronautics, Cape Town, South Africa, on October 3-7, 2011.

### **Effects of Photobiomodulation in Osteoclast Formation *in Vitro*: A Pilot Study**

#### **Abstract**

##### **Introduction:** Near-Infrared Light Emitting Diode (NIR-LED)

photobiomodulation has been found to be effective in improving wound healing, bone regeneration, mitochondrial function, and attenuating cellular oxidative stress. Little is known regarding the use of NIR-LED and the formation of osteoclasts, which break down bone. **Purpose:** Determine if the use of NIR-LED 670nm photobiomodulation may attenuate or amplify osteoclast differentiation in the RAW264.7 cell line. **Methods:** RAW264.7 cells were cultured for 24 hours and induced to differentiate into osteoclasts, using the cytokine Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL). Cultures were divided into groups according to RANKL dose (0, 2.5, 10, 25, 50 ng/ml) and different energy densities (2.25, 4.5, 45 J/cm<sup>2</sup>), treated with light either once or on four consecutive days using the WARP™ 75 (Quantum Devices, Barneveld, WI). Osteoclast-like cells were stained for Tartrate-Resistant Acid Phosphatase (TRAP). **Analysis:** Multinucleated, TRAP<sup>+</sup> cells were scored as osteoclasts, counted manually by microscopy. Results were expressed as means and standard deviations, and groups were compared by one-way ANOVA with posthoc Tukey. **Results:** RANKL-induced osteoclast formation by RAW264.7 cells occurred as expected in all experiments. Light-treatment alone had no observable effect. A single light-treatment at 4.5 J/cm<sup>2</sup> with

RANKL added (10 - 50ng/ml) suggested a biostimulatory effect upon osteoclastogenesis compared to controls, as multiple light-treatments compared to single light-treatment were less stimulatory in the energy density  $4.5 \text{ J/cm}^2$  and RANKL dose 25ng/ml group. The effects of the NIR-LED treatment on osteoclastogenesis are RANKL dose and light-intensity specific. **Conclusion:** Effects of NIR-LED treatment on osteoclastogenesis are RANKL dose and light-intensity specific. NIR-LED light-treatment affects RANKL-induced osteoclast formation suggestive of a biostimulatory effect, and multiple light treatments compared to single light treatment may biostimulate osteoclastogenesis less.

### **Introduction**

Older adults often experience complications of muscle (Degens & Alway, 2003, 2006; Trappe, 2009) and bone loss during the process of aging (Khosla & Riggs, 2005), after prolonged bed rest during hospitalization (Crepaldi & Maggi, 2005), and/or from self-limiting behaviors due to the fear of falling after returning home after hospitalization (Fletcher & Hirdes, 2004). Microgravity during space flight simulates these complications in astronauts. Research concerning these skeletal health problems that develop and validation of countermeasures to the pathological consequences of microgravity during long-duration space flights involves interventions and procedures designed to mitigate health issues and performance hazards present in a space environment (Clement, Buckley, & Paloski, 2007). It is highly likely that lessons learned from this research could be applied to the development of innovative technologies for interventions that may improve bone cell function and skeletal health in at-risk older adults. Some researchers investigating bone loss in microgravity environments believe that there is the potential for translational research that could affect the pathology of bone

loss in the aging population (Rittweger, 2007). Collaboration between clinical and bench science researchers may develop, enhancing the use of new technologies that may mitigate bone loss for the older adult population to prevent fracture (Rittweger, 2007).

**Translation of NASA Technologies.** Quantum Devices Incorporated

(Barneveld, WI) developed a NASA technology, entitled High Emissivity Aluminiferous Lighting Substrate <sup>TM</sup> (HEALS), to grow plants for long-term space flight. In 2000, they received a Space Technology Hall Of Fame Award for their *Innovation of Light Emitting Diodes for Medical Applications* (National Astronautical Space Agency, 2008; Quantum Devices Incorporated, 2004). They successfully developed a commercial device, the Warfighter's Accelerated Recovery by Photobiomodulation (WARP 10), a portable Near Infrared Light Emitting Diode (NIR-LED), used in combat to provide first aid for minor injuries and pain. Further, this application is proposed to be helpful in improving medical care for long-term space flight (Whelan et al., 2001; Whelan, 2000). It may be beneficial to explore other medical implications for the use of the WARP 10 as an innovative intervention that may improve bone cell function and skeletal health. Thus, proposed use of this technology was to study the effects of NIR-LED treatment on osteoclast cell differentiation.

**Statement of the Problem**

**Defining of Osteoporosis.** One major area of musculoskeletal health research is the study of the pathogenesis of osteoporosis. The conceptual definition for osteoporosis was determined to be a skeletal disease of compromised bone strength. A disease characterized by low bone mass (bone mineral density) and/or structural deterioration of bone tissue (microarchitecture), leading to bone fragility and an increased susceptibility

to fractures, especially of the hip, spine, and wrist (European Foundation for Osteoporosis and Bone Disease & National Osteoporosis Foundation of USA, 1997; National Institutes of Health Consensus Development Panel on Osteoporosis, 2001). The operational definition for osteoporosis was determined by a set of diagnostic measurements. Currently, there is no reliable measurement for bone strength; however, bone mineral density (BMD), a surrogate measurement, estimates approximately 70% of bone strength (Suh & Lyles, 2003).

Bone structure reflects the quality of the bone tissue, such as the architecture, turnover, accumulated damage to the bone tissue and the amount of mineralization (European Foundation for Osteoporosis and Bone Disease & National Osteoporosis Foundation of USA, 1997). It has been reported that microarchitectural bone changes may be independently associated in bone weakness, but not associated with a change in BMD (Harvey, Dennison, & Cooper, 2013). Osteoporosis is the most common bone disorder (National Institutes of Health Consensus Development Panel on Osteoporosis, 2001), a growing worldwide public health problem due to the increasing numbers of individuals over the age of 65 (Suh & Lyles, 2003) and is reported to affect an estimated 44 million Americans (National Osteoporosis Foundation, 2010). It has been reported that bone degradation increases risk for fall-induced fractures (Grahm Kronhed, Blomberg, Lofman, Timpka, & Moller, 2006) and specifically hip fracture, a well-documented problem within this population (Lim, Hoeksema, & Sherin, 2009) which has markedly increase health care cost (World Health Organization Scientific Group, 2003).

It is projected that by the year 2030, the United States (U.S.) population of

older adults will double to 71.5 million (U.S. Department of Health and Human Services Administration on Aging, 2006). In 2007, data from the U.S. Census Bureau estimated that there are more than 84,000 centenarians in the U.S., and their population is projected to increase sevenfold by 2040 (U.S. Census Bureau & U.S. Department of Commerce Economics and Statistics Administration, 2007). This aging population is at risk for disability, morbidity, and mortality related to bone loss which is an identified risk factor for fracture resulting from a fall (Hall, Williams, Senior, Goldswain, & Criddle, 2000; Ostir, Ottenbacher, & Markides, 2004).

Fifty-five percent of people aged 50 years and older are affected by osteoporosis (National Osteoporosis Foundation, 2010 [revised 2013]). There has been only a modest decline in the rate of osteoporosis over the past 15 years despite the development of interventions using medications and weight-bearing exercise. According to the World Health Organization, osteoporosis has the potential to become an epidemic by the middle of the 21st century, from an incidence of 1.7 million in 1990 to a projected 6.3 million by 2050 (World Health Organization, 1999).

**Bone Physiology.** In brief, the physiology of bone homeostasis includes the activity of four cell types responsible contribute to the formation and maintenance of bone: osteoprogenitor cells, osteocytes, osteoclasts, and osteoblasts (Porth, 2009). Osteoprogenitor cells are undifferentiated cells that are the source of all bone cells except osteoclasts which originate from monocyte/macrophages within the hemopoietic lineage. Osteocytes are mature bone cells that maintain the bony matrix. In addition, they may respond to bone tissue strain and recruit osteoclasts to areas of bone requiring bone repair/remodeling (Lanyon, 1993). There are interconnected passageways, canaliculi,

found throughout the calcified matrix that contain extracellular fluid (Hadjidakis & Androulakis, 2006) that provide a communication network between the neighboring osteocytes and blood system for the exchange of nutrients and metabolites because diffusion does not occur in calcified bone (Porth, 2009).

Osteoclasts and osteoblasts are two types of cells within mineralized bone that contribute to mineral homeostasis (Lemaire, Tobin, Greller, Cho, & Suva, 2004). They are responsible for building and maintaining bone structure. Osteoclasts are bone-resorbing cells that originate from monocyte/macrophages within the hemopoietic lineage, and their activity is regulated by osteoblasts, bone-forming cells, and numerous hormones and other growth factors, macrophage colony-stimulating factor (M-CSF), interleukin-1 (IL-1), and tumor necrosis factor (TNF) (Hock et al., 2001). Osteoblasts are differentiated mesenchymal stem cells, responsible for laying down the new bone matrix that becomes mineralized, thus replacing old bone previously reabsorbed by osteoclasts (Hadjidakis & Androulakis, 2006; Hock et al., 2001).

There are two pathways that induce osteoclastogenesis. On the osteoblast stromal cell membrane, Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) receptor, binds to the osteoclast precursor cell (macrophage) RANK receptor. When this occurs, RANK signaling activates the transcription factor Kappa-B to differentiate into osteoclasts (Kumar, 2010). The other pathway occurs with the osteoblast secretion of M-CSF and the osteoclast precursor cell (macrophage) M-CSF receptor. When joined, tyrosine kinase activity is stimulated, also important to osteoclast formation. This signaling occurs in tandem with the RANKL/RANK pathway. Further bone marrow stromal/osteoblast cells secrete osteoprotegerin (OPG), a decoy receptor for RANKL,

preventing RANKL from binding with the osteoclast precursor cell's RANK receptor.

This prevents bone absorption and attenuating osteoclast differentiation (Kumar, 2010).

In healthy remodeling tissue of the adult skeleton, 5-10% of existing bone at specific bone sites is replaced every year, but it is not replaced uniformly throughout the skeleton (World Health Organization Scientific Group, 2003). As to not alter the bone's shape, bone resorption and formation occur within the same space (resorption lacunae) of the bone. Bone remodeling occurs in both spongy or trabecular (cancellous) tissue and dense/compact bone (cortical) tissue, which together comprise intact bone. The percentage of cancellous and cortical tissues in a given bone varies according to the type of bone. Vertebrae are particularly rich with cancellous tissue, while the hip and wrist are rich with cortical composites of bone (Wyngaarden, Smith, & Bennett, 1992).

Normal bone remodeling is a coupling process, a balance between bone gain and loss, where bone absorption and bone formation are balanced (North American Menopause Society, 2010). Bone remodeling begins with bone resorption in both cortical and cancellous bone by osteoclasts over a period of 30-40. Osteoclasts attach to the mineralized bone surface and resorb bone via secretion of hydrogen ions and cathepsin K (Raisz, 1999, 2008), creating irregular, scalloped cavities named Howship lacunae in trabecular bone or cylindrical Haversian canals in cortical bone. Osteoblasts lay down a new organic extracellular matrix in two stages: ossification, formation of unmineralized bone (osteoid), and calcification that becomes mineralized with calcium and phosphate and fills the resorption lacunae over approximately 150 days (E. Eriksen, 2010; Raisz, 1999).

**Bone Pathology.** Osteoporosis arises from an imbalance in osteoclast/osteoblast homeostasis (uncoupling) in remodeling bone (Lemaire et al., 2004; Manolagas & Jilka, 1995). Bone loss in osteoporosis has been described in a two-stage model. The first stage is the loss of sex steroids (estrogen) resulting in rapid trabecular bone loss by 20-30% and in cortical bone loss by 5-10% (Khosla & Riggs, 2005) due to an increase in osteoblast/osteoclast activity (Parfitt, 1990). Osteoclastogenesis is proportionally greater than osteoblastogenesis during this period of time (Jilka, Weinstein, Takahashi, Parfitt, & Manolagas, 1996). The duration of this first stage differs according to individual variables, but does not last indefinitely (Jilka et al., 1996). This stage usually lasts 5-10 years in women (Drake & Khosla, 2013). Stage two follows approximately 8-10 years postmenopausal which is a period of time of slow continuous bone loss due to aging where cortical and trabecular bone loss occurs at an equal rate until the end of the life span (Drake (Drake & Khosla, 2013; Parfitt et al., 1983). Men also experience a slow process of bone loss from middle age to the end of the lifespan that is similar to the second stage of bone loss seen in postmenopausal women (Drake & Khosla, 2013). Aging, in addition to loss of sex steroids or parathyroid hormone (Parisien et al., 1992; Steiniche et al., 1989), has been associated with bone loss and a decrease in the thickness of the bone wall and a decrease growth factors. These growth factors are the precursors for osteoblastogenesis (Ho et al., 1987) and directly influence osteoclastogenesis (Lips, Courpron, & Meunier, 1978; Parfitt et al., 1983).

There are four major pathways for the pathogenesis of osteoporosis: failure to achieve optimal peak bone mass and strength, accelerated bone loss due to increased bone absorption, inadequate bone remodeling, and increased falls (Raisz, 2008). Bone

loss (Crepaldi & Maggi, 2005) is one of the general characteristics of aging, along with increased mortality, vulnerability to disease (Cristofalo, 1988), progressive deterioration of tissues (Looker, Melton, Harris, Borrud, & Shepherd, 2010; Shock, 1985), and other conditions. Bone loss is a problem that worsens as people age. Osteoporosis arises from the out-of-balance osteoclast/osteoblast activity in remodeling bone. Because vertebrae are particularly rich in cancellous tissue (Wyngaarden et al., 1992), vertebral compression fractures are the most commonly associated osteoporosis bone injury (Neyhart & Gibbs, 2002). Hip and wrist fractures commonly result from cortical composite weakening; occurring later in life than is usually the case with vertebral fractures (Wyngaarden et al., 1992). Therefore, bone strength is impaired, predisposing older adults to fractures when they fall. Thus, there are numerous risk factors associated with bone loss.

**Falls and Fractures.** Serious injury occurs in 10-15 % of falls (Centers for Disease Control and Prevention, 2006). In the U.S., osteoporosis causes 1.5 million fractures every year. There is considerable research that associates osteoporosis with fall-induced fractures (Kendler et al., 2010; Schwartz, Nevitt, Brown, & Kelsey, 2005; World Health Organization Scientific Group, 2003). The lifetime risk for osteoporotic fractures (vertebral, wrist, and hip) is estimated to be 40% which is similar to the lifetime risk for coronary heart disease (World Health Organization Scientific Group, 2003). Vertebral compression fractures are the most commonly associated osteoporosis bone injury. Unlike vertebral fractures, hip and wrist fractures commonly result from cortical composite weakening that can occur later in life (Wyngaarden et al., 1992). Ninety percent of osteoporotic-induced fractures, specifically hip fractures among older adults in

the U.S., are caused by falls (Cummings, Kelsey, Nevitt, & O'Dowd, 1985; Grisso et al., 1991).

**Falls Morbidity and Mortality.** Hip fracture mortality rates have been reported to be three times higher for individuals with hip fracture as opposed to the mortality rate of the general population (Kannegaard, van der Mark, Eiken, & Abrahamsen, 2010). The rate of mortality associated with hip fractures within the first year is estimated to be 20% - 35% (Goldacre, Roberts, & Yeates, 2002; North American Menopause Society, 2010) and is greatest immediately after the fracture (Center, Nguyen, Schneider, Sambrook, & Eisman, 1999). Long-term care is required for approximately 25% of women, and 50% have some form of immobility (North American Menopause Society, 2010). Currently, the majority of hip fractures occur in Europe and North America. Over the next 50 years, due to demographic changes in the number of older adults living in developing countries, 75 % of the world's total hip fractures will occur in developing countries (World Health Organization, 1999).

**Healthcare Expenditures.** The World Health Organization reported in 2003 that osteoporosis-induced fractures burden society by depleting available resources. In 1992, in the state of California, it was estimated that 62.4% of expenditures were used for inpatient care, 28.2% for long-term care, and 9.4% for outpatient care to treat osteoporotic-induced fractures (Ray, Chan, Thamer, & Melton, 1997). However, these figures do not take into account indirect costs such as lost wages or the decreased productivity of caregivers and the patient (National Institutes of Health Consensus Development Panel on Osteoporosis, 2001). Researchers using the 1999-2005 Chronic Conditions Warehouse dataset (Iowa Foundation for Medical Care, 2008) reported that

the U.S. Medicare beneficiaries receiving payments for osteoporotic-induced fracture incurred a cost of \$7,788 (95% CI, \$7,550-\$8,025) for an average wrist fracture. The cost to treat an average open hip fracture was \$31,310 (95% CI, \$31,007-\$31,547) per beneficiary (Kilgore et al., 2009). Further, in 2005, it was estimated that the annual cost to treat osteoporosis associated fractures will increase 50% by year 2025— \$17 to \$25 billion—due to the growth of the aging population (Burge et al., 2007).

Due to the cost treating osteoporotic fall-induced fractures, the development of effective interventions meant to slow the progression of or reverse bone loss could be instrumental in decreasing the financial burden of healthcare for the aged, as well as enhancing the quality of life for aging persons by helping them avoid long-term care placement. Implementing a new intervention, such as the use of photobiomodulation (light-treatment), may have the potential to preserve bone density and prevent bone loss.

## **Background**

**Biological Aging & Oxidative Stress.** The theory of biological aging has been associated with cellular oxidative stress and mitochondrial dysfunction (Melov, 2000), and this may induce bone loss (Srinivasan & Avadhani, 2007). Oxidative stress can result from the generation of excess free radicals (Beckman & Ames, 1998) which causes an imbalance with cellular antioxidants which in turn damages cellular components and function (Sheweita & Khoshhal, 2007). Specifically, free radicals are unpaired electrons on the electron orbitals or electron shells of a molecule. In this unstable state, the free radicals are highly reactive with other molecules, and can instigate oxidative cell destruction. The oxygen molecule has an atypical placement of electrons within electron

orbitals, and during chemical reactions, oxygen prefers to accept one electron at a time rather than two electrons. Accepting two electrons at a time would keep the oxygen molecule in a stable state. When electrons are accepted one at a time, the oxygen molecule becomes unstable and superoxide ( $O^{\cdot -}$ ) and hydrogen peroxide ( $H_2O_2$ ) are generated. These reactive oxygen species undergo further biochemical cellular reactions, and the result is that an extremely reactive molecule, such as hydroxyl radicals ( $OH^{\cdot}$ ), is produced (Beckman & Ames, 1998) and affects mitochondrial function, responsible for cellular energy production.

**Mitochondrial Dysfunction.** It is suggested that mitochondrial dysfunction via oxidative stress may instigate premature apoptosis (programmed cell death) and alter mechanical signaling in bone (Booth & Criswell, 1997; Hock et al., 2001; Yalin et al., 2005). In a recent study using the RAW264.7 cell culture model (osteoclast precursor cells), without the addition of osteoclast differentiation factors, hypoxia-mediated mitochondrial stress increased reactive oxygen species (increased oxidative stress), which resulted in osteoclastogenesis (the formation of bone-resorbing cells) (Srinivasan & Avadhani, 2007).

**Proposed Intervention.** Photobiomodulation—referred to in the literature as low-level laser therapy, cold-laser therapy, or laser biostimulation—was introduced in the 1980s. Laser “light-treatment” uses light in the infrared to near-infrared region of the absorption spectrum (630-1000nm), such as a NIR-LED, and known to affect numerous cell functions that are dependent on the energy production in the mitochondria (Desmet et al., 2006; Karu, Piatibrat, & Kalendo, 1987). It has been postulated that the mechanism of the effect of a low-power laser works at the cellular level due to changes

in components of the electron transport chain system within the mitochondria (Karu, 1988). The electron transport system of the cell is directly related to the production of Adenosine triphosphate (ATP), the primary energy compound for the cell. These discoveries have been investigated using a NASA invented NIR-LED technology as previously mentioned (J. T. Eells et al., 2004; Sommer, Pinheiro, Mester, Franke, & Whelan, 2001; Whelan et al., 2008).

**NIR-LED Treatment.** Light-treatment in numerous studies has been documented to have beneficial cellular level mechanisms that reduce oxidative stress, improve mitochondrial function, and prevent premature apoptosis (Desmet et al., 2006; Eells et al., 2004; Karu et al., 1987). NIR-LED (630-1000nm) photobiomodulation—an FDA-approved treatment—has been found to be effective in improving wound (Whelan et al., 2003; Whelan, 2001), improving bone regeneration (Pinheiro et al., 2009), improving mitochondrial function (Wong-Riley, Bai, Buchmann, & Whelan, 2001), attenuating cellular oxidative stress (Desmet et al., 2006; Eells et al., 2004; Karu et al., 1987), decreasing inflammation and pain (Whelan et al., 2003), aiding in recovery of ischemic cardiac injury (Oron, 2006; Oron et al., 2001), and attenuating retinal/optic nerve degeneration (Eells, 2007; Eells, 2004; Liang et al., 2006).

**NIR-LED Cellular Effects.** Specifically, NIR-LED treatment, using an absorption spectrum of 670nm, activates the mitochondrial photo acceptor molecule cytochrome c oxidase (CO), specifically, Complex IV (COX IV) of the electron chain transport system (Karu, 1999). If cytochrome c (COX) does not properly function, the cell electron transport system performs poorly, which alters the production of ATP, and premature cell death can occur. Therefore, the use of light as a treatment to improve

oxidative metabolism and mitochondrial function in bone tissue may prevent bone demineralization and preserve cell life.

Research has been completed that has increased our understanding of NIR-LED effects with osteoblast cell function (in vitro) (Renno, McDonnell, Parizotto, & Laakso, 2007; Yamada, 1991), bone graft biomaterials (in vivo) (Pinheiro et al., 2008; Pinheiro et al., 2009; Torres, dos Santos, Monteiro, Amorim, & Pinheiro, 2008), and bone formation (in vivo) (Blaya, Guimaraes, Pozza, Weber, & de Oliveira, 2008). In one study that investigated osteoblastogenesis, it was found that low-level laser irradiation in cell culture significantly increased osteoblast proliferation and differentiation (Stein, Benayahu, Maltz, & Oron, 2005), another study found that photobiomodulation increased bone formation (Torres et al., 2008), increased the amount of well-organized bone trabeculi (Pinheiro et al., 2008), and enhanced vertical regeneration of bone (Blaya et al., 2008).

The evidence that NIR-LED increases *osteoblastogenesis* and bone formation is documented in the literature, but little is known regarding the effects of NIR-LED on *osteoclastogenesis*. Osteoblastogenesis and osteoclastogenesis need to be coordinated—i.e., achieve “coupling” (Rodan & Martin, 1981)—to attain a healthy balance (homeostasis) between bone gain and bone loss. It is with aging that the “uncoupling” of the bone remodeling cycle occurs, resulting in increased bone resorption (Kiel, Rosen, & Dempster, 2008; Lemaire et al., 2004). It is documented in the literature that osteoblastogenesis is increased with NIR-LED (Stein et al., 2005). Therefore, in order to develop effective and safe interventions for a vulnerable population, such as older adults, it is imperative to investigate the cellular-level effects of NIR-LED to determine

if osteoclastogenesis is amplified or suppressed due to uncoupling.

### **Research Questions and Hypotheses**

Our primary goal in this study was to determine the effects of NIR-LED treatment on osteoclast cell differentiation (osteoclastogenesis) in cell-cultured RAW264.7 cells using the WARP 75™ NIR-LED light. Our specific research questions and hypotheses were as follows:

**Question 1.** Is there a difference in osteoclast cell count between single light-treated Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL)-induced cell cultures as compared to non-light-treated controls? **Hypothesis.** Single light-treatment of RANKL-induced cell cultures will attenuate osteoclastogenesis.

**Question 2.** Is there a difference in osteoclast cell count between light-treated RANKL-induced cell cultures treated at different energy densities of light-treatment (2.25, 4.5 and 45 J/cm<sup>2</sup>) compared to non-light-treated controls? **Hypothesis.** Light-treatment of RANKL-induced cell cultures at a higher energy density (45 J/cm<sup>2</sup>) will have a greater attenuation of osteoclastogenesis compared to lower energy densities (2.25 and 4.5 J/cm<sup>2</sup>).

**Question 3.** Is there a difference in osteoclast cell count between single light-treated RANKL-induced cell cultures compared to multiple light-treated RANKL-induced cell cultures? **Hypothesis.** Multiple light-treatments of RANKL-induced cell cultures will have a greater attenuation of osteoclastogenesis compared to single light-treated RANKL-induced cell cultures.

### **Materials and Methods**

**Experiment Timeline.** This pilot study is a description of two experiments using

the WARP™ 75 light source. RAW 264.7 cells (obtained from the American Type Culture Collection [ATCC]) were plated on cell culture Day 0 and then incubated at 37° Celsius for 24 hours. RANKL was added to cell culture medium on Day 1 at specific experimental doses 0, 25, and 50ng/ml. Light-treatment began on Day 2 for all experiments regardless of whether the experiment was testing single or multiple light-treatments. Experiments that tested multiple light-treatments had repeated exposure on Days 3-5 for a total of 4 light-treatments. The culture medium was changed and fresh RANKL was added to the medium on Day 4 in all experiments. On Day 7, cells were stained for Tartrate-Resistant Acid Phosphatase (TRAP), and were prepared for cell counting.

The first experiment tested only a single light-treatment; the second experiment tested multiple and single light-treatments. All experiments tested energy densities of 4.5 and 45 J/cm<sup>2</sup>, but the second experiment tested an additional energy density of 2.25 J/cm<sup>2</sup> to explore an energy density light-treatment dose response. Lastly, experimental controls were tested for RANKL dose response at 0, 25, and 50ng/ml in all experiments, and experimental controls were tested in the first experiment for RANKL dose response at RANKL dose 0, 2.5, 10, 25 and 50ng/ml.

**Cell Plating Procedure.** On Day 0, RAW 264.7 cells were plated at  $2 \times 10^4$  cells per well ( $4 \times 10^4$  cell/ml) in 15 wells of a 24-well plate (well diameter: 1.77mm) for each experimental condition. The WARP™ 75 (Quantum Devices, Barneveld, WI) illumination surface covered 15 wells (well rows A-C by columns 2-6). Some experimental conditions did not require the full 15 wells; in those cases, only 10 wells were used. Plates were divided into experimental treatment groups according to

experimental condition: number of light-treatments (1 or 4), energy density (varied by experiment: 2.25, 4.5 or 45 J/cm<sup>2</sup>), and RANKL dose (varied by experiment: 0, 2.5, 10, 25, or 50 ng/ml) with respective negative controls. The cells were cultured for 24 hours prior to RANKL induction. At the end of the 24-hour period, RANKL was added to each well at 0, 2.5, 10, 25 or 50ng/ml, according to the experimental design.

**Light-treatment Procedure.** A continuous wave NIR-LED (WARP™75) with a wavelength of 670nm, power intensity of 60mw/cm<sup>2</sup>, and energy density of 5 J/cm<sup>2</sup> was used in the experiments because it was shown in previous studies to produce positive cellular effects (Desmet et al., 2006; Eells, 2007; Eells, 2004; Karu, 1988; Liang, Whelan, Eells, & Wong-Riley, 2008; Pinheiro et al., 2002; Whelan et al., 2003; Whelan et al., 2008; Wong-Riley et al., 2001; Wong-Riley et al., 2005). The dosage of light-treatment was determined by applying the following formula: Power (mW/cm<sup>2</sup>) x Time (seconds) ÷ 1,000 = Energy Density (J/cm<sup>2</sup>) in order to deliver 4.5 J/cm<sup>2</sup> and 45J/cm<sup>2</sup>. In order to ensure delivery of an accurate energy density of 4.5 J/cm<sup>2</sup> and 45 J/cm<sup>2</sup>, light-treatment was timed to 80 seconds to deliver 4.5J/cm<sup>2</sup> and to 800 seconds to deliver 45 J/cm<sup>2</sup>.

Light-treatment began on cell culture Day 2 (Yamada, 1991; Stein, 2005) under a sterile laminar hood with the dish lid in place. Because one of the objectives of a particular experiment was to test multiple light-treatments versus single light-treatment, additional light-treatments were performed, once per day on three consecutive days.

### Analysis

TRAP staining (SIGMA #387) was the method used for manual osteoclast cell counting (Nakano, Toyosawa, & Takano, 2004). Using a visual cell counting

classification tool, those multinucleated cells that were stained light pink/purple were identified by light microscopy (inverted microscope, 10x magnifications; Nikon Eclipse TS100) and counted. Mean osteoclast cell counts per well and standard deviations were calculated by taking the sum of multinucleated TRAP+ osteoclast cells for each well and dividing by the well surface area, expressed as cells per  $\text{cm}^2$ . To determine light-treatment effects, the difference between osteoclast cell counts in treatment plates and controls was calculated. Results are expressed as means and standard deviations, and groups were compared by one-way ANOVA with posthoc Tukey ( $p < 0.05$ ).

### **Results**

Our experimental groups were organized according to the number of light-treatments, energy density, and RANKL dose. Our first experiment tested single light-treatments at energy densities of  $4.5 \text{ J/cm}^2$  and  $45 \text{ J/cm}^2$  at RANKL doses of 0, 2.5, 10, 25, and 50ng/ml; it demonstrated the experimental RANKL dose response curve, which resulted in the expected stepwise two-fold increase in biological signal (see Table 1). In treatment groups, stepwise dose response occurred approximately two fold between energy densities  $4.5$  and  $45 \text{ J/cm}^2$  at RANKL dose 2.5ng/ml. However, in the RANKL group 25 and 50 ng/ml at energy densities of 0, 4.5, and  $45 \text{ J/cm}^2$  were practically inversed. The 10ng/ml RANKL group, only modestly increased at energy densities of 0, 4.5, and  $45 \text{ J/cm}^2$  (see Table 2).

One-way ANOVA comparing different energy densities for the single light-treatment were computed. In the first experiment, significant difference found between groups indicated an increased biostimulatory effect in energy density  $45 \text{ J/cm}^2$  and RANKL dose 2.5ng/ml compared to controls and energy density  $45 \text{ J/cm}^2$  was more

effective in amplifying osteoclast formation compared to 4.5 J/cm<sup>2</sup> (F (2,12)= 61.17, p<.0001). Further, energy densities of 0, 4.5 and 45 J/cm<sup>2</sup> at RANKL dose 10ng/ml (F (2, 12) = 8.04, p < .006) an increase in osteoclastogenesis was found compared to no-light treatment. (see Table 2). Energy densities of 0 J/cm<sup>2</sup> (M = 484.4, SD = 87) and 4.5 J/cm<sup>2</sup> (M = 485.2, SD = 53) yielded the same biostimulatory effect in the RANKL dose 50ng/ml experiment compared to the energy density groups of 45 J/cm<sup>2</sup> (M = 419.4, SD = 130). (see Table 2)

Our second experiment tested the effect of single light- treatment at energy densities of 2.25, 4.5, and 45 J/cm<sup>2</sup> at RANKL doses of 0, 25, and 50ng/ml; it also compared multiple light-treatments versus a single light-treatment at an energy density of 4.5 J/cm<sup>2</sup>. In this experiment, the expected dose response curve yielded a stepwise increase in the 25ng/ml RANKL dose compared to control, however the stepwise response in the 50ng/ml was only increased modestly. (see Table 3) This may be attributed to possibly the minimum dose threshold was met by RANKL dose 25ng/ml, but at the 50ng/ml dose, biological signal may drop.

In treatment groups, dose response in RANKL dose of 25 ng/ml and 50ng/ml occurred in the 2.25 and 4.5 J/cm<sup>2</sup> treatment groups; however, the single light-treatment (45 J/cm<sup>2</sup>) group and the multiple light-treatment (4.5 J/cm<sup>2</sup>) group did not yield the expected two-fold response. This finding may be attributed to the minimal energy density threshold being met as biological signal is lost at the 45 J/cm<sup>2</sup>. This decrease may also be attributed to light treatment effect. (see Table 4)

The energy density of 4.5 J/cm<sup>2</sup> was the most effective in amplifying osteoclastogenesis (M = 491.4, SD = 65) in the RANKL dose 25ng/ml experiment

compared to 45 (M = 289.2, SD = 77) and 2.25 J/cm<sup>2</sup> (M = 274.4 SD =92), as the 45 J/cm<sup>2</sup> was less effective in stimulating osteoclastogenesis compared to the 4.5 J/cm<sup>2</sup> (see Table 4). However, the first experiment in the RANKL dose 10ng/ml experiment, equal amplification was seen in both energy densities 4.5 (M = 68.8, SD = 13) and 45 J/cm<sup>2</sup> (M = 69.6, SD =11) as compared to the control (M = 45.6, SD = 7). (see Table 2)

In order to determine which pairs of means were significantly different, a post hoc Tukey honestly significant difference (HSD) test was used. Significant differences were found between the groups, no-light treatment group and energy density 4.5 J/cm<sup>2</sup> at RANKL dose 25ng/ml. Energy density 4.5 J/cm<sup>2</sup> significantly amplified osteoclastogenesis compared to no light treatment and was more effective in osteoclast formation than the 2.25 J/cm<sup>2</sup> (F (3,16) = 4.52, p <.0176). Further significance differences between groups were found in energy density 4.5 J/cm<sup>2</sup> and no light treatment, 2.25 and 45 J/cm<sup>2</sup> in RANKL dose 25ng/ml (F (3,16) = 12.51, p < .0001). (see table 4) Osteoclastogenesis increased in the energy density of 4.5 J/cm<sup>2</sup> compared to no-light treatment and was more effective than the 2.25 J/cm<sup>2</sup>. A decrease in osteoclastogenesis also was seen with 45 J/cm<sup>2</sup> compared to 4.5 J/cm<sup>2</sup> (see Table 4).

Further, a one-way ANOVA comparing single versus multiple light treatments for energy density 4.5 J/cm<sup>2</sup> and RANKL dose 0, 25and 50ng/ml were computed. A significant difference was found between single and multiple light treatment as well as no light treatment compared to single light treatment in the RANKL dose 25ng/ml groups (F (2, 11) = 24.8, p < .0001) (see Table 5). In RANKL dose 25ng/ml, biostimulation in the multiple light-treatment group was less (M = 258.6, SD = 30.9) compared to single light-treatment (M = 511.5, SD = 52.3). The post hoc Tukey HSD

test indicated a significant difference between single versus multiple light treatment ( $p = .01$ ) and single versus no light treatment ( $p = .01$ ).

### **Discussion**

Developing interventions to preserve bone density and to prevent or reverse the pathogenesis of osteoporosis (bone loss) is important because osteoporosis is a major public health problem estimated to affect 44 million Americans (National Osteoporosis Foundation, 2010). The U.S. population of older adults will be approaching 70 million by 2020; the financial burden to treat osteoporosis in older adults and osteoporotic-induced fractures will be cost prohibitive and burdensome to the health care system (World Health Organization Scientific Group, 2003).

Since the mid-1990s, overall there has been a decline in the incidence of low femur BMD (Looker et al., 2010). Because health care expenditures for osteoporosis associated fractures by 2025 will double (Burge et al., 2007), it may be helpful to determine the most efficacious point in time to implement an intervention to prevent/delay the onset of elemental bone loss (osteopenia). Commonly, studies investigate interventions for post-menopausal women when loss of BMD has mostly occurred. The most common preventive osteoporosis non-pharmacological intervention is weight bearing exercise (Howe et al., 2011; Schmitt, Schmitt, & Doren, 2009). Pharmacological interventions include prescribing calcium and vitamin D supplements (Elders et al., 1991; Rahmani & Morin, 2009; Shea et al., 2002; Weaver & Heaney, 2008), estrogen/progestin (Cauley et al., 2003) and bisphosphonates (Bonnick et al., 2007; Pols et al., 1999). The motivation to investigate the effects of NIR-LED light-treatment on osteoclastogenesis is to begin a program of research

developing a possible non-invasive intervention that may be appropriate for perimenopausal and postmenopausal women.

We investigated the effects of NIR-LED light-treatment using the WARP 75™ on osteoclastogenesis in the RAW 264.7 cell line. The purpose of this study was to pilot test a new protocol designed to answer the broad research question: Does NIR-LED photobiomodulation affect RANKL-induced osteoclastogenesis in a cell culture model? The integrity of our experiments and controls were evaluated by successful results of RANKL-induced osteoclast formation by RAW cells. Our controls (negative RANKL cell cultures) and the use of light treatment alone did not result in a measured effect on osteoclast formation.

Our first hypothesis predicted that a single light-treatment of RANKL-induced cell cultures would inhibit osteoclastogenesis; however, only two out of 19 **single light-treatment** experimental groups actually inhibited osteoclastogenesis compared to the control and were non-significant. In fact, two out of the 19 single light-treatment experimental groups significantly *increased* osteoclastogenesis. (see Table 2 and 4).

Our second hypothesis predicted that light-treatment at a higher energy density would be less stimulatory toward osteoclast formation than lower energy densities; this was found true, since in our first experiment energy density 45 J/cm<sup>2</sup> stimulated osteoclastogenesis less than the 4.5 J/cm<sup>2</sup> in the RANKL dose 25 and 50ng/ml group. On the other hand, in the 10ng/ml group, there was not a difference in effect between 4.5 and 45 J/cm<sup>2</sup>, but in the 2.5ng/ml group there was a significant difference between 0, 4.5 and 45 J/cm<sup>2</sup> groups indicating an increased biostimulatory effect with increased

energy density. The second experiment demonstrated  $45 \text{ J/cm}^2$  was less biostimulation compared to  $4.5 \text{ J/cm}^2$  in the RANKL dose 25 and 50ng/ml group.

Our third hypothesis predicted that multiple light treatments would stimulate osteoclastogenesis less than single light-treatments. It was found that multiple light-treatments compared to single light-treatment was less stimulatory in amplifying osteoclastogenesis at energy density  $4.5 \text{ J/cm}^2$  and RANKL dose 25ng/ml group, as shown in Table 5.

In order to evaluate our results, examples of low level laser light treatment models in the literature that investigated osteoclastogenesis using a gallium aluminum arsenide (Ga-Al-As) semiconductor laser (Aihara, Yamaguchi, & Kasai, 2006) and others investigating osteoblastogenesis with a helium neon (He-LE) laser (Stein et al., 2005; Yamada, 1991) were consulted. Further, two NIR-LED animal models studies were found (Blaya et al., 2008; Torres et al., 2008).

The two cell culture models investigating osteoblastogenesis used helium neon (He-LE) laser light in the near-infrared light spectrum and reported amplification of osteoblastogenesis. The two animal studies that investigated bone as a living tissue and histologically identified osteoblast and osteoclast activity, collectively, found that near-infrared light treatment enhanced bone formation (Torres et al., 2008) and bone architecture (Blaya et al., 2008). This is similar to our results of biostimulation with NIR-LED light treatment.

Aihara and colleagues (2006) studied rat osteoclast precursor cells using a continuous wave 810nm Ga-Al-As semi-conductor laser, with a power intensity of 50nW. Cell cultures were inducted with a RANKL dose of 10ng/ml and exposed to

light treatment for 1, 3, 6, or 10 min/day for 8 consecutive days, delivering energy densities of 9.99, 27.99, 55.98, and 93.30 J/cm<sup>2</sup>, respectively. They report a 1.3-fold increase in osteoclastogenesis in the 27.99 and 55.98 J/cm<sup>2</sup> groups (Aihara et al., 2006). In contrast, our NIR-LED continuous wave 670nm, power intensity 60mw, tested single light-treatment at energy densities at 2.25, 4.5, and 45 J/cm<sup>2</sup> at RANKL doses 0, 2.5, 10, 25, and 50ng/ml, as well as multiple light-treatments (4 consecutive days) at an energy density of 4.5 J/cm<sup>2</sup>. We report an increase in osteoclastogenesis with a similar energy density; however, this was demonstrated with one single light-treatment. Aihara and colleagues (2006) suggest photobiomodulation and higher energy densities amplify osteoclastogenesis. Overall, we found that a single light-treatment increased osteoclastogenesis, but when comparing the 4.5 J/cm<sup>2</sup> group to the highest energy density group, 45 J/cm<sup>2</sup>, the 4.5 J/cm<sup>2</sup> group was more effective in increasing osteoclastogenesis and multiple light-treatments stimulated osteoclastogenesis less compared to a single light-treatment. These contrasting results may be attributed to the use of different types of light sources and our experimental model testing single-light treatment.

Yamada (1991) studied the clonal osteoblastic MC3T3-E1 cell line using single light treatment with a continuous wave 632.8nm, power intensity of 3.03mW/cm<sup>2</sup> He-Ne laser at energy densities 0.01, 0.1, and 1.0J/cm<sup>2</sup>. Light treatment was administered on cell culture day 2, as compared to our continuous wave 670nm, power intensity of 60mW/cm<sup>2</sup>, NIR-LED light at an energy density of 2.25, 4.5, and 45cm<sup>2</sup> administered on cell culture day 2. They found a significant increase in

osteoblastogenesis ( $p < 0.05$ ) at energy densities of 0.01 to 1.0 J/cm<sup>2</sup> (Yamada, 1991). Stein and colleagues used human osteoblast cells for cell culture. A single light-treatment was administered on cell culture days 2 and 3 with a 632nm, power intensity 180mW/cm<sup>2</sup> He-Ne laser, at energy densities 0.14, 0.43, and 1.43 J/cm<sup>2</sup>. They also found a significant increase in osteoblastogenesis ( $p < 0.05$ ) (Stein et al., 2005). Both of these studies used low-energy densities, suggesting that lower energy densities had a biological effect on osteoblastogenesis; thus, we tested an energy density of 2.25, which was not found to significantly increase or decrease osteoclastogenesis.

We chose to test higher energy densities than in the osteoblastogenesis studies because they were comparable to the successful 670 nm NIR-LED light treatment studies used in a human model (Whelan et al., 2003) and in a neuronal cell culture model (Wong-Riley et al., 2001; Wong-Riley et al., 2005). Further, these studies by Whelan (2003) and Wong-Riley (2001, 2003) used the same NIR-LED light source manufacture (Quantum Devices, Barneveld, WI) as ours. However, our NIR-LED light source was a commercial product rather than the scientific grade NIR-LED.

Bone loss occurs rapidly during early menopause; osteoclastogenesis is, thus, proportionally greater than osteoblastogenesis (Eriksen, 1990; Jilka et al., 1996) followed by an age-related steady continuous loss of bone until the end of the life span (Parfitt, 1990). Loss of estrogen in women results in rapid trabecular bone loss by 20-30% and in cortical bone loss by 5-10% (Khosla & Riggs, 2005; Parfitt, 1990). However, it has been hypothesized that it may not be the mismatch of osteoclastogenesis/osteoblastogenesis, but a decrease in the number of stromal/osteoblastic cells (Manolagas & Jilka, 1995). A study (Jilka et al., 1996)

investigating the validity of a senescence-accelerated osteopenic mouse model for the study of metabolic bone disease reported a correlation between low bone mineral density and impaired osteoblastogenesis due to a low number of stromal/osteoblastic cells. The impairment of osteoblast formation resulted in a secondary impairment of osteoclastogenesis. When adding exogenous stromal/osteoblast cells from neonate mouse calvaria cells to the aging and osteopenic *ex vivo* marrow cell cultures, osteoclastogenesis was restored (Jilka et al., 1996). This may suggest that the imbalance between osteoblast/osteoclast activities may be due to the number of bone cells.

If osteoclastogenesis activity is thought to be proportionally greater than osteoblastogenesis during the aging process, then as indicated by our results, as well as those of Aihara and colleagues, NIR light treatment increases osteoclastogenesis. Therefore, if osteoblastogenesis and osteoclastogenesis both increase by light treatment and the bone remodeling cycle is already “uncoupled”, that is, imbalanced due to aging, NIR-LED treatment could result in the same disrupted bone remodeling process seen before light-treatment. The literature suggests that osteoblastogenesis and osteoclastogenesis increase with low-energy light treatment, as we found. However, it remains, based on our limited data, that the multiple light-treatments of NIR-LED increases or decreases osteoclastogenesis, as opposed to multiple light-treatments, increased osteoclastogenesis with a Ga-AL-Gs diode laser. Regardless, both light sources deliver low-energy irradiation in the near-infrared range.

In this study, we investigated the effects of NIR-LED treatment solely on osteoclastogenesis, but it would be useful to investigate both osteoblastogenesis and osteoclastogenesis under the same experimental conditions to compare their rates of

amplification or inhibition with NIR-LED treatment. After the effects of NIR-LED light-treatment are determined by testing each cell type in culture, the next step would be to investigate the effects of NIR-LED light-treatment on the bone remodeling process, because the biological effects of NIR-LED light-treatment on a system of homeostasis may be different from the biological response found in separate osteoclast and osteoblast cell cultures.

The long-range goal of this program of research is to discover and test interventions that may increase bone strength and integrity in the older adult population to prevent bone fracture (specifically hip fractures) and improve skeletal health. There is not sufficient evidence to draw a firm conclusion regarding the effects of NIR-LED light-treatments on osteoclastogenesis in these pilot experiments. However, the majority of the experimental groups yielded an increase in osteoclastogenesis. Furthermore, these data must be interpreted with caution, since they are pilot data and meant to assist us in designing other experiments. Several limitations to this study are discussed below; they include the use of a commercial light source rather than a scientific grade light source, the possibility of cell culture heat shock, and investigator subjectivity in manual osteoclast cell counting.

**Commercial NIR-LED Light Source.** The light sources used in these experiments are commercial grade and approximately calibrated at the factory for energy density, wavelength, and light intensity. The scientific grade light source has the advantage of controls for fine-tuning these variables. The commercial grade light source used in the experiments turned off automatically every 80 seconds, requiring manual restarting of the light during the 45 J/cm<sup>2</sup> experiments. This may have resulted in

measurable effects on osteoclast formation similar to pulsing light-treatment (Karu, 1992, 1993). The manual restarting of the light-treatment (WARP<sup>TM</sup> 75) caused a brief temperature surge in testing during development of the protocol, but the cell culture medium never rose above 37<sup>o</sup> C. Temperature surges could be considered pulsing light-treatment, which have been shown in the literature to give a greater biological effect than continuous wave light-treatment (Karu, 1992). Given the existence of temperature surges, heat shock cannot be ruled out; if heat shock did occur, the cultured cells may have been stressed by the abrupt change in temperature resulting, in an effect that was not brought about by the light-treatment. What is needed to control for these possible confounding variables is a light source utilizing a built-in timing mechanism and the capability to manually tune specific energy densities and light intensities. This would give the investigator greater control to test specific irradiation wavelengths, energy densities, and light intensities and to prevent heat shock by using consistent low radiation intensity (670nm) (Karu, 1992; Letokhov, 1991), 1991). In future experiments, obtaining a scientific grade continuous wave NIR-LED would be necessary for ensuring reliable results.

**Manual Cell Counting.** Lastly, the manual cell counting technique is a subjective process relying on visual interpretation of color and identification of cell nuclei. A visual cell counting classification tool was developed to guide in the cell counting process (see Appendix G). To be counted as an osteoclast cell, all cells were TRAP+ with a pink/purple or dark purple bordering on black (if nuclei were still visible). To determine the faintest acceptable pink, the stain color should match the RANKL control pink stain. The counted cells should be stained darker than the control faint pink.

The cell was counted if there were three or more nuclei present. Clumps of TRAP+ mono-nucleated cells were not counted, and cells connected by cell processes were counted as separate cells. The plates were blinded and the investigator took frequent breaks. Alternative methods for osteoclast quantification are described in the literature: computer-imaging software that quantifies osteoclast by color, shape, and nuclei; and by a TRAP5b assay (Alatalo, Halleen, Hentunen, Monkkonen, & Vaananen, 2000; Halleen et al., 2000). The computer software captures multiple images within specific boundaries demarcated by a gridded disk in the cell culture well, and counts osteoclasts based on these specifications. This method of quantifying osteoclastogenesis is less time consuming than manual cell counting.

Another way to confirm manual cell count reliability is the use of spectroscopy as a rapid screening method to determine osteoclast differentiation from a collected cell culture medium has been demonstrated with the serum marker TRAP5b (Halleen et al., 2000). The advantage to utilizing a TRAP5b assay is it changes data collection from a binary value to total biological activity value. For example, osteoclasts with greater numbers of nuclei are more effective in removing old bone and recruited macrophages could be measured. This is another method to compare cell count to biological signal. One method may be more valuable than the other or affirming of experiment results.

### **Conclusion**

It is clear that the effects of the light on osteoclastogenesis are RANKL-dose and light-intensity specific. We conclude at this time that a NIR-LED single light-treatment may amplify osteoclastogenesis, and multiple light treatments may impair RANKL-induced osteoclastogenesis. The use of NIR-LED light therapy may be a beneficial

intervention to return the bone remodeling process to homeostasis and preserve bone integrity.

It remains unknown how NIR-LED light therapy affects the intricate physiological signaling between osteoblasts and osteoclasts, as well as what effects it may have on bone remodeling and absorption. NIR-LED may decrease oxidative stress, preventing premature apoptosis, which is thought to occur with aging.

Continued experiments are planned with the use of a scientific-grade NIR-LED light source that will allow for greater control of energy density and light intensity. Repeated experiments will produce a larger sample size and enable a more substantial statistical analysis. Understanding that aging impairs osteogenesis (bone remodeling) due to an imbalance or “uncoupling” of osteoclastic/osteoblast activity, interventions aimed at correcting this pathogenesis will further the science to provide knowledge in reaching the goal of developing an intervention that may restore physiological function, preserve or improve bone quality, improve bone mineralization with the hope of preventing bone fragility and fracture, and ultimately improve the bone architecture to withstand mechanical insult such as a fall.

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Table 1

*Experiment 1**Dose Response Curve RANKL Dose 0, 2.5, 10, 25 and 50ng/ml with NO LIGHT TREATMENT*

RANKL Dose	0 ng/ml	2.5 ng/ml	10 ng/ml	25 ng/ml	50ng/ml	F	P < .05
	M (SD)	M (SD)	M (SD)	M (SD)	M (SD)		
0 J/cm <sup>2</sup>	0.9 (1)	25.4 (2)	45.6 (7)	250.6 (53)	484.4 (87)	102.22	0.0001

Table 2

*Experiment 1**Single Light Treatment for RANKL Dose 2.5, 10, 25 and 50ng/ml with Energy Density of 0, 4.5, 45 J/cm<sup>2</sup>*

Single Light Treatment Energy Density J/cm <sup>2</sup>	0 J/cm <sup>2</sup>	4.5 J/cm <sup>2</sup>	45 J/cm <sup>2</sup>	F	P < .05
	M (SD)	M (SD)	M (SD)		
0 ng/ml	0.9 (1)	0.8 (1)	0	1.33	0.3000
2.5ng/ml	25.4 (2)	24.8 (5)	51.2 (6)	61.17	0.0001*
10ng/ml	45.6 (7)	68.8 (13)	69.6 (11)	8.04	0.0060*
25ng/ml	250.6 (53)	226.2 (32)	194.2 (34)	2.41	0.1318
50ng/ml	484.4 (87)	485.2 (53)	419.4 (130)	0.78	0.4803

\* = significant

Table 3

*Experiment 2**Dose Response Curve RANKL Dose 0, 2.5, 10, 25 and 50ng/ml with NO LIGHT TREATMENT*

RANKL Dose	0 ng/ml	25 ng/ml	50 ng/ml	F	P < .05
	M (SD)	M (SD)	M (SD)		
0 J/cm <sup>2</sup>	0 (0)	254.4 (39)	276.8 (58)	69.86	0.0001

Table 4

*Experiment 2**Single Light Treatment for RANKL Dose 0, 25 and 50ng/ml with Energy Density of 0, 2.25, 4.5, 45 J/cm<sup>2</sup>*

Single Light Treatment Energy Density J/cm <sup>2</sup>	0 J/cm <sup>2</sup>	2.25 J/cm <sup>2</sup>	4.5 J/cm <sup>2</sup>	45 J/cm <sup>2</sup>	F	P < .05
	M (SD)	M (SD)	M (SD)	M (SD)		
0 ng/ml	0	0	3 (3)	1 (1)	4.52	0.0176*
25ng/ml	245.4 (40)	274.4 (92)	491 (65)	289.2 (77)	12.51	0.0001*
50ng/ml	276.8 (58)	316 (64)	336.8 (82)	297.6 (33)	0.86	0.4831

\* = significant

Table 5  
*Single versus Multiple Light Treatments*  
*Energy Density 4.5 J/cm<sup>2</sup> and RANKL Dose 0, 25 and 50ng/ml*

Number of Treatments	Single	Multiple	None		
	<i>M</i> (SD)	<i>M</i> (SD)	<i>M</i> (SD)	F	P < .05
0 ng/ml	3.4 (3)	0.8 (1)	0	4.12	0.0434*
25ng/ml	491(65)	344 (70.6)	245.4 (39)	21.41	0.0001*
50ng/ml	336.8 (81)	358 (61.7)	276.8 (58)	1.92	0.1890

\* = significant

## **CHAPTER FIVE - DISCUSSION, CONCLUSIONS AND IMPLICATIONS**

The purpose of this dissertation was to pilot test a new protocol designed to answer the broad research question: Does Near-Infrared Light Emitting Diode (NIR-LED) treatment affect Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) induced osteoclastogenesis in a cell culture model? This study was conducted at the NASA Ames Bone and Signaling Laboratory and consisted of four protocol development experiments (Chapter Three) and two experiments for collection of data (Chapter Four). In order to gain a robust understanding for the pathogenesis of bone loss, specifically osteoporosis, three extensive reviews of the literature were completed. The first required an understanding of the cellular mechanisms of NIR-LED and a search for existing NIR-LED light treatment protocols using a cell culture model, specifically with RAW 264.7 cells. Based upon this knowledge, the second and third searches were conducted to translate bench research into applicable knowledge for generalist nurses resulting in two manuscripts that informed nursing practice regarding bone physiology and pathology and nursing implications. The manuscripts describe gaps in the literature, provide literature updates and implications for future research.

### **Synthesis of the Manuscripts**

There are various updated clinical practice guidelines and many concerned researchers, however, the point remains that by 2030, the US older adult population is set to double with a significant portion over the age of 85 (U.S. Department of Health and Human Services Administration on Aging, 2006), increasing the incidence of falls. The costs to treat osteoporosis induced fractures estimated at \$22 billion (Blume & Curtis, 2011). Most hip fractures result in hospitalization, and within the first year,

approximately 37% of men and 26% of women will die from complications associated with these fractures (Kannegaard, van der Mark, Eiken, & Abrahamsen, 2010). Of the individuals who survive the first year many do not return to their pre-fall functional status and suffer from fear, anxiety, and depression, all of which dramatically impacts their quality of life (Eastwood et al., 2002; Harvey et al., 2013; National Institutes of Health Consensus Development Panel on Osteoporosis [NIH-CDPO], 2001; National Osteoporosis Foundation [NOF], 2010 [revised 2013]; Ziden et al., 2008). Ultimately, the overall gap found in the osteoporosis literature was the inability to effectively identify and treat patients prior to a fracture. Although osteoporosis research has been extensive over the past two decades, only a modest impact has been shown in decreasing fracture rates; thus, the societal economic impact to treat remains high and will only increase as the population ages (Bilezikian & Cusano, 2013; Bonjour, Schurch, Chevalley, Ammann, & Rizzoli, 1997; Burge et al., 2007; Challen, 1997; Chan, Uzer, & Rubin, 2013; Cohen & Shane, 2013; Cummings et al., 2009; Looker, 2010; Looker, Melton, Borrud, & Shepherd, 2012; Looker et al., 1997; Looker et al., 2008).

Two manuscripts were generated in order to understand nursing implications and to identify gaps in the literature. The first manuscript provides the generalist nurse with a review of the literature defining osteoporosis from a physiological perspective; reviewing bone anatomy and physiology, overviewing the pathogenesis of osteoporosis, osteoporosis outcomes, and presenting new treatment insights applicable to bone physiology. The second manuscript's focus is a review of nursing implications pertinent to osteoporosis such as bone loss and fall risk assessment, interventions, prevention, and monitoring. These manuscripts identified the need for the experimental study.

Therefore, results of the pilot data from the NASA Ames Bone and Signaling Laboratory extrapolated from the developed bench research protocol (Chapter Three) testing the effects of NIR-LED on osteoclastogenesis are reported in the third manuscript.

The first manuscript provided insight into understanding the etiology and consequences of osteoporosis. The generalist nurse was presented with the most current definition of osteoporosis, an overview of bone physiology and pathology, including its causes, and negative health outcomes, including the societal financial burden to treat osteoporotic induced fractures. Further, new methods to evaluate bone architecture volume quantitative computed tomography (vQCT), micro-computed tomography ( $\mu$ CT) and Trabecular Bone Score (TBS) (Silva et al., 2014) were discussed. Newer medications were discussed such as parathyroid bone anabolic (Teriparatide [Forteo®]), Receptor Activator of Nuclear Factor Ligand (RANKL)-inhibitor (Denosumab [Prolia® or XGEVA®]), and experimental drugs; Calcilytics, stimulate PTH secretion *in vivo* [animal study model] (JTT-305), and a newly discovered molecular pathway antagonist, Wnt/ $\beta$ -catenin, responsible for osteoblastic differentiation and recruitment (AMG 785).

The second manuscript provided the generalist nurse with the knowledge base to appropriately assess and identify osteoporosis, implement tailored interventions, provide disease monitoring and understand pertinent nursing implications for practice and policy. Ultimately, nurses are active members of the multidisciplinary team and are positioned to assist patients in understanding bone loss. Several gaps in the literature were highlighted: few prospective studies exist to understand the specifics of the pathogenesis of bone loss in the young and peri-menopausal women (Seifert-Klauss et al., 2012). This raises the question; are clinicians screening for bone loss too late in the lifespan? Also, the

majority of investigators have overlooked osteoporosis in men, as well as significantly under diagnosing osteoporosis in general. In fact, some believe early diagnosis is overlooked due to other comorbidities concomitantly occurring because they receive more medical consideration, such as cardiac disease, simply because early osteopenia occurs asymptotically (Rachner, Khosla, & Hofbauer, 2012). Too often the diagnosis of osteoporosis is made after a fracture. Thus, interventions are aimed towards preventing further bone loss and consequential fractures, not efforts towards early diagnosis, consequently not preventing the first fracture (Rachner et al., 2012). Osteoporosis must be recognized as a disease that affects individuals across their entire life-span.

The main study of this dissertation tested the effects of NIR-LED photomodulation on osteoclast formation investigating for a possible intervention that may have benefits for bone health. This research could be applicable to improving bone health for long-term space flight and general population. A protocol was developed over a series of four experiments in preparation for a two experiment studies that constituted the pilot study. The NASA award winning light treatment device used in this study was created by Quantum Devices Incorporated, originally named, High Emissivity Alumiferous Lighting Substrate™ (HEALS), used to grow plants for space flight. Using this technology, Quantum Devices Incorporated developed a commercial device, Warfighter's Accelerated Recovery by Photobiomodulation (WARP™ 10 & 75), a portable NIR-LED, used in combat to provide first aid for minor injuries and pain (National Astronautical Space Agency, 2008; Quantum Devices Incorporated, 2004; Whelan, 2004). The size and portability of the NIR-LED was ideal for testing effects in a cell culture model.

RANKL-induced osteoclast formation by RAW264.7 cells occurred as expected in all experiments. Light-treatment alone had no observable effect. A single light-treatment at  $4.5 \text{ J/cm}^2$  with RANKL added (10 - 50ng/ml) suggested a biostimulatory effect upon osteoclastogenesis compared to controls, as multiple light-treatments compared to single light-treatment were less stimulatory in the energy density  $4.5 \text{ J/cm}^2$  and RANKL dose 25ng/ml group. The effects of the NIR-LED treatment on osteoclastogenesis are RANKL dose and light-intensity specific. Concluding, NIR-LED light-treatment affects RANKL-induced osteoclast formation, but multiple light treatments compared to single light treatment may stimulate osteoclastogenesis less. Although pilot data results must be cautiously interpreted, it was determined that osteoclastogenesis was affected by NIR-LED light treatment. This was the first study that investigated the effects of NIR-LED light treatment on osteoclastogenesis.

### **Limitations**

Several study limitations existed. Challenges existed with producing consistent negative control dose response curves. Experiment one was successful, but experiment two, the RANKL stepwise dose response curve did not occur as expected. In fact, the RANKL dose 25 and 50ng/ml had approximately the same biological effect. Thus, the results of the first experiment yields more reliable results suggesting that single light treatment is biostimulatory to osteoclastogenesis. Because the controls in experiment two were inversely related, caution must be used in interpreting results. Single light treatment appeared to be biostimulatory and multiple light treatments were less stimulatory compared to controls.

Evaluating the RANKL dose response curves, it appears that the RANKL dose 50ng/ml may meet the minimum dose threshold and adding a higher dose of RANKL dose 75ng/ml would be advantages to more fully evaluate the dose response. The energy density dose response curve is variable. However, based on experiment one, the energy densities of 2.25 J/cm<sup>2</sup> appears to be possibly meeting the minimal threshold, and then increased biostimulation at the 4.5 J/cm<sup>2</sup> is stepwise, but 45 J/cm<sup>2</sup> biostimulation decreases. Continuing testing energy densities in this stepwise dosing would be beneficial to replicate.

These pilot data are to be interpreted with caution as the protocol has not consistently produced trending results. However, with pilot data, dose response curves can be adjusted and statistical control can be evaluated. Each experiment is unique and the end result is to produce physiologically meaningful data. Ultimately, a successful program of research should progress from cell culture into an animal model and then into clinical studies, thus bringing bench research to the bedside. Further, in cell culture animal and human cells will biologically respond differently to a same RANKL dose and energy density. Once the protocol produces trending reliable results, and the most important independent variables are identified, then at least three repeated experiments must be conducted producing robust data appropriate to support or refute the hypothesis. Because the experiments compare multiple variables between groups, better statistical control of these comparisons could benefit from using the Holm-Bonferroni method (Abdi, 2010). This type of analysis controls the family-wise error rate (probability of type I errors) and is more powerful than the Bonferroni correction. Lastly, a power

analysis should be calculated to ensure enough data is collected to reach significance (Faul, Erdfelder, Lang, & Buchner, 2007).

Several limitations were encountered using a commercial light source rather than a scientific grade light source, the possibility of cell culture heat shock, and investigator subjectivity in manual osteoclast cell counting. The light sources used in these experiments were commercial grade calibrated at the factory for energy density, wavelength, and light intensity. A scientific grade light source has controls to fine-tune variables and fans to cool the light.

The commercial grade light source used in the experiments turned off automatically every 80 seconds, requiring manual restarting of the light during the 45 J/cm<sup>2</sup> experiments. This may have resulted in measurable effects on osteoclast formation or inhibition similar to pulsing light-treatment which has a certain parameter of time set between the light's on/off times (Karu, 1992). Manually restarting the WARP™ 10 may have caused a brief temperature surge during protocol development, but the cell culture medium never rose above 37<sup>0</sup> C, the temperature maintained by the incubator. These temperature surges may be considered pulsing light-treatment, shown superior to continuous wave light treatment dependent on cell type to inhibit or stimulate cells (Hashmi et al., 2010). With this said, these possible temperature surges, could result in heat shock; if heat shock did occur, the cultured cells may have been stressed by the abrupt change in temperature resulting in an effect that was not brought about by the light-treatment.

Modifications to the experimental protocol are required for future research studies addressing these confounding variables created by the commercial light source. By using

a light source with a built-in timing mechanism and capability to manually tune specific energy densities and light intensities, giving greater control to test specific irradiation wavelengths, energy densities, and light intensities, and to prevent heat shock by using consistent low radiation intensity (670nm) (Karu, 1992; Letokhov, 1991). In future experiments, obtaining a scientific grade continuous wave NIR-LED would be necessary for ensuring reliable results.

Lastly, the manual cell counting technique is a subjective process relying on visual interpretation of color and identification of cell nuclei. A visual cell counting classification tool was developed to guide in the cell counting process (see Appendix G). To be counted as an osteoclast cell, all cells were TRAP+ with a pink/purple or dark purple bordering on black (if nuclei were still visible). To determine the faintest acceptable pink, the stain color should match the RANKL control pink stain. The counted cells should be stained darker than the control faint pink. The cell was counted if there were three or more nuclei present. Clumps of TRAP+ mono-nucleated cells were not counted, and cells connected by cell processes were counted as separate cells. The investigator took frequent breaks. Alternative methods for osteoclast quantification are described in the literature: computer-imaging software that quantifies osteoclast by color, shape, and nuclei; and by a TRAP5b assay (Alatalo, Halleen, Hentunen, Monkkonen, & Vaananen, 2000; Halleen et al., 2000). The computer software captures multiple images within specific boundaries demarcated by a gridded disk in the cell culture well, and counts osteoclasts based on these specifications. This method of quantifying osteoclastogenesis is less time consuming than manual cell counting.

Using spectroscopy as a rapid screening method to determine osteoclast total

biological signal from the collected cell culture medium has been demonstrated with the serum marker TRAP5b (Halleen et al., 2000). The advantage to utilizing a TRAP5b assay is it changes data collection from a binary value to total biological activity value. For example, osteoclast cell with multiple nuclei yield a higher biological signal and may be more effective in removing old bone. These data compared to cell count are a beneficial intra reliability measure.

Overall, three different perspectives of the bone loss literature were reviewed. The first two manuscripts provide implications for nursing practice, public policy, and future research; specifically health system wide comprehensive patient education programs implemented across the life-span. The third manuscript presented pilot data that must be interpreted with caution, but it can be determined that the study provided a solid robust experimental protocol suitable for continued research. Continued experiments are planned with the use of a scientific-grade NIR-LED light source that will allow for greater control of energy density and light intensity. Repeated experiments will produce a larger sample size and enable a more substantial statistical analysis. It remains unknown how NIR-LED light therapy affects the intricate physiological signaling between osteoblasts and osteoclasts, as well as what effects it may have on bone remodeling and absorption, but new literature does provide some insight.

### **Implications**

Implications for practice, policy, research and nursing research are offered based on the results of this study. Nurses are able to evaluate a patient's health care needs holistically and integrate all physiological systems related to the human condition. For example, a bone health scientist could make a suggestion for older adults to follow a

vegan diet in order to preserve bone health. This dietary lifestyle is readily available along the Pacific coast, and may be an ineffective intervention, because a vegan diet is not as prevalent in the Midwest. Based upon the nurse's prior rural home health experience within the farming community, these older adults would not be willing to remove dairy, eggs and meat from their diet based simply upon the social norms within their personal culture. Therefore, implementing an educational program to follow a vegan diet would most likely not be effective in a rural farming community.

**Practice.** Nurses have the opportunity to impact osteoporosis screening, management and medication adherence through patient education. Because bone loss is a multifaceted healthcare concern, the nurse can view the problem of attaining and maintaining bone health across the human life-span. Nurses have the most frequent patient contact, are trusted among many patient populations (Dinç & Gastmans, 2013), embrace the implementation and improvement of evidence based practice (Lee et al., 2013), and are an integral part of the multidisciplinary health care team (Wilkes et al., 2014). Nurses have the opportunity to guide women and men to improve bone health and reduce or reverse bone loss, thus preventing fractures and mortality and improving the quality of life of older adults through thoughtful risk and clinical assessment, attentively monitoring and implementing tailored interventions (Teng, Curtis, & Saag, 2009).

Effective nurse driven interventions have shown to be economical (\$50 per patient), successful using 70 minutes of one-on-one nurse case management by simply providing patient education regarding the risks and benefits of bisphosphonate therapy, arranging BMD testing, and assisting if needed to obtain prescriptions from their primary care provider (Majumdar et al., 2007). With this said, nurses in outpatient clinic settings,

can develop a comprehensive bone health educational programs that provides intervention at the four points of the lifespan: maternal/infant health, pediatric, perimenopause/menopause (women's health) and post-menopause (gerontology).

**Policy.** In order for a comprehensive bone health program to be effective, hospital system must employ more registered nurses(RNs) in their clinic systems to provide assessment and work in collaboration with advanced practice nurses to provide data to support screening perimenopausal women beginning at age 45 for bone loss.

Between 2008-2013 only (9%) and (7%) RNS were employed in outpatient clinics compared to (62%) and (60%) of medical assistants, respectively (Bureau of Labor Statistics, 2008a, 2008b, 2013a, 2013b). Many of these nurses are performing below their scope of practice while unlicensed assistive personnel are performing above their scope of practice (American Academy of Ambulatory Care, 2012). As health care services shift from inpatient settings to outpatient setting, access to RNs will be significantly less for the majority of the patient population (Bureau of Labor Statistics, 2013a).

Peri-menopause has been a focus in the literature since the mid-1990s, however prevention and screening for osteopenia or evaluating perimenopausal women for bone loss is lacking. Bone loss is reported to be stable during pre-menopause, but accelerates significantly in late perimenopause (Finkelstein et al., 2008). In the 5-7 year span of perimenopause and menopause, approximately, 12% of total bone mass is lost which is equal to one T-score (-1 SD) of measured bone density by DEXA which corresponds to a diagnosis of osteopenia (Armas & Recker, 2012). Most interventional studies focus on post-menopausal women, at that point in the life-span the proverbial "horse is out of the

barn”. Menopausal transition biomarker research is needed to identify the perimenopausal women. The best clinical setting to improve bone health awareness across the lifespan would be in the outpatient clinic setting for both generalist and advanced practice nurses to increase screening, patient education, and management.

**Research.** A collaboration and translation of technologies applicable to astronaut health may be useful in the general population. Currently, the International Space Station uses NIR-LED to grow plants, but some day this technology may be applicable to bone health. It has already been translated as a technology used in the general population for pain relief (Quantum Devices Incorporated, 2004) and used for wound healing in the military (Whelan et al., 2004).

According to the National Institutes of Health, translational research can be defined as a two stage process; the application of innovative laboratory research into preclinical studies to design human clinical trials that results in the implementation of best practices into the community with objectives to achieve cost-effectiveness in prevention and treatment (National Institutes of Health, 2007). Research is transferred from the laboratory’s bench (basic research) to the patient’s bedside (clinical research) then clinical study and trial findings transferred into the community and practice settings to improve health outcomes (Rubio et al., 2010).

One example of translational research in collaboration with NASA was recently presented at the International Osteoporosis Foundation - International Society for Clinical Densitometry. These researchers from Johnson Space Center, Wyle; aerospace contractor, Mercy Health, Cincinnati, and Lausanne University Switzerland investigated a new measurement for bone strength, trabecular bone score (TBS) of the lumbar spine in

long-duration spaceflight astronauts (Smith et al., 2014). They calculated for changes in DEXA and TBS scores in three different bone loss countermeasure (intervention) groups. Changes in mineral content, cortical vs. trabecular bone, may suggest independent effects of bone loss according to group intervention. These data support the continued need to investigate TBS as a significant measurement to evaluate bone architecture.

**Education.** Opportunities for nurses engaged in basic science research discovery are available. The University Of Maryland School Of Nursing recently posted a nurse physiologist research scientist tenure track faculty position. Further, access to laboratories for nurse physiologists can be found directly in the Schools of Nursing at Yale University and University of Pennsylvania. However, it still remains difficult to find a good fit with a university that provides adequate support for basic science research without relying on external laboratory space for the nurse scientist.

Most positions require at least one year of post-doctoral education (University of Maryland School of Nursing, 2014). The Robert Wood Johnson Foundation supports a Nurse Faculty Scholar Award providing research funding to junior faculty creating the next generation of nurse leaders (Robert Wood Johnson Foundation, 2014).

More regionally, Medical College of Wisconsin Center for Clinical and Translational Institute provides information regarding resources for specialized research laboratories at partner institutions; Blood Center of Wisconsin, Children's Hospital of Wisconsin, Froedtert Hospital, Marquette University, Medical College of Wisconsin, Milwaukee School of Engineering, University of Wisconsin-Milwaukee and Zablocki VA Medical Center (Medical College of Wisconsin Center for Clinical and Translational Institute, 2014). These resources may allow a bench research nurse scientist network and

collaborate with those scientists with laboratory facilities if one is not available at their primary institution. It is important that in becoming a basic science nurse scientist, a commitment is made not only by higher educational institutions to employ basic science nurse researchers but more importantly to foster relationships and mentor other doctoral nursing students.

Implementing curriculum to prepare an Integrative Nurse Physiologist into the Nursing Doctorate of Philosophy should be considered. Currently, there is not an academic pathway for this course of academic study and typically, post-doctoral study is required for employment in current nursing faculty positions. It would be most advantageous to accommodate immediate employment upon completion of the PhD, thus, providing colleges of nursing an applicant pool of nurse physiologists.

The curriculum should complement the student's program of research and allow for specialization. For example, flexibility in course choice would be most beneficial to the student. Some appropriate courses to include, but not limited to, are cell biology, clinical lab sciences, advanced anatomy and physiology, and chemistry. Before implementing this academic pathway, resources such as laboratory facilities and research funding for bench research opportunities must be secured. This may be established through College of Nursing endowment funding and field sites with bench research scientists. Nurse physiologists benefit the research community because their participation among multidisciplinary members provides perspectives regarding the human condition, and fosters creativity for research questions that involve multiple disciplines. They are the great collaborators, communicators, and teachers; and they tend to be the healthcare expert and caring agents among the team. Many times their role is essential for bringing

the team together to together to discuss solutions to problems, and interpreting results then moving science forward.

### **Future Research**

The main focus of this study was to test a biomedical device for effects in cell cultured osteoclasts. Much laser light treatment research has advanced, particularly in the field of dentistry. In the past, the most common use for lasers was seen in surgical techniques to cut, coagulate, or ablate tissue. The laser's mechanism of action in this instance is to destroy tissue and provide precise surgical incisions. This is achieved by heating the tissue at a high irradiation intensity and energy density. The use of low level laser treatment is the utilization of a low power laser or light emitting diode in the irradiance range of (1mW – 500MW) (Huang, 2009).

Recent search for NIR-LED treatment in combination with osteoporosis yielded no studies. However, searching low level light treatment, a few new studies were found. As with any use of a bio-technology, beneficial or harmful effects may occur in different cell types. As stated biological aging related to human aging has been associated with cellular oxidative stress (OS) and mitochondrial dysfunction (Melov, 2000), and this may induce bone loss (Srinivasan & Avadhani, 2007). Mitochondrial DNA (mtDNA) indicates that mutations of the mitochondrial genome are vulnerable to oxidative damage (Linnane et al., 1989) and may cause degenerative diseases and aging. NIR-LED light treatment has beneficial effects in reducing cellular oxidative stress, improving mitochondrial function and preventing premature cell apoptosis (Karu, 1999; Desmet et al., 2006; Wong-Riley et al., 2001). The following are updates to the NIR-LED literature.

As previously discussed, beneficial effects were found in improving wound healing (Eells et al., 2004; Whelan et al., 2003), bone regeneration (Pinheiro et al., 2009), decreasing inflammation and pain, aiding in recovery of ischemic cardiac injury (Oron, 2006; Oron et al., 2001), and attenuating retinal/optic nerve degeneration (Eells et al., 2007; Eells et al., 2004; Eells et al., 2003; Liang et al., 2006). Recent animal model research has demonstrated continued positive effects in bone repair activating osteogenic factors (Barbosa et al., 2013; Tim et al., 2014). Of interest, bisphosphonates-related osteonecrosis of the jaw (BRONJ), a well-known adverse side effect of long-term use of bisphosphonates, is an area of bone exposed in the maxillofacial region that does not heal within two months (Khosla et al., 2007). Use of surgical laser-assisted (low level laser treatment) in BRONJ patients aided bone healing positively compared to traditional medical treatment (Vescovi et al., 2012). Further, NIR-LED treatment was used in two transgenic Alzheimer's mouse animal models and found cerebral degeneration was reversed or alleviated (Purushothuman, Johnstone, Nandasena, Mitrofanis, & Stone, 2014). Further, improvement in cognitive function in two traumatic brain injury patients improved with forehead and scalp NIR-LED light treatment (Naeser, Saltmarche, Kregel, Hamblin, & Knight, 2011). Detrimental effects of NIR-LED treatment were found in a Meckel Syndrome animal model reporting that cystic kidneys did not improve with light therapy as a twofold increase in BUN was reported (Lim et al., 2011). It remains unknown how NIR-LED light therapy affects the intricate physiological signaling between osteoblasts and osteoclasts, as well as what effects it may have on bone remodeling and absorption.

**Continued Program of Research.** Provided once the experiment protocol produces trending reliable results and the most important independent variables are identified, three repeated experiments will be conducted to produce robust data appropriate to support or refute the hypothesis. However, understanding more fully that aging impairs osteogenesis (bone remodeling) due to an imbalance or “uncoupling” of osteoclastic/osteoblast activity, interventions aimed at correcting this pathogenesis will further the science to provide knowledge in reaching the goal of developing an intervention that may restore physiological function, preserve or improve bone quality, improve bone mineralization with the hope of preventing bone fragility and fracture, and ultimately improve the bone architecture to withstand mechanical insult such as a fall.

It is known that sex steroid deficiency (loss of estrogen) stimulates osteoblast and osteocyte apoptosis leading to fewer osteocytes in the bone matrix (Almeida & O'Brien, 2013; Manolagas, 2010) because estrogen stimulates osteoprotegerin (OPG) production and is vital to the regulation of osteoclastogenesis. As a result, menopausal transition and post-menopause, RANKL cytosol concentrations are greater than the OPG generation due to loss of estrogen (Tella, Gallagher, Tella, & Gallagher, 2013). Therefore, osteoclastogenesis is appropriately regulated by OPG, and osteoclastogenesis is increased due to higher concentration of RANKL. Low level light treatment is known to have the above mentioned benefits to improve cellular oxidative stress, mitochondrial function, and improved gene transcription. Further, transcription factor nuclear factor B (NF- $\kappa$ -B), regulated by changes in cellular redox state is activated with low level light treatment producing protective and stimulatory gene products (Huang et al., 2011). This is the same nuclear factor activated by the joining of the osteoblast RANKL and macrophage

RANK receptor. There may be a link between light treatment and osteoclast formation. A future research question may be to explore if LLLT induces osteoclast formation via the activation macrophage nuclear factor B (NF- $\kappa$ -B) in the absence of RANKL in cell culture.

### **Discussion**

Given the opportunity to work collaboratively with NASA was a unique experience for a doctoral nursing scientist. Participating with a bench-research scientific team, a nursing perspective was welcomed. Traditionally the specialty of space nursing involves assisting flight surgeons in pre-flight preparation similar to outpatient patient care. Another role is the management and medical monitoring of specialized space-related research, for example managing and monitoring subjects in bed-rest and sleep deprivation studies. Nursing has multiple perspectives to bring to bench research teams.

To summarize the major tenets of the considerable amount of available bone loss literature, the current incidence of osteoporosis and fractures remains high, and is soon to become a worldwide epidemic while many countries fail to consider this as a major health problem. It is evident that estimates of cost to treat osteoporotic-induced fractures are expected to at least double by 2040 placing a great societal financial burden to care for the anticipated 70 million older adults by 2030. Access to quality and timely health care services may become difficult due to the shortage of healthcare providers, specifically, geriatric certified (Centers for Medicare & Medicaid Services, 2005; Hartford Center of Geriatric Nursing Excellence at the Arizona State University College of Nursing & Healthcare Innovation, 2008). Nurses have the most frequent patient contact, are trusted among many patient populations, and embrace the implementation

and improvement of evidence based practice. Therefore, nursing has an opportunity to fill the foreseeable gaps in services by enhancing their knowledge base to efficiently identify and implement appropriate interventions for the prevention and treatment for osteoporosis across the life-span. Skeletal health researchers have one common goal, which is to gain an in-depth understanding of how to prevent or reverse bone loss in order to prevent fractures and mortality, thus improving the quality life of older adults (Teng et al., 2009).

### **Conclusions**

This pilot study accomplished two objectives. First the development of a successful experiment protocol and second, concluding that NIR-LED affects osteoclastogenesis. A challenge exists on how to effectively impact the identification and treatment of osteoporosis in order to prevent initial osteoporotic induced fractures. Osteoporosis and osteopenia are grossly underdiagnosed and usually are not assessed until a fracture occurs. Fracture is a main causal factor for morbidity and mortality in the older adult population and the most common trigger point for osteoporosis identification and treatment; however, only modest results in improving osteoporosis management have been achieved through patient and clinician education, but nurse driven interventions have been found to be effective. Further research is warranted to investigate fully the effects of NIR-LED light treatment and possible implications as an intervention to prevent or preserve bone health.

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## **List of Figures**

Figure 1

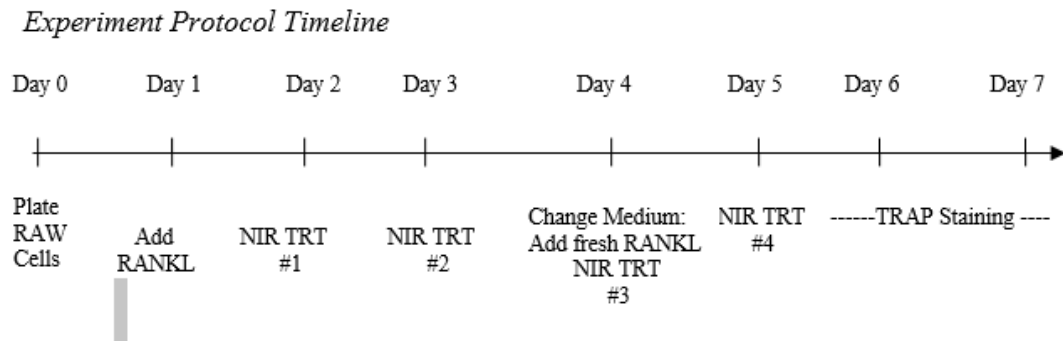
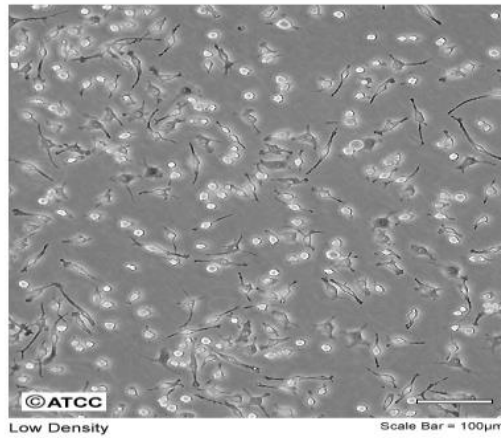


Figure. 2

*RAW 264.7 Cell Appearance*

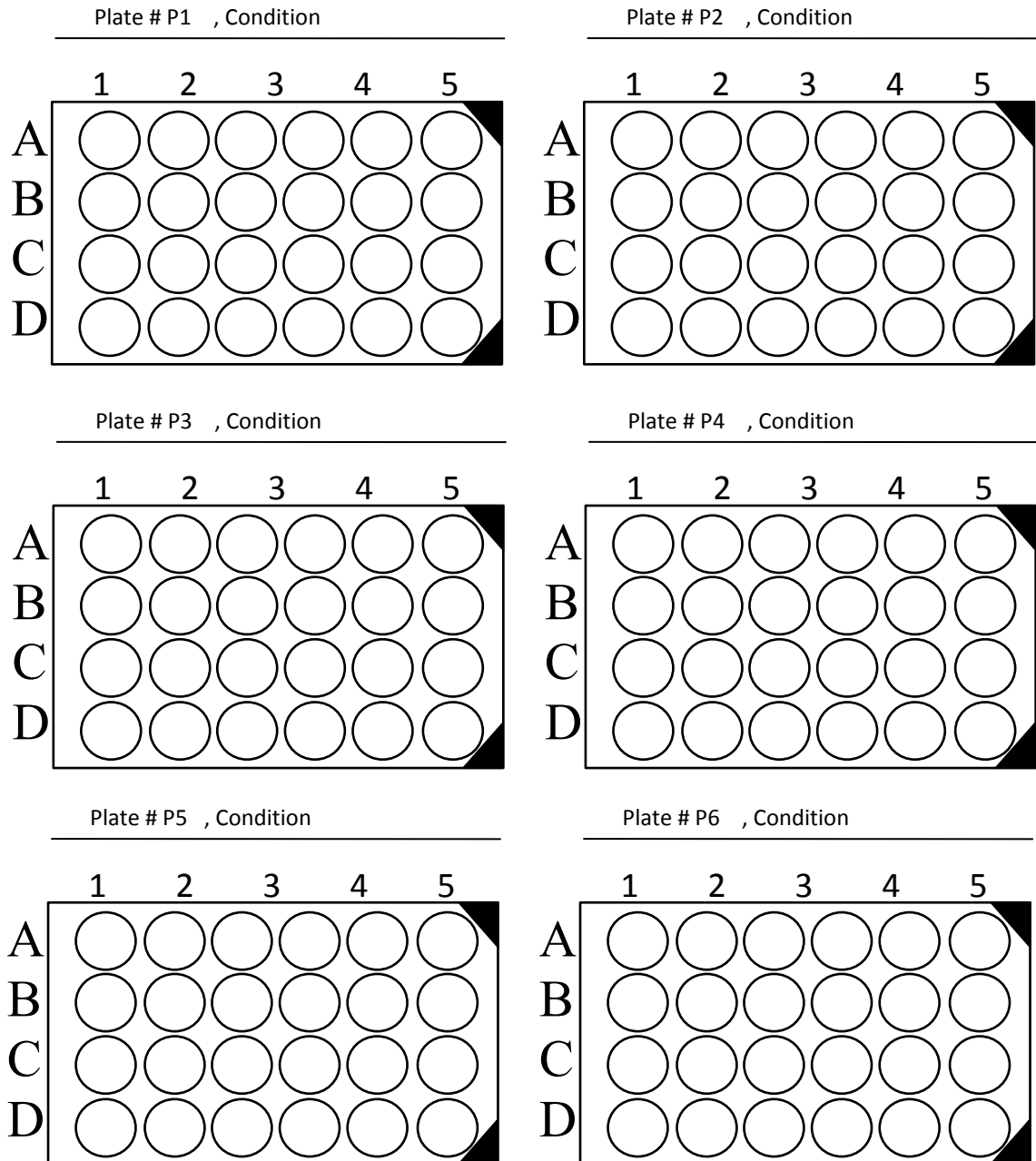
ATCC Number: **TIB-71**  
 Designation: **RAW-264.7**



(American Type Culture Collection [ATCC], 2009)

Figure 3  
*Cell Plate Template*

E1388

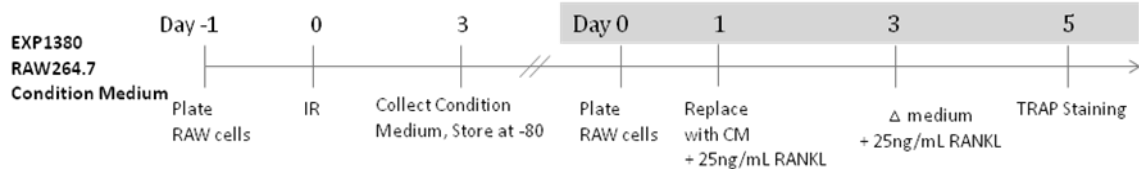


## Appendix

Appendix A  
Conditioned Medium Experiment

## AEXP1381 Conditioned Medium Experiment on RAW264.7 Cells

**Goal:** Follow Exp1377, to collect more CM for pit assay, also use the irradiated cells to repeat the replating exp, and possibly test different inhibitors



### I. Part A. Conditioned Medium Collection

#### Day-1. Cell Plating (density of $7 \times 10^4$ cells/mL)

##### 1. Cell Collection

- \_\_\_ aspirate medium
- \_\_\_ add 5mL pre-warmed medium to flask
- \_\_\_ use a sterile cell scraper gently remove cells from flask
- \_\_\_ transfer cell suspension into a sterile conical tube
- \_\_\_ centrifuge at 800rpm for 5 min
- \_\_\_ resuspend cells in 10mL medium
- \_\_\_ use 1mL pipetman to break up clumps by pipetting up and down

##### 2. Hemocytometer Counting

- \_\_\_ take 5uL of cell suspension, and mix with 45uL of trypan blue (dilution: 1/10, due to higher dilution if too concentrated.)
- \_\_\_ load the chamber with 20uL diluted cells suspension
- \_\_\_ count viable cells in 4 x  $1\text{mm}^2$ -squares with 10X objective under a microscope
- \_\_\_ calculate the number of cells/mL

$$\text{Cell Density} = D1 = (\text{___}/4) \times 10 \times 10^4 = \text{___} \text{ cells/mL}$$

$$\text{Total Number of Cells in 10mL cell suspension} =$$

$$(D1) \times 10 = \text{___} \text{ cells}$$

- \_\_\_ add another 10mL of medium to cell suspension, mix well

##### 3. Make 80 mL cell stock with density of $7 \times 10^4$ cells/mL

- \_\_\_ add \_\_\_\_\_ uL cell suspension
- \_\_\_ mix with \_\_\_\_\_ mL  $\alpha$ -MEM+10%FBS

##### 4. Plating in T-75

- \_\_\_ pipet up and down several times to get an even suspension
- \_\_\_ add 12mL of cell suspension to each T-75
- \_\_\_ check under microscope
- \_\_\_ incubate at  $37^\circ\text{C}$

**Day 0: Irradiation (this procedure will be carried by Ruth.)**

1. Irradiation step will be carried out by Ruth (0Gy and 2Gy)
1. Immediately after IR, collect and replace with 12mL fresh medium.  
Centrifuge medium collected at 1000rpm for 10 min, then store at -80°C.  
⇒ About \_\_\_\_\_ conditioned medium per flask was collected.
2. Incubate flasks at 37°C.

Flask# (CM#)	IR dose (Gy)	Δ medium after IR	IR Time (min), Position 3, Rotate
1, 2, 3	0Gy	Δ	Sham IR
4, 5, 6	2Gy	Δ	2.19 min

**Day 3: Culture Medium Collection**

1. Prepare and label 6 x sterile and pyrogen-free centrifuge tubes
2. Combine and transfer condition medium from flasks to centrifuge tubes
3. Centrifuge tubes at 1000rpm for 10 minutes
4. Collect supernatant and transfer to sterile, pyrogen-free tubes  
Store at -80°C

## Appendix B

### Culture Growth Medium Preparation

**RAW 264.7 Culture Growth Medium Preparation:**

Manufacture HyClone procedure was followed (HyClone, 2003).

1. Removed 500ml bottle of FBS from -80°C freezer and placed in refrigerator to thaw overnight. Completed the thawing of serum the following day by placing serum in a 37°C water bath (Polysciences) with a water level higher than the serum level in the bottle. Mixed by inversion.
2. Once serum was completely thawed, incubated for an additional 15 min to allow serum to equilibrate with the 37°C bath.
3. Raised the temperature setting of the bath to 56°C and continued heating the bath and serum until the bath reached 56°C (approximately 35 minutes). During this incubation period, mixed the serum by inversion every 10 min.
4. Once bath reached 56°C, incubated serum for 30min mixing the serum bottle every 10 min.
5. Removed serum from water bath and allowed to cool at room temperature for 30 min. Reset water bath to the 37°C mark.
6. Cleaned all surfaces in the cell culture hood, pipetman and conical tube holder with 70% ethanol. Then cleaned external surfaces of ten 50ml conical tubes and the 500ml bottle of FBS from the water bath with 70% ethanol (ETOH) to sterilize them to prevent contamination of cultured cells. Aliquoted 50ml of treated serum into ten 50ml

conical tubes (Fisher Scientific: catalog number 0553860) and stored at 4°C or freeze at -20°C.

7. After the FBS was heat inactivated and filtered (Fisher Catalog number: 0974028C), the FBS may be added to the growth culture medium (Fisher Scientific, 2010b). Removed one 50ml conical FSB (Fisher Scientific: catalog number 0553860) from freezer and thawed in 37°C water bath (Polysciences).
8. Cleaned external surfaces of the vial and hood with 70% ethanol (ETOH) to prevent contamination of cultured cells
9. Removed 500ml bottle of  $\alpha$ -MEM from the refrigerator and filtered through a 500ml filter Nalgene, 500/250ml capacity (surfactant-free cellulose acetate (SFCA) membrane 0.2 $\mu$ m: Fisher catalog number: 0974028C) followed by filtering 50ml of FBS (10% concentration).

**a4. RAW264.7 Cell Thawing:** The procedure for propagation of RAW 264.7 cells was as follows:

1. Prepared a water bath (Polysciences) to 37°C and pre-warmed  $\alpha$ -MEM-10% FBS for approximately 15min in water bath.
2. Thawed cells with a constant gentle agitation in the 37°C water bath.
3. Cleaned all surfaces in the cell culture hood, pneumatic pipette-man (BrandTech Accu-Jet) and conical tube holder with 70% ethanol. Cleaned external surfaces of two 50 ml conical tubes (Fisher Scientific: catalog number 0553860) to be used in the transfer of RAW 264.7 cells into cell culture dishes with 70% ethanol. Placed the

two conical tubes in the hood and labeled one tube “cell suspension” and the other “medium” with cryo marker (marker will not be removed if sprayed with 70% ethanol). Added 10ml of culture medium to “medium” conical tube.

4. Under the sterile hood, flamed lid of vial of RAW 264.7 cells. Sterilized the lid prior to opening.
5. Under the hood, gently transferred thawed cells to 50ml conical tube.
6. Added warmed culture medium to “cell suspension” labeled conical tube in a drop-wise fashion such that 10ml is added over about 2 min then added the rest a little faster, gradually diluting cells and preservative.
7. Centrifuged for 5 min at 100g (730 rpm Beckman GS-6KRI).
8. Discarded the supernatant (medium above visible ring of centrifuged cells) and re-suspended the cells in 10 ml of fresh medium for culture.
9. Aspirated cells with pipetman to break up cells and gently expelled them into three 10 cm tissue-culture treated Polystyrene petri dishes (Corning, Fisher Catalog number: 0877222) (Fisher Scientific, 2010a) in recommended cell suspension ratios 1:20, 1:10, 1:5 cell.
10. Cell suspension Ratios:
  - Added culture medium and adjusted accordingly to ratio for a total of 10ml per culture dish
    - 1:20 2ml cells/8ml = 20% concentration
    - 1:10 1ml cells/9ml = 10% concentration

— 1:5 0.5ml cells/9.5ml = 5% concentration

Cells are then ready for propagation (the multiplication and division of cells by natural reproduction)

## Appendix C

### Procedure for Cell Passaging

### Procedure for Cell Passaging

The procedure to passage RAW 264.7 was as follows:

1. Cleaned all surfaces in the cell culture hood, pneumatic pipette-man (BrandTech Accu-Jet) and conical tube holder with 70% ethanol. Then cleaned external surfaces of two 50 ml conical tubes (Fisher Scientific: catalog number 0553860) to be used in the transfer of RAW 264.7 cells into cell culture dishes with 70% ethanol. Labeled one tube “cell suspension” and the other “medium” with cryo marker.
2. Removed 500ml bottle of  $\alpha$ -MEM-10% FBS from the refrigerator and cleaned with 70% ethanol and place in hood. Transferred 50ml of medium to the conical tube labeled “medium”. Removed and placed in water bath.
3. Prepared a water bath (Polysciences) to 37°C and pre-warmed  $\alpha$ -MEM-10% FBS. When warmed to 37°C (approximately 10 min), cleaned outside of conical tube with 70% ethanol prior to placing back in hood.
4. While warming medium in water bath, gathered 10ml pipette tip (BD-catalog number 309604) and cell scraper (Falcon: catalog number 353085). Placed by sterile technique two 10cm tissue-culture treated Polystyrene petri dishes (Corning, Fisher Catalog number: 0877222) (Fisher Scientific, 2010a) in the hood.

5. Labeled petri dishes with initials, date, the number of times cells have been passaged, name of cell line (RAW 267.4) and cell concentration suspension ratios of 1:10 and 1:5 cell with cryo marker.
6. Took incubated petri dishes of RAW 264.7 cells from incubator and visualized under inverted microscope (inverted microscope, 10x magnification; Nikon Eclipse TS100). Determined which cell culture dish had the highest cell confluence. Saved other cell culture dish in reserve.
7. Under hood, aspirated old culture medium from petri dish with suction and replaced with 10 ml fresh medium.
8. Took cell scraper and gently whisked cells up off of the petri dish surface moving in a clockwise fashion.
9. Aspirated 10ml from petri dish and placed in conical tube label “cell suspension”, Centrifuge for 5 min at 800rpm (Beckman GS-6KRI).
10. While centrifuging, placed 9.5 ml and 9 ml of medium into petri dishes respectively.
11. Took conical tube from centrifuge and discarded the supernatant and re-suspended with cells in 10ml of fresh medium. Pipetted up and down in conical tube to break up cell clumps, then gently expelled 1ml of cell suspension into the labeled 1:10 petri dish and 0.5ml into the 1:5 label petri dish.
  - Cell suspension Ratios:
  - 1:10 1ml cells/9ml = 10% concentration
  - 1:5 0.5ml cells/9.5ml = 5% concentration

## Appendix D

### Cell Plating Procedure

## Cell Plating Procedure

The cell plating density was calculated by the following procedure:

1. Calculate number of required experiment wells

**Control Plates: 5 wells per condition Total Wells: 50**

### 4.5 J/cm<sup>2</sup> Energy Density Control Plate

Plate 1:	0-0-0	0-0-2.5	0-0-10	15 wells
Plate 2:	0-0-0	0-0-25	0-0-50	10 wells

### 45 J/cm<sup>2</sup> Energy Density Control Plate

Plate 1:	0-0-0	0-0-2.5	0-0-10	15 wells
Plate 2:		0-0-25	0-0-50	10 wells

**Experimental Plates: 5 wells per condition Total Wells: 50**

### 4.5 J/cm<sup>2</sup> Energy Density Exp Plate

Plate 1:	1-4.5-0	1-4.5-2.5	1-4.5-10	15 wells
Plate 2:		1-4.5-25	1-4.5-50	10 wells

### 45 J/cm<sup>2</sup> Energy Density Exp Plate

Plate 1:	1-45-0	1-45-2.5	1-45-10	15 wells
Plate 2:		1-45-25	1-45-50	10 wells

2. Total wells plated

\_\_\_\_\_ Controls: 50 wells  
 \_\_\_\_\_ Experimental: 50 wells  
 \_\_\_\_\_ Total **100 wells**

4. Used 24 well plates @ 0.5 ml/well culture medium

- a. Equation:

Total needed medium:   x   of wells \* 0.5 ml =   y   mls  
 — 100 wells \* 0.5ml = 50 mls [make 55 mls of cell stock (additional 5mls for reserve)]

- b. Cell plating density equation:

$$\frac{4 \times 10^4 \text{ cells}}{1 \text{ ml}} * \frac{0.5 \text{ ml}}{\text{well}} = 2 \times 10^4 \text{ cells/well}$$

**Day 0: Cell Plating:** Density of  $2 \times 10^4$  cells/well RAW264.7 cells were passaged 17

times in  $\alpha$ -MEM-10%FBS

1. Cleaned all surfaces in the cell culture hood, pneumatic pipette-man (BrandTech Accu-Jet) and conical tube holder with 70% ethanol. Then cleaned external surfaces of three 50 ml conical tubes (Fisher Scientific: catalog number 0553860) to be used in the transfer of RAW 264.7 cells into cell culture dishes with 70% ethanol. Labeled these three tubes “Cell Suspension”, “Plate Cell Suspension” and “Medium” with cryo marker.
2. Removed 500ml bottle of  $\alpha$ -MEM-10% FBS from the refrigerator and clean with 70% ethanol and place in hood. Transfer 50ml of medium to the conical tube labeled “medium”. Remove and place in water bath.
3. Prepared a water bath (Polysciences) to 37°C and then pre-warmed  $\alpha$ -MEM-10% FBS. When medium is warmed to 37°C (approximately 10 min), cleaned external surface with 70% ethanol prior to placing back in hood.
  - While warming medium in water bath, gathered 10ml pipette tip (BD- catalog number 309604) and cell scraper (Falcon: catalog number 353085). Placed by sterile technique two 10cm tissue-culture treated Polystyrene petri dishes (Corning, Fisher Catalog number: 0877222) (Fisher Scientific, 2010a) in the hood.
  - Used cryo marker, labeled petri dishes with initials, date, the number of times cells have been passaged, name of cell line (RAW 267.4) and cell concentration suspension ratios of 1:10 and 1:5 cells.

- Took incubating petri dishes of RAW 264.7 cells from incubator and visualized under inverted microscope (inverted microscope, 10x magnification; Nikon Eclipse TS100). Decided which cell culture dish had the highest cell confluence. Saved other cell culture dish in reserve.
- Under hood, aspirated old culture medium from petri dishes with suction and replaced with 10 ml fresh medium. Took cell scraper and gently whisked cells up off of the petri dish surface moving in a clockwise fashion.
- Aspirated 10ml from petri dish and placed in conical tube label “cell suspension”, Centrifuge for 5 min at 800rpm (Beckman GS-6KR1).
- While centrifuging, placed 9.5 ml and 9 ml of medium into petri dishes respectively.
- Took conical tube from centrifuge and discarded the supernatant and re-suspended with cells in 10ml of fresh medium. Pipetted up and down in conical tube to break up cell clumps, then gently expelled 1ml of cell suspension into the labeled 1:10 petri dish and 0.5ml into the 1:5 label petri dish.
  - Cell suspension Ratios:
    - 1:10 1ml cells/9ml = 10% concentration

$$\text{--- } 1:5 \quad 0.5\text{ml cells}/9.5\text{ml} = 5\%$$

concentration

Save “Cell Suspension” conical to prepare plating cell suspension procedure.

4. Hemocytometer Counting:

- took 10uL of cell suspension, and mixed with 20ul of trypan blue (used to stain cells) (dilution: 1/10, go to higher dilution if too concentrated.)
- loaded the hemocytometer chamber with approximately 10uL diluted cell suspension
- counted viable cells in  $4 \times 1\text{mm}^2$  squares with 10x objective under a microscope: squares need to be  $> 50$  cells.
- calculated the number of cells/ml

#cells in  
hemocytometer  
↓

$$\text{Cell Density} = \left( \frac{214}{4} \right) = 53.5 * 10 \text{ (1/10 dilution)} * 10^4 = 5.35 \times 10^6 \text{ cells/ml}$$

↑  
Dilution factor

5. Cell stock (Plating Cell suspension)

Made 55 ml cell stock with density of  $4 \times 10^4$  cells/ml

- 100 wells \* 0.5ml = 53.5 mls [make 55 mls of cell stock]

$$55\text{ml} \times \frac{4 \times 10^4}{1\text{ml}} = 2.2 \times 10^6 \text{ cells/ml}$$

↑  
Cell plating density

Total amount of cell suspension needed → 
$$\frac{2.2 \times 10^6 \text{ cells} \mid 1 \text{ ml}}{X \mid 5.35} \leftarrow \text{Cells/ml for hemocytometer}$$

= .411 ml or 411ul

- added 411ul cell suspension
- mixed with 54.5 ml  $\alpha$ -MEM+10% FBS in labeled “Plate cell suspension” conical

6. Plated 25 wells in Control 24 well cell culture plates:

Divided plates by experimental treatment groups according to the RANKL dose (0, 2.5, 10, 25 or 50 ng/ml), and the energy density (4.5 J/cm<sup>2</sup>, 45 J/cm<sup>2</sup>) with respective negative controls.

Plate 1: (4.5 J/cm<sup>2</sup> controls)  
 1<sup>st</sup> control plate: 15 wells  
 [A2 – A6]: 0-0-0  
 [B2 – B6]: 0-0-25  
 [C2 – C6]: 0-0-50

Plate 2: (4.5 J/cm<sup>2</sup> controls)  
 2<sup>nd</sup> control plate: 10 wells  
 [A2 – A6]: 0-0-2.5  
 [B2 – B6]: 0-0-10

Plate 3: (45 J/cm<sup>2</sup> controls)  
 1<sup>st</sup> control plate: 15 wells  
 [A2 – A6]: 0-0-0  
 [B2 – B6]: 0-0-25  
 [C2 – C6]: 0-0-50

Plate 4: (45 J/cm<sup>2</sup> controls)  
 2<sup>nd</sup> control plate: 10 wells  
 [A2 – A6]: 0-0-2.5  
 [B2 – B6]: 0-0-10

7. Plated in 25 wells of treatment 24 well cell culture plates:

Plate 5: (4.5 J/cm<sup>2</sup> treatment plates)  
 1<sup>st</sup> treatment plate: 15wells  
 [A2 – A6]: 1-4.5-0  
 [B2 – B6]: 1-4.5-25

[C2 – C6]: 1-4.5-50

Plate 6: (4.5 J/cm<sup>2</sup> treatment plates)

2<sup>nd</sup> treatment plate: 10 wells

[A2 – A6]: 1-4.5-2.5

[B2 – B6]: 1-4.5-10

Plate 7: (45 J/cm<sup>2</sup> treatment plates)

1<sup>st</sup> treatment plate: 15wells

[A2 – A6]: 1-45-0

[B2 – B6]: 1-45-25

[C2 – C6]: 1-4.5-50

Plate 8: (45 J/cm<sup>2</sup> treatment plates)

2<sup>nd</sup> treatment plate: 10 wells

[A2 – A6]: 1-45-2.5

[B2 – B6]: 1-45-10

- pipetted up and down several times for an even suspension
- added 0.5ml cell suspension to each well
  - expelled cells gently alongside of well wall, and swirled plate for an even cell suspension
- visualized cells under microscope to determine an even cell suspension
- placed in incubator for 24hrs

## Appendix E

Receptor Activator of Nuclear Factor Ligand Induction (RANKL)

Induction Procedure



3. Cleaned all surfaces in the cell culture hood, pneumatic pipette-man (BrandTech Accu-Jet) and conical tube holder with 70% ethanol. Prepared five 2ml cryotubes: removed from sterile packaging and placed in hood. Labeled each tube with RANKL dose. Tube 1-5 labeled 50, 25, 10, 2.5, and 0 ng/ml, respectively. Then cleaned external surfaces of two 50 ml conical tubes (Fisher Scientific: catalog number 0553860). Labeled both "medium".
4. Removed 500ml bottle of  $\alpha$ -MEM-10% FBS from the refrigerator and cleaned with 70% ethanol and placed in hood. Transferred 50ml of medium to the conical tubes labeled "medium". Removed and placed in water bath.
5. Prepared a water bath (Polysciences) to 37°C and pre-warmed  $\alpha$ -MEM-10% FBS. When medium was warmed to 37°C (approximately 10 min), cleaned external surface of conical with 70% ethanol prior to placing back in the hood.
6. While water bath is warming medium, removed 10 $\mu$ l aliquot of RANKL from the -70°C freezer. Slowly thawed 10 $\mu$ l aliquot of RANKL stock (1250ng/ml concentration) over ice for approximately 15 minutes. Monitored closely because cytokines derogate rapidly with warmth. When RANKL volume was visually mobile in the cryotube, i.e. thawed, centrifuged for 3 seconds to collect RANKL into one collected volume in the bottom of the cryotube. The aliquot was now ready for dilution with culture medium.
7. Added 25 $\mu$ l of culture medium to the 10 $\mu$ l of RANKL stock, thus yielding 35 $\mu$ l volume. Mixed thoroughly with pipetman.

Changed pipette tips in-between mixing serial dilutions as to not alter the concentration of the RANKL dose. Note all final volumes of RANKL intermediate are greater than 450 $\mu$ l needed for RANKL induction.

8. Placed final volume amount in tubes 2-5. Placed 765 $\mu$ l into tube 1 to begin dilution.
9. Aspirated 35 $\mu$ l RANKL stock and added to tube 1 to create the RANKL intermediate.
10. Aspirated 338 $\mu$ l RANKL intermediate from tube 1 and added to tube 2.
11. Aspirated 225 RANKL intermediate from tube 2 and added to tube 3.
12. Aspirated 113 RANKL intermediate from tube 3 and added to tube 4.
13. Aspirated 20 $\mu$ l of RANKL intermediate from tube 2 and added to corresponding RANKL dose well. Repeated until 20 wells have been filled. Repeated procedure for tubes 1, 4, and 3. (Changed pipette tips when changing to the next RANKL concentration as not to alter the RANKL concentration.)
14. When adding RANKL intermediate to each well
  - tilted culture plate towards self
  - did not touch sides of well or bottom of well to avoid disturbing cultured cells
  - gently pipetted up and down several times into the medium and swirled plate to get even mixing
  - visualized cells under microscope to determine if there was any disruption of plated cells

- placed in incubator for 24hrs

### **RANKL Induction Plate Labeling**

<b>Tube 1 : 50ng/ml</b>				
<b>Plate Number</b>	<b>Energy Density J/cm<sup>2</sup></b>	<b>RANKL Dose ng/ml</b>	<b>Number of Wells</b>	<b>Well Number</b>
1	4.5 (controls)	50	5	[C2 – C6]: 0-0-50
3	45 (controls)	50	5	[C2 – C6]: 0-0-50
5	4.5 ( treatment)	50	5	[C2 – C6]: 1-4.5-50
7	45 ( treatment)	50	5	[C2 – C6]: 1-45-50

<b>Tube 2: 25</b>				
<b>Plate Number</b>	<b>Energy Density J/cm<sup>2</sup></b>	<b>RANKL Dose ng/ml</b>	<b>Number of Wells</b>	<b>Well Number</b>
1	4.5 (controls)	25	5	[B2 – B6]: 0-0-50
3	45 (controls)	25	5	[B2 – B6]: 0-0-50
5	4.5 ( treatment)	25	5	[B2 – B6]: 1-4.5-50
7	45 ( treatment)	25	5	[B2 – B6]: 1-45-50

<b>Tube 3: 10ng/ml</b>				
<b>Plate Number</b>	<b>Energy Density J/cm<sup>2</sup></b>	<b>RANKL Dose ng/ml</b>	<b>Number of Wells</b>	<b>Well Number</b>
2	4.5 (controls)	10	5	[B2 – B6]: 0-0-10
4	45 (controls)	10	5	[B2 – B6]: 0-0-10
6	4.5 ( treatment)	10	5	[B2 – B6]: 1-4.5-10
8	45 ( treatment)	10	5	[B2 – B6]: 1-45-10

<b>Tube 4: 2.5ng/ml</b>				
<b>Plate Number</b>	<b>Energy Density J/cm<sup>2</sup></b>	<b>RANKL Dose ng/ml</b>	<b>Number of Wells</b>	<b>Well Number</b>
2		2.5	5	[A2 – A6]: 0-0-2.5
4		2.5	5	[A2 – A6]: 1-4.5-2.5
6		2.5	5	[A2 – A6]: 1-45-2.5
8		2.5	5	[A2 – A6]: 0-0-2.5

## Appendix F

### Tartrate-Resistant Acid Phosphatase Staining Protocol

#### (TRAP)-Staining Protocol

**TRAP Staining Protocol:** using SIGMA Acid Phosphatase Leukocyte Kit (prod #387)

Solution not in kit, but required

- Acetone
- Formaldehyde, 37% (F1635 formaldehyde solution from SIGMA)

### 1. *Preparation of Solutions*

— Warm up dH<sub>2</sub>O to 37C ( 200ml)-

**A. Prepare fixation Solution:** (for cell cx only, use polypropylene or glass containers and pipettes)- keep at room temp- in glass beaker

— Citrate solution:	25ml	18.75	12.5ml	8.3ml	6.25ml
— Acetone:	65ml	48.75ml	32.5ml	21.6ml	16.25ml
— 37% Formaldehyde	<u>8ml</u>	<u>6ml</u>	<u>4ml</u>	<u>2.6ml</u>	<u>2ml</u>
<b>TOTAL</b>	<b>98ml</b>	<b>73.5ml</b>	<b>49ml</b>	<b>32.5ml</b>	<b>24.5ml</b>

**0.5 ml/well**

**B. Prepare the Fast Garnet Solution:** use glass pipettes, Mix in 1.5ml centrifuge tube

— Fast Garnet GBC	0.75ml	0.5ml	0.35ml
— Sodium Nitrite Sol	<u>0.75ml</u>	<u>0.5ml</u>	<u>0.35ml</u>
<b>TOTAL</b>	<b>1.5ml</b>	<b>1ml</b>	<b>0.7ml</b>

—→ Mix gently inversion for 30sec —→ let stand for 2min

**C. Make the staining solution:** (using a glass beaker): NEXT Experiment make up 74.25 & 34.65 for 100 wells

May mix in a polypropylene conical tube if amount is under 50ml.

— dH <sub>2</sub> O, 37C	67.5ml	45ml	31.5ml
— Fast Garnet Sol	1.5ml	1ml	0.7ml
○ (From <b>B</b> )			
— Naphtol AS-BI phosphate	0.75ml	0.5ml	0.35ml
— Acetate Sol	3.0ml	2.0ml	1.4ml
— Tartrate Sol	<u>1.5ml</u>	<u>1.0ml</u>	<u>0.7ml</u>
<b>TOTAL</b>	<b>74.25ml</b>	<b>49.5ml</b>	<b>34.65ml</b>

**D.** Warm the Staining Solution in 37C water bath, protect from light.

## 2. Staining

### A. Staining cell culture plates

- Aspirate media
- Wash the plate using PBS Ca<sup>+</sup>, Mg<sup>+</sup> (2 times)
- Add the fixation solution from (from above 1A), enough to cover the cells (e.g. 1ml per well for 12 well plates or 0.7ml per well for 24 well plates)

—→ wait for 30sec —→ Rinse thoroughly with dH<sub>2</sub>O (3 times)

Note: Do not let sample dry out

— Add the Staining Solution (from step 1C), enough to cover the cells (same as fixation sol) —→

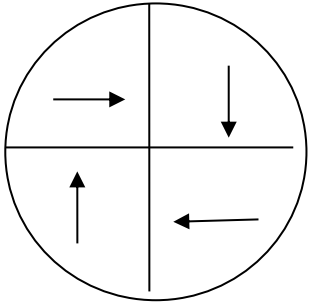
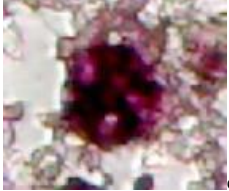
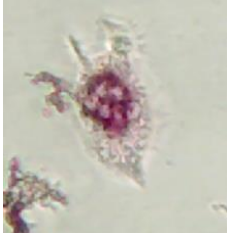
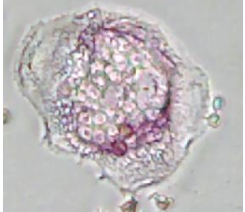

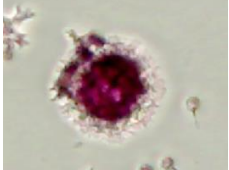
— Incubate in 37C (protected from light) for 10min but check q 5 min



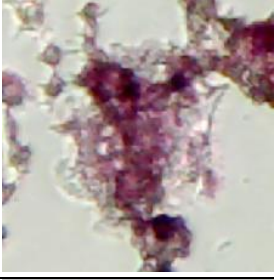
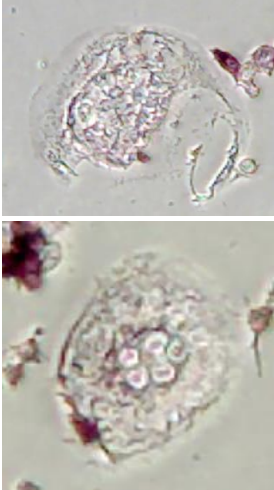
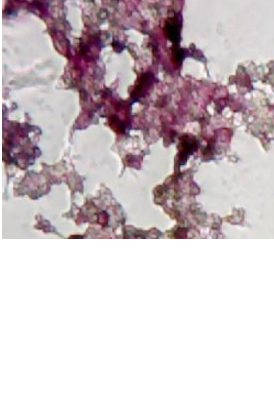
— Rinse thoroughly with dH<sub>2</sub>O (3times)

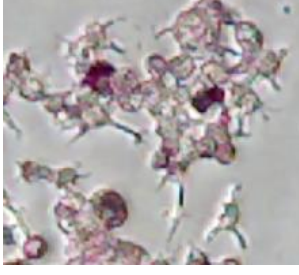
Air dry and evaluate with microscope – store plates protected from light

## Appendix G

### Cell Counting Classification Tool

Example/source E1384 NIR #3 RAW Cells 090713 TRAP Stain Counting Rules: Lisa Anderson-Antle	Rule	Est.
 <p style="text-align: right;">Counted</p> <p>RUQ and LLQ Established 10/20/09</p>	<p>Use 10x Magnification on cell cx room microscope.</p> <p>Entire well counted: Divided into 4ths. Counted OC that touched the arrowed margins Cells must be: First—TRAP+ Then—Have 3+ nuclei</p>	<p>8/8/2009</p>
 <p>e1385_NIR #4 Plate 1-4b</p>  <p>e1385_NIR #4 Plate 1-5b 0-0-25</p>	<p>These are nuclei. Found in cell clump, if defined cell margins and 3 nuclei but did not count.</p>	<p>8/8/2009</p>
 <p>e1385_NIR #4 Plate 1-4b 0-0-25</p>  <p>e1385_NIR #4 Plate 1-4b</p>  <p>e1385_NIR #4 Plate 1-5c 0-0-25</p>	<p>Pale purple; counted numerous Lg OC throughout all plates, decision to count</p> <p>Counted</p> <p>Counted dark purple</p> <p>Deep Purple-pink; counted</p>	<p>8/8/2009</p>

	<p>e1385_NIR #4 Plate 1-4c 0-0-25 m</p>	<p>Counted OC with processes</p>	
	<p>e1385_NIR #4 Plate 1-4b 0-0-25</p>	<p>Dark Pink; counted</p>	
	<p>e1385_NIR #4 Plate 1-5b 0-0-25</p>	<p>OC under cell clump; counted</p>	
	<p>e1385_NIR #4 Plate 1-4b 0-0-25</p>	<p>Clear cytoplasm, no distinct pink or purple around nuclei—this is not TRAP+ Did not count</p>	<p>8/8/2009</p>
	<p>e1385_NIR #4 Plate 1-4b 0-0-25</p>	<p>Did not count clumps on side walls not counted  Scattered TRAP+ areas; not counted  Stained Cell clump: did not count</p>	<p>8/8/2009</p>

	e1385_NIR #4 Plate 1-4b 0-0-25	Fusing not defined borders; not counted	
---	--------------------------------	---	--

E1385 NIR #4: TRAP Stain-OC Counting Rules

24 well plates: 8 plates; 36 wells. 2 wells per plate; wells 3b & 4b  
control plates [3a – 3e] [4a – 4e] [5a – 5e]

## Appendix H

### Tables

Table 1.

*Cell Plate Labeling Example: 5 wells per Condition Labeled by Number of Light Treatments – Energy Density - RANKL Dose*

Plate Number	Row 1	Row 2	Row 3	Number of Wells Used
<u>4.5 J/cm<sup>2</sup> Energy Density Control Plate</u>				
Plate 1	0-0-0	0-0-2.5	0-0-10	15 wells
Plate 2	0-0-0	0-0-25	0-0-50	10 wells
<u>45 J/cm<sup>2</sup> Energy Density Control Plate</u>				
Plate 1	0-0-0	0-0-2.5	0-0-10	15 wells
Plate 2	*	0-0-25	0-0-50	10 wells
<u>4.5 J/cm<sup>2</sup> Energy Density Experimental Plate</u>				
Plate 1	1-4.5-0	1-4.5-2.5	1-4.5-10	15 wells
Plate 2	*	1-4.5-25	1-4.5-50	10 wells
<u>45 J/cm<sup>2</sup> Energy Density Experimental Plate</u>				
Plate 1:	1-45-0	1-45-2.5	1-45-10	15 wells
Plate 2:	*	1-45-25	1-45-50	10 wells

\* Well not utilized

Table 2.

*Serial Dilution for RANKL Intermediate Stock Concentration*

Tube Number	Dilution Factor	Volume of Previous Stock/ $\mu$ l	Add Medium Volume/ $\mu$ l	Beginning Tube Volume/ $\mu$ l	Total Final Volume/ $\mu$ l	RANKL Intermediate Stock Concentration
1: 50ng/ml	1:1	35ul		765	800	1.25 x 10 <sup>3</sup> ng/ml
2: 25ng/ml	1:2	800	338	338	765	0.63 x 10 <sup>3</sup> ng/ml
3: 10ng/ml	1:2.5	765	225	338	676	0.68 x 10 <sup>3</sup> ng/ml
4: 2.5ng/ml	1:4	676	113	337	563	0.31 x 10 <sup>3</sup> ng/ml
5: 0 ng/ml	0		0	450	450	

Table 3  
*Summary of Experiment Variables by Experiment Number*

Experiment Number	Number of Light Treatments	WARPTM 10 Light Source	Energy Density J/cm <sup>2</sup>		RANKL Dose ng/ml		
			4.5	45	0	25	50
e1380	1	*	*	*	*	*	*
	4	*	*	*	*	*	*
e1383	1	*	*	*	*	*	*
	4	*	*	*	*	*	*
e1384	1	*	*	*	*	*	*
e1385	1	*	*	*	*	*	*

\* Variable utilized in the experiment

Appendix I  
Letters of Permission



April 15, 2014

Lisa Anderson-Antle, MSN(R), GCNS/BC, APNP, RN  
Doctoral Candidate  
University of Wisconsin-Milwaukee College of Nursing  
NASA Student Ambassador

Photos of the RAW 264.7 cells, ATCC® No. TIB-71™ are being provided to you as a courtesy by ATCC for one-time use. All rights to these photos remain with ATCC. Any other use of the photos is forbidden without written consent.

Photo credit should read: **Photo courtesy of ATCC.**

I agree to the terms above.

Lisa Anderson - Antle

Name (printed)

Signature

6/2/2014

Date

## **CURRICULIUM VITAE**

**LISA L. ANDERSON-ANTLE \* RN, PHD CANDIDATE,  
GCNS(R)-BC, APNP**

**CURRICULIUM VITAE**

---

**EDUCATION**

---

2003-current	University of Wisconsin-Milwaukee <i>Doctoral Candidate, College of Nursing</i> <i>Anticipated Award Date: December 2014</i>	Milwaukee, WI
1992-1995	Saint Louis University <i>Masters of Science in Nursing Research,</i> <i>Gerontological Clinical Nurse Specialist</i>	St. Louis, MO
1986-1991	University of Wisconsin-Madison <i>Bachelors of Science in Nursing</i> <i>President, Student Nurses Association 1990-1991</i>	Madison, WI
1985-1986	Beloit College <i>Porter Scholars Program</i>	Beloit, WI

**AWARDS**

---

2010	<b>NASA Student Ambassador</b>
Fall 2009	<ul style="list-style-type: none"> <li>▪ Wisconsin Space Grant Graduate Fellowship</li> <li>▪ Porth Nurse Physiologist Scholarship</li> <li>▪ Exploration Space Mission Directorate/Wisconsin Space Grant Internship <u>NASA Ames Research Center: Bone and Signaling Laboratory</u> Mentor: Ruth Globus, PhD</li> <li>▪ <b>NASA Student Ambassadors Program:</b> Nominated by the International Astronautical Congress</li> </ul>
Summer 2009	<ul style="list-style-type: none"> <li>▪ <b>Exploration Space Mission Directorate/Wisconsin Space Grant Internship</b> <u>NASA Ames Research Center: Bone and Signaling Laboratory</u> Mentor: Ruth Globus, PhD</li> </ul>

Summer 2008

- **NASA Science & Technology Institute: United Negro College Fund-Special Programs**

NASA Ames Research Center: Psychophysiology Laboratory

Mentor; Patricia Cowings PhD

---

**SKETCH OF WORK HISTORY** *(FULL LIST AVAILABLE UPON REQUEST)*

**ACADEMIC RESEARCH**

2009: Summer & Fall, NASA Ames Research Center

***Doctoral Research Assistant***

Responsibilities: Completed supervised independent research

2008: Summer, NASA Ames Research Center

***Research Assistant-Medical Monitor***

Responsibilities: Human Sleep Deprivation Study; medical monitoring

2007 – 2008 University of Wisconsin-Milwaukee: College of Health Sciences

***Research Assistant***

Responsibilities: Multi-disciplinary team participation; in vivo development and experimentation

2006-2007 University of Wisconsin-Milwaukee: College of Nursing, Milwaukee, WI

***Clinical Assistant Professor***

**Project Director:** RO1 NIH randomized interventional study

Responsibilities:

- Implementation , budget accountability, management & supervision
- Database validity/reliability and statistical interpretation
- Validity of instrumentation and data collection

2004-2005 University of Wisconsin-Milwaukee: College of Nursing, Milwaukee, WI

***Research Coordinator***

Responsibilities: Implementing and coordinating a multi-grant/research site study

**ACADEMIC HIGHER EDUCATION**

2010-2012 George Williams College of Aurora University

***Adjunct Faculty***

- 2005-2006 Columbia/St Mary's Health System College of Nursing, Milwaukee, WI  
*Assistant Professor*
- 2003 Fall Southwest Missouri State University Department of Nursing, Springfield, MO  
*Clinical Supervisor*
- 2001-2003 Southwest Baptist University: Department of Nursing, Springfield, MO  
*Adjunct Faculty*
- 1997-1998 Lester E. Cox Health Systems: College of Nursing, Springfield, MO  
*Assistant Professor of Nursing*

### **ADVANCED PRACTICE NURSING**

- 2007 – 2008 In Your Home Internal Medicine & Geriatrics SC, Milwaukee, WI  
*Gerontological Advance Practice Nurse*
- 2004-2005 Evercare Wisconsin: United Health Group, Milwaukee, WI  
*Nurse Practitioner*
- 2000-2003 Gerontological Consulting Services, Springfield, MO  
*Gerontological Advanced Practice Nurse*
- 1996-1997 Lester E. Cox Health Systems, Springfield, MO  
*Gerontological Clinical Nurse Specialist*

### **FIELDS OF INTEREST**

- Space Biomedical Countermeasures; Skeletal and Bone Health-Translational Medicine
- Bio-Gerontological Preventive Aging Research: Photobiomodulation
- Population Health Research: Gerontological Community/Home Care & Institutional Based Long-Term Care

### **PROFESSIONAL ORGANIZATIONS**

- The American Society for Bone and Mineral Research
- American Nurses Association
- Sigma Theta Tau International Nursing Honor Society
- Wisconsin Nurse Association
- American Medical Directors Association
- Midwest Nursing Research Society:
- Missouri Nurses Association: Board member (select years) 1992- 2003

- Missouri League of Nursing
- National Conference of Gerontological Nurse Practitioners
- The American Geriatric Society
- The Gerontological Society of America
- Student Nurses Association: President 1990-1991

## **PUBLICATIONS**

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Marek, K. D., & Antle, L. L. (2008) Medication management of the community-dwelling older adult. In R. Hughes (Ed.), *Advances in Patient Safety & Quality – An Evidence-based Handbook for Nurses*. Rockville, MD: Agency for Healthcare Research and Quality.

Antle, L. A. (2004). Advanced practice nurse controlled substance authority. *The Missouri Nurse*, 1, 11-12, 25.

Antle, L. A. (1995). *Defining the Role of Advanced Practice Nursing: Clinical Nurse Specialists' and Nurse Practitioners' Perceptions of Their Roles*. Unpublished Masters Thesis, St. Louis, MO, St. Louis University.

## **CONFERENCE PRESENTATIONS**

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Anderson-Antle, L. L., & Globus, R. (2011, October 3-7). *Effects of Photobiomodulation and Osteoclast Formation in Vitro: a Pilot Study*. Paper Presented at the 62<sup>nd</sup> International Astronautical Congress: African Astronautics, Cape Town, South Africa. Submitted.

Anderson-Antle, L. L., Cowings, P., Toscano, W., Orasanu, J., & DeRoshia, C. W. (2009, October 12 – 16). *Consequences of sleep deprivation on performance and mood states*. Paper presented at the 60th International Astronautical Congress: Space for Sustainable Peace and Progress, Daejeon Republic of Korea.

Anderson-Antle, L. L., & Globus, R. (2010). *Near Infrared Laser Emitting Diode Photobiomodulation and Osteoclast Formation In Vitro*. Poster presented at the 34th Midwest Nursing Research Society: “Nursing Research: Bench to Bedside”. Kansas City, Missouri.

Anderson-Antle, L. L., Globus, R. (2009). *Near Infrared Laser Emitting Diode Photobiomodulation and Osteoclast Formation In Vitro*, Poster Presentation at the Ames’ 70<sup>th</sup> Anniversary Celebration. NASA Ames Research Center, Moffett Field, California.

Anderson-Antle, L. L., Cowings, P., and Toscano, W. (2008). *Consequences of Sleep Deprivation on Performance & Mood States*, Poster Presentation at the NLSI Lunar Science Conference. NASA Ames Conference Center, Moffett Field, California.

Anderson-Antle, L. L., Bauer, D., Gopalakrishnan, S., Lark, D.S., Sandoval, C., Eells, J. T., and Snyder, A.C. (2008). *Streptozotocin-Induced Diabetic Animal Model Recapitulates Many Features of Frailty in Older Adults with Diabetes*. Poster Presentation Experimental Biology 2008, “Today’s Research: Tomorrow’s Health”. San Diego, California.

Marek, K. D., Antle, L. L. (2006). *Pharmacy Screen Results and Consumer Satisfaction of a Home Care Medication Management Program*, Paper Presentation at the 30<sup>th</sup> Annual Midwest Nursing Research Society Research Conference “*Linking Research and Practice: A Roadmap for the Future*”, Milwaukee, Wisconsin.

Antle, L. L. (May, 2005). *Clinical Outcomes of Aging in Place: Karen Dorman Marek PhD, MBA, RN, FAAN*, Paper Presentation at the 7th Annual Southeastern Wisconsin Nursing Research Conference “*Building Bridges to Research Based Practice*”. Pewaukee, Wisconsin.

Antle, L. (2001). *Role of the Advanced Practice Nurse in Long Term Care*. Paper presented at the Southwest Missouri Geriatric Symposium, Springfield, Missouri.

Antle, L. (1995). *Defining the Role of Advanced Practice Nursing: Clinical Nurse Specialists’ and Nurse Practitioners’ Perceptions of Their Roles*. Paper presented at the Missouri Nurses Association “*Coming together in Advanced Practice*”. Columbia, Missouri.

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## LICENSES AND CERTIFICATIONS

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Registered Nurse: Wisconsin

Registered Nurse: California *inactive*

Registered Nurse: Missouri *inactive*

Advanced Practice Nurse: Missouri *inactive*

Advanced Practice Nurse Prescriber: Wisconsin

Gerontological Clinical Nurse Specialist: American Nurse’s Credentialing Center