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BISPHOSPHONATES AND BONE MICRODAMAGE

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science
in Biomedical Engineering in the College of Engineering
at the University of Kentucky

By

William Alexander Caruthers

Lexington, KY

Director: Dr. David Pienkowski

Lexington, KY

2012

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ABSTRACT OF THESIS

BISPHOSPHONATES AND BONE MICRODAMAGE

Osteoporosis is a significant healthcare issue due to the increasing elderly population. Bisphosphonates are used to treat osteoporosis by reducing the rate of resorption, increasing bone mineral density (BMD) and thereby reducing fracture risk. Long-term bisphosphonate treatment, however, has been associated with low-energy fractures. Bone microdamage may provide a partial explanation for one of the mechanisms responsible for these fractures since it has been shown to reduce bone toughness, fracture resistance, and bone strength. The goal of this study was to quantify the changes in bone microdamage parameters with the duration of bisphosphonate treatment. This study selected, stained, and histomorphometrically analyzed 40 iliac crest bone biopsies from controls and female patients with osteoporosis treated with bisphosphonates for varying durations (up to 12 years). All subjects were matched for age and low turnover. The results showed that microcrack density and microcrack surface density were significantly greater in patients who took bisphosphonates for at least 5 years compared to those who took bisphosphonates for less than 5 years or not at all. These results reveal novel, clinically relevant information linking microdamage accumulation to long-term bisphosphonate treatment without influences from age or turnover.

KEYWORDS: bone microcracks, alendronate treatment, anti-resorptive treatment, fragility fractures

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12-10-12

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CHAPTER I: GLOBAL INTRODUCTION

The older population in America is expanding rapidly. In 2030 it is predicted the number of Americans over 65 years of age will grow to nearly 72.1 million, up from only 39.6 million in 2009 ⁽¹⁾. The medical consequences of this increase will be a heightened awareness for preventing or treating aging-related disorders including cardiovascular disease, cancer, and musculoskeletal issues. The most notable musculoskeletal issue is loss of bone mass and resulting propensity to incur bone fractures, i.e. osteoporosis.

Osteoporosis

The medical and economic issues accompanying osteoporosis have become increasingly concerning for at-risk adults as they age. Nationally, osteoporosis affects an estimated 10 million Americans, and another 34 million are likely to develop osteoporosis as indicated by their low bone mass ⁽²⁾. Osteoporosis is defined as a skeletal fragility condition caused by decreased bone mass and deterioration of bone microarchitecture, consequently increasing the risk of bone fracture ⁽³⁾. Bone fractures are a strong concern for the aging population because of their association with long-term disability, psychosocial impairment, and overall reduced quality and quantity of life. Within one year of hip fracture, one third of patients are admitted to a nursing facility, and the fatality rate exceeds 20% for all hip fracture cases ⁽²⁾. There are an estimated 1.5 million osteoporotic fractures per year, and that number is likely to increase in the coming decades. Costs associated with osteoporosis treatment, fractures, and post-fracture care are a financial burden not only to patients but also to Medicare and the US as a whole, exceeding \$20 billion nationally ⁽²⁾. For example, one third of all fracture patients suffered from hip fractures, and their total individual cost for the year averaged at over \$39,000 ⁽²⁾.

Bone strength, or the ability of bone to withstand loading without failure, has been a key biomechanical parameter when discussing osteoporosis and understanding fracture risk. Several properties influence overall bone strength from macro and microscopic perspectives such as BMD and bone microarchitecture, among others. Bone mineral density (BMD) is quantified as the amount of mineral per area of bone and is

largely associated with bone strength since mineral stiffens the bone matrix. It is common practice to measure a patient's BMD to determine whether the patient is at risk for developing osteoporosis, has osteoporosis, and if the patient can benefit from anti-resorptive treatments. Patients are clinically defined as having osteoporosis if their BMD scores are "2.5 standard deviations below the mean for young, healthy adult women at any site." ⁽⁴⁾ However, although BMD is a strong indicator for fracture risk, BMD alone cannot predict which individuals will fracture ^(5, 6). Alterations in bone mineralization will also affect bone strength. Hypermineralized bone may be more brittle, occasionally caused by oversuppressed bone turnover. Inversely, low mineralization, osteomalacia, reduces bone stiffness and strength ⁽⁷⁾.

Bone microarchitecture is another factor that affects bone strength and resulting fracture risk. This refers to the connectivity of the trabecular "lattice" of cancellous bone. Bone resorption via post-menopausal osteoporosis will weaken the microarchitecture by reducing the trabecular thickness and reducing the number of connections between trabeculae. Understandably, a thinner and less dense trabecular lattice will withstand less compressive force. In fact, characteristics of bone microarchitecture have a stronger correlation with bone strength and are a better descriptor of fracture risk compared to BMD ⁽⁸⁾.

Bisphosphonates

Current osteoporosis treatments rely on suppressing bone turnover. This serves the purpose of reducing bone resorption and subsequent weakening of the bone matrix responsible for the heightened fracture risk in osteoporotic patients. Anticatabolic agents reduce excessive osteoclastic activity common in postmenopausal osteoporosis, and bisphosphonates have been the most prescribed anticatabolic agent over the past several decades. Bisphosphonates alone do not actively build bone tissue to strengthen bone; they simply suppress bone resorption that counteracts new bone formation by osteoblasts ⁽⁹⁾.

The pyrophosphate chemical structure of bisphosphonate gives it a high affinity for bone mineral. In fact, the skeleton will retain large amounts of bisphosphonate seemingly without saturation ⁽¹⁰⁾. They will naturally bind to hydroxyapatite crystals exposed at bone remodeling sites which are especially abundant in high turnover bone ⁽¹¹⁾. The bisphosphonate molecule contains phosphate and hydroxyl groups responsible for its affinity for bone; however its two side chains make bisphosphonate especially appealing for clinical applications. The side chains, as seen in Figure 1.1 ⁽¹²⁾, allow bisphosphonate to act as a drug carrier to treat skeletal diseases since it can bind strongly to bone or be easily excreted when unbound. The presence of nitrogen in the R2 side chain increases the potency of bisphosphonate and alters its mechanism of action ⁽¹⁰⁾. All modern bisphosphonates contain a nitrogen-based side chain including alendronate, risedronate, ibandronate, zoledronate, et al.

Once bound to the exposed bone mineral, bisphosphonates are taken up by osteoclasts attempting to resorb bone tissue, where the drug negatively alters osteoclast activity or can cause apoptosis. The more potent nitrogen-containing bisphosphonates affect osteoclasts by inhibiting FPP synthase, a key enzyme in the mevalonate pathway responsible for producing cholesterol and isoprenoid lipids such as geranylgeranyl diphosphate (GGPP) ⁽¹³⁾. These isoprenoid lipids are considered building blocks for many metabolites and are essential for GTPases, signaling molecules responsible for cytoskeletal arrangement ^(13, 14). The loss of these GTPase signaling molecules causes apoptosis in osteoclasts. By these actions bisphosphonates can effectively inhibit osteoclastic bone resorption of hydroxyapatite. A helpful side effect of bisphosphonate treatment is that it also possesses the ability to inhibit apoptosis of osteoblasts and osteocytes. This secondary function may enhance the therapeutic efficacy of bisphosphonate treatment in addition to osteoclastic activity inhibition ⁽¹⁵⁾.

Bisphosphonates are typically prescribed to patients with BMD scores at 2.5 standard deviations below normal (t-score: -2.5), the clinical definition of osteoporosis. However, it is also advised that bisphosphonates be prescribed to patients with BMD t-scores between -1.0 and -2.5, defined as osteopenia, if they suffered from low-energy fractures or have a family history of poor bone health and fragility fractures. They are

considered likely to develop further fractures and will benefit from bisphosphonate therapy. For a patient with no history of fractures, fracture risk is based on BMD: osteoporotic patients should receive treatment, whereas osteopenic patients should not yet receive treatment. Essentially, since BMD is not the only predictor of fracture risk, as a non-osteoporotic BMD score can still warrant bisphosphonate use if the patient experiences rare low-energy fractures, since reducing fracture risk is the primary objective of bisphosphonates ⁽¹⁶⁾.

The patient's rate of bone turnover before taking bisphosphonates can be a cause for concern. Bone turnover suppression therapy will adequately suppress high turnover osteoporosis and reduce the elevated rate of bone resorption as intended. However, it will also suppress low turnover osteoporosis often excessively to the point where the skeleton is unable to stimulate repair, and bone microdamage accumulates. This has raised many questions about the long term effects of turnover suppression therapy and if there is a direct link between oversuppression of turnover and low-energy fractures indicating severe bone brittleness.

Rare fractures

Although it is well documented that bisphosphonate therapy widely reduces fracture risk and improves BMD, many publications report patients receiving atypical, spontaneous, non-traumatic fractures while on long term treatments of bisphosphonate ⁽¹⁷⁻²¹⁾. Shin et al. ⁽²¹⁾ reported a 63-year-old Korean woman who suffered non-traumatic **diaphyseal** fractures in both femurs while on bisphosphonates for 5 years despite lots of ambulatory activity and no prior fracture history. Additionally, she experienced delayed healing after bone fixation surgery as a result of the oversuppression of both turnover and subsequent repair caused by prolonged bisphosphonate use. Sellmeyer ⁽²⁰⁾ described a 58-year-old Caucasian woman who took bisphosphonates for 10 years after experiencing a foot stress fracture with test results indicating an osteopenic BMD. After 10 years of therapy, she complained of thigh pain and suffered a complete subtrochanteric femur fracture while stepping down a stair. She similarly experienced delayed healing due to bisphosphonate-induced turnover suppression. Odvina et al. ⁽¹⁸⁾ examined 9 osteoporotic patients on alendronate therapy for 3-8 years who suffered non-traumatic fractures while

taking the medication. All patients displayed severely suppressed bone turnover with evidence that alendronate was the culprit. Fractures occurred in areas uncommon to osteoporosis, including the femoral shaft, ischium, and pubic bone. Also, fracture healing was impaired or absent in 6 patients who continued taking alendronate after fracturing.

Not only are there many incidences of rare fractures in long-term bisphosphonate-treated patients, but also certain characteristics of these fractures are common among them. There exists an association between alendronate use and low-energy, transverse femur fractures through thickened diaphyseal cortices⁽²²⁾. Neviaser et al.⁽²²⁾ reviewed 70 patients with low-energy fractures, and 25 of them were being treated with alendronate. Seventy-six percent of the 25 exhibited simple, transverse fractures with a unicortical beak in a thickened cortex. Only 2% of patients not being treated with alendronate shared the same fracture pattern. Moreover, patients taking alendronate who exhibited the pattern have been taking the drug for significantly longer than those who did not exhibit the same pattern (6.9 versus 2.5 years of use).

Lenart et al.⁽²³⁾ agree that this pattern is highly associated with bisphosphonate use. Significantly more patients in Lenart's study with subtrochanteric/femoral shaft fractures were taking bisphosphonates compared to the patients with intertrochanteric/femoral neck fractures. It is concluded that these unusual fractures may result from unrepaired, propagating stress fractures and accumulated microdamage due to suppressed turnover from the duration of alendronate use.

Safety and Duration of Use

Due to the many studies reporting atypical femur fractures in patients taking bisphosphonates for long periods, questions have arisen about the safety of bisphosphonates and optimal duration of use. It is well understood that bisphosphonates improve BMD and reduce fracture risk in most patients; however, the existence of rare fracture incidences during long-term bisphosphonate use merits a re-examination of how long these drugs should be prescribed to ensure patient safety. After all, the intent of taking bisphosphonate is to improve bone quality and reduce the risk of fractures.

Unfortunately, stating an exact length of time for safe bisphosphonate use is difficult. Numerous clinical factors such as patient age, BMD, fracture history, bone turnover rates, activity levels, and others all contribute to bisphosphonate's potential to reduce fracture risk. Plus, BMD and turnover are constantly changing during therapy. Therefore, it has been recently advised to perform more thorough and individualized patient assessments of these variables to determine the proper duration of use by weighing the benefits versus potential risks^(16, 24, 25).

Although no definite duration is considered completely safe from atypical fractures, several studies indicate 3-5 years is long enough for the average osteoporosis patient to take bisphosphonates to improve bone quality with little risk of atypical femur fractures, with some doctors recommending stoppage after 5 years if the patient's BMD has significantly improved⁽²⁴⁻²⁶⁾. Watts and Diab⁽²⁷⁾ suggest an optimal-use window as large as 5-10 years for high-risk patients; however, that is certainly at the upper limit. Meijer et al⁽²⁸⁾ analyzed fracture rates in 14,750 women taking bisphosphonate for osteoporosis, and more than half stopped during the first year. Compared to that group, those that took it for 3-4 years had significantly fewer fractures, but those that took it for 5-6 years had slightly more fractures. Similarly, Ott challenged a 2000 study about the skeletal benefits of 7 years of continuous alendronate by highlighting the fact that vertebral fractures were 3 times higher in years 6-7 compared to years 1-3^(29, 30). Additionally, other studies found that femur bone density increases then reaches a plateau after 3 years of bisphosphonate use^(9, 31). These findings support the notion that stopping bisphosphonate after 3-5 years will generally provide an adequate therapeutic response without increasing secondary fracture risk related to long-term bisphosphonate use.

Microdamage

Microdamage accumulation may explain the link between oversuppression of bone turnover and fracture occurrence. Microdamage is defined as microscopic cracks in bone around 30-150 μm in length caused by cyclic fatigue stress. The presence of minor microdamage is not inherently detrimental as it is caused by normal loading activities. In

fact, a small amount of microdamage is considered beneficial since the formation and propagation of microscopic cracks are manifested releases of energy that could otherwise cause the bone to catastrophically fail ⁽³²⁾. Microdamage acts as a stimulus, through osteocyte apoptosis near the damage ⁽³³⁾, to enable healthy bone to continually repair itself via turnover at a rate that sufficiently keeps up with microdamage formation ^(34, 35). On the other hand, when microdamage formation exceeds the rate at which bone can be repaired, bone quality can be compromised. Bone with low or suppressed turnover, for example, cannot repair the microdamage quickly enough to keep up with microdamage formation. Therefore, turnover suppression causes microdamage to accumulate and thereby alter the mechanical properties of bone ⁽³⁶⁾.

Greater microdamage accumulation significantly reduces bone toughness, fracture resistance, and bone strength ^(36, 37) resulting in strength and stiffness losses that are likely to increase fracture risk ⁽³⁸⁾. Also, microdamage density in both cortical and trabecular bone tends to increase with age, likely related to less remodeling and lower trabecular volume in older patients ^(39, 40).

The parameters used to quantify microdamage are crack number (Cr.N), crack length (Cr.L), crack density (Cr.D), and crack surface density (Cr.S.D). Cr.N is the number of cracks found in each specimen, although this parameter is more useful when the area of bone in each specimen is taken into account. Cr.L is the length of the crack along its path measured in μm . Cr.D is defined as the number of cracks per mm^2 of bone area. Lastly, Cr.S.D is defined as the total length of all cracks in a specimen per mm^2 of bone area. Cr.D and Cr.S.D are the most commonly used parameters to quantify microdamage accumulation, as they account for the varying bone areas, especially when studying trabecular bone. The length of microcracks has been suggested to be an important factor in fatigue resistance since bone that allows cracks to form but not grow should be resistant to fracture ⁽⁴¹⁾. On the contrary, other studies showing significant changes in microdamage accumulation report no change in crack length ^(42, 43).

Microdamage Detection

The most common procedure for identifying bone microdamage is Burr and Hooser's en bloc basic fuchsin staining method ⁽⁴⁴⁾ which evolved from previous microdamage detection methods ^(35,45). This procedure uses a series of graded alcohols in solution with basic fuchsin to stain *in vivo* microcracks before sectioning. The graded alcohols serve to slowly dehydrate the bone tissue to avoid causing *ex vivo* cracks associated with quick dehydration. After sectioning, thin slices of the sample are examined under light microscopy to identify microcracks and measure the previously mentioned crack parameters to quantify the amount of microdamage.

Over the past two decades, a variety of approaches have been developed to identify microdamage as an alternative to using only light microscopy. Although the light microscopy method has proven validity, its microcrack detection involves varying the depth of focus, light intensity, and magnification to correctly distinguish stained cracks from artifactual ones ⁽⁴⁶⁾. For example, Lee et al. ⁽⁴⁶⁾ showed that fluorescence microscopy could be used to aid in detecting microcracks. Samples were stained via the same en bloc basic fuchsin staining method; however, slices were examined using both light microscopy and epifluorescence. Results between the two had no statistical differences, proving fluorescence microscopy can be a reliable alternative to light microscopy. Another technique is laser scanning confocal microscopy (LSCM). This process focuses laser light onto the bone surface and excites fluorochromes within the stained microcracks to produce a three-dimensional, high resolution representation of each microcrack ⁽⁴⁷⁾.

Beyond the application of microcrack detection for the purpose of quantifying microdamage accumulation, staining techniques have also been used to track the propagation of microcracks. Multiple stains, in this case chelating fluorochromes, bond to the exposed hydroxyapatite present in microcracks and are visible using fluorescent microscopy ⁽⁴⁸⁾. To track microcrack growth, bone samples are mechanically stressed in cyclic compression to initiate microdamage in several sequential stages. In between stages, the samples are stained with a different chelating fluorochrome in a specific order based on bonding affinity to properly label cracks ⁽⁴⁹⁾. The result is an assortment of

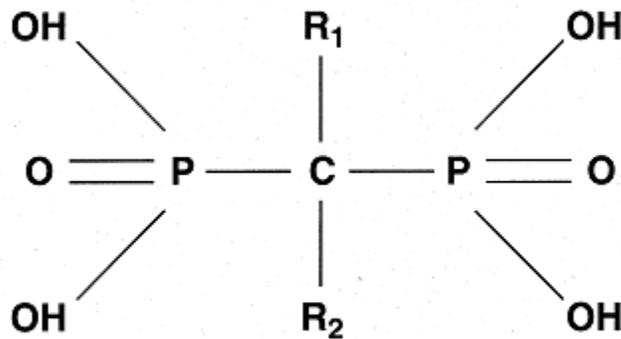
multi-colored microcracks that reveal the shape, time, and direction of crack propagation based on the progression of stain colors.

Thesis Research Goal

Reports of fragility fractures in patients on bisphosphonate treatments raise concerns about this class of drug's long-term safety. The link between microdamage accumulation and bisphosphonate use has mainly been explored in animal models using accelerated treatment dosages, and research using human bone is extremely limited. Therefore, the purpose of this study is to address these prior limitations by studying human bone from patients which used bisphosphonates for actual clinical treatment of varying (0-12 years) durations.

The specific aim of this research is to quantify microdamage in iliac crest bone biopsies of osteoporotic patients to determine if there is an association between microdamage accumulation and long-term bisphosphonate use. The hypothesis is that long-term bisphosphonate use is associated with greater microdamage accumulation compared to short-term and no bisphosphonate use.

Figure 1.1: Basic chemical structure of bisphosphonate.⁽¹²⁾



CHAPTER II: MANUSCRIPT

Microdamage in Bisphosphonate Treated Human Bone.

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Disclosure

The authors state that they have no conflicts of interest.

Summary

Bisphosphonates (BPs) have served as an effective short-term treatment for osteoporosis; however, prolonged BP use may adversely affect bone quality. The goal of this study was to analyze iliac crest bone biopsies from postmenopausal osteoporotic women to test the hypothesis that there is no difference in microdamage accumulation among: 1) patients treated with long-term BP use (≥ 5 yr, $n=15$), 2) patients treated with short-term BP use (< 5 yr, $n=14$), or 3) untreated, age- and turnover-matched osteoporosis patients ($n=11$). Bone samples from each of these three subject populations were stained en bloc with basic fuchsin then sectioned for microdamage analysis by using light and fluorescent microscopy. Microdamage was quantified by measuring microcrack length (Cr.L), density (Cr.D), and surface density (Cr.S.D) and was compared between groups. Cr.D and Cr.S.D were 76% and 87% greater (Cr.D, $p=0.01$; Cr.S.D, $p=0.02$) in the long-term BP group compared to the short-term BP group and were 27% and 29% greater (Cr.D, $p=0.02$; Cr.S.D, $p=0.04$) compared to the control group. No differences in crack length were detected among these groups.

Keywords: bone microcracks, alendronate treatment, anti-resorptive treatment, fragility fractures

Introduction

Bisphosphonates (BPs) have been used for nearly 2 decades as a proven anti-resorptive therapy to treat osteoporosis by reducing osteoclastic activity, thus increasing BMD and reducing fracture risk in women with postmenopausal osteoporosis. This inhibition of bone resorption by osteoclasts is maintained throughout the treatment duration⁽⁵⁰⁾. BP treatment for up to 3-5 years has been shown to effectively reduce fracture risk^(31, 50). Long-term (> 5 years⁽⁹⁾) BP use has been the subject of growing concern in recent years as it may induce oversuppression of bone turnover and impair the biomechanical properties of bone. Reports have surfaced regarding patients experiencing non-traumatic fractures while on BP therapy for 5-12 years⁽¹⁷⁻²¹⁾. In fact, the American Society for Bone and Mineral Research found that 94% of patients with atypical femur fractures had been taking BP treatments, most for over 5 years⁽⁵¹⁾.

Changes in bone microdamage may be partially responsible for the link between long-term BP use and atypical femur fractures. BPs not only reduce bone turnover but they also suppress targeted remodeling essential for repairing microdamage⁽⁵²⁾. Accumulated microdamage as a result of prolonged BP treatment has been shown to reduce canine bone toughness, essential for fracture prevention⁽³⁶⁾. Data exist showing a difference in microdamage in BP-treated patients, but there is no distinction between short- and long-term BP use, and the rate of bone turnover could not be determined in the control subjects⁽⁵³⁾. The present study seeks to improve upon prior studies by comparing long-term BP patients to age-matched and turnover-matched groups of short-term BP-treated patients and untreated control patients.

The specific aim of this research was to determine if the duration of BP treatment is associated with changes in bone microdamage in patients with postmenopausal osteoporosis.

Materials and Methods

Study Design

This laboratory study was designed to compare the effects of long-term BP treatment versus short-term or no BP treatment on microdamage accumulation in human bone from postmenopausal osteoporosis patients by using histological microdamage analysis. Subject groups were matched for age and turnover to eliminate their effects on bone microdamage and isolate only the effects from the duration of BP treatment. BP treatment duration (independent variable) was analyzed as a function of microcrack length, density, and surface density (dependent variables).

Subject Inclusion and Exclusion Criteria

Anterior iliac crest bone biopsies were obtained from low-turnover, postmenopausal, osteoporotic, Caucasian female patients between 41–74 years of age with no history of smoking or diabetes. Most treated patients took Fosamax (alendronate), whereas others took risedronate, ibandronate, or zoledronate. Biopsies were separated into three groups: 11 patients with no previous BP therapy, 14 patients with less than 5 years of BP therapy (2.66 ± 1.1 yr duration), and 15 patients with 5 years or more BP therapy (8.57 ± 2.6 yr duration), with no differences in age or turnover between groups.

Patients were excluded if they had: a diagnosis of osteogenesis imperfecta or other genetic bone disease, osteomalacia, hyperparathyroid bone disease, chronic kidney disease, endocrine abnormalities, diabetes, Paget's disease of bone, malignancies, history of drug or alcohol abuse, teriparatide, SERMs, sex steroids, or any other medications known to alter bone metabolism. The protocol of this IRB approved study adhered to the Declaration of Helsinki.

Specimen Preparation

Bone specimens were previously embedded in MMA and were immersed in 2-methoxyethyl acetate for 3-4 weeks while agitated until the plastic was completely removed. Staining solutions were made from solutions of 1% basic fuchsin (JT Baker,

B660-03, Phillipsburg, NJ) in 80%, 90%, and 100% ETOH and stirred overnight. Each biopsy was stained en bloc using a previously established protocol ⁽⁴⁴⁾ in the following solutions under vacuum:

1. 48h: 70% ETOH
2. 2h: 1% basic fuchsin in 80% ETOH
3. Change solution
4. 2h: 1% basic fuchsin in 80% ETOH
5. Repeat steps 2-4 for 1% basic fuchsin in 90% ETOH
6. Repeat steps 2-4 for 1% basic fuchsin in 100% ETOH
7. Rinse in 100% ETOH to remove excess stain

Stained bone specimens were re-embedded in MMA and then sectioned by using a diamond-bladed band saw (Model 300, EXAKT, Oklahoma City, OK) into 4-5 slices, each 150-300 μ m thick.

Microdamage Assessment

Light and fluorescence microscopy connected to histomorphometry software (OsteoMeasureXP V1.01, OsteoMetrics, Decatur, GA) was used to measure established crack parameters in trabecular bone: crack length (Cr.L, μ m), crack density (Cr.D, cracks/mm²), and crack surface density (Cr.S.D, total Cr.L/mm²). Cr.D shows how many cracks exist within a given bone area, and Cr.S.D quantifies the total lengths of all cracks in that area. The thinnest slice for each specimen was viewed under 200x magnification (Axioplan 2 Imaging, Carl Zeiss, Thornwood, NY) to examine an optical field of view of 485 x 365 μ m. Starting at the center of each specimen, 50 optical fields containing bone were viewed for microdamage analysis totaling 8.85mm² of tissue. Crack number (Cr.N, #) standard deviation stabilized after 50 optical fields, indicating this amount of bone analysis provided an accurate representation of the specimen's microdamage. Stained microcracks were identified by their sharp borders, stain penetration through crack walls, visibility while altering depth of field, and intermediate size being larger than canaliculi but smaller than vascular channels ⁽⁴⁵⁾. Microcracks longer than 30 μ m were recorded because this was the lower limit for reliable microcrack detection ⁽⁴⁹⁾. All measurements were performed by a single observer blinded to specimen group affiliation.

Bone Histology: Activation Frequency

Bone samples were processed without mineral removal and were embedded in methylmethacrylate following tetracycline double-labeling. Serial sections of 4- to 7 μ m thickness were cut and stained with modified Masson-Goldner trichrome. Unstained sections were prepared for fluorescent and polarized light microscopy ⁽⁵⁴⁾. Histomorphometry was done at standardized sites in cancellous bone to obtain activation frequency (Ac.f, cycles/yr).

Statistical Analysis

Data were tested for normality by using the Shapiro-Wilk test. Normally distributed data were analyzed by using the one-way ANOVA test. Non-normally distributed data were analyzed by using Kruskal-Wallis and Mann-Whitney U tests. The Pearson's R test was used to test correlations of normally distributed microcrack parameters with BP duration, age, and Ac.f. The Spearman rank test was used to test correlations of non-normally distributed data. All computations were done by using SPSS version 20 (IBM SPSS Inc, Chicago, IL).

Results

Microcrack density and surface density in bone from patients receiving long-term BP treatment were significantly greater compared to both short-term BP treatment and control groups (*Table 1*). Microdamage parameters were not normally distributed, and thus the median Cr.D in long-term BP treated patients was 76% greater ($p = 0.009$) compared to short-term BP treated patients and 27% greater ($p = 0.016$) compared to controls. Similarly, the median Cr.S.D in long-term BP treated patients was 87% greater ($p = 0.016$) than short-term BP treated patients and 29% greater ($p = 0.040$) than controls. When comparing the control group to short-term BP patients, there were no differences in microdamage parameters. With 80% power at a 95% confidence level, in order to see differences in microdamage parameters between the control and short-term BP groups, the number of subjects in each group must equal 39,470 for Cr.L, 320 for Cr.D, and 1,723

for Cr.S.D. Intraobserver variation accounted for differences of less than 2% in microdamage parameter measurements.

Crack density correlated with BP treatment duration ($\rho = 0.36$, $p = 0.023$, *Fig. 2.1*), whereas Cr.S.D and mean Cr.L showed no correlation to BP duration (*Fig. 2.2, 2.3*). Cr.D was unrelated to the age of the subjects ($\rho = 0.27$, $p = 0.096$, *Fig. 2.4*) or the rate of bone turnover ($\rho = 0.04$, $p = 0.843$, *Fig. 2.5*). The same applies for the Cr.S.D. and mean Cr.L parameters.

Discussion

The two chief findings of this study are that postmenopausal osteoporotic women who took BP for at least 5 years had greater density of microcracks compared to those who took BP for less than 5 years or not at all and that the mean length of these cracks could not be associated with changes in length as a function of BP treatment duration. These results, obtained from an age and turnover-matched population, reveal novel, clinically relevant information about bone microdamage and BP treatment durations. This suggests that prolonged BP use is associated with mechanisms responsible for crack initiation, but perhaps not propagation. Although microdamage has been shown to weaken bone tissue ⁽³⁶⁾, the inability to observe a change in mean crack length may indicate that these microcracks, regardless of density, are not propagating and coalescing. These findings add new information regarding bone microdamage and BP treatment duration.

Few microdamage studies of human bone treated with BPs are reported in the literature. A prior study compared BP patients to an unmatched control group composed of cadaver specimens with unknown clinical histories and revealed that more than half of the treated and control specimens contained no microdamage ^(53, 55). Although it claims BP has no effect on microcrack frequency, the ability of that study to verify the relationship between BP use and microdamage remains unclear. Another study showed increased microdamage accumulation in women treated with alendronate for an average of 5 years ⁽⁴³⁾. Both the increase in crack surface density and unchanged crack length

resulting from BP use agree with the findings of the present study. An additional study also supports the present finding that BP treatment had no effect on crack length as previously stated ⁽⁴²⁾. The present study is the only human study to compare microdamage from prolonged BP therapy to short-term BP therapy as well as no-BP controls. The addition of the short-term BP group may help further identify the deleterious effects of long-term BP on bone health and help better distinguish a safer duration of BP use for treating postmenopausal osteoporosis.

The fact that only the long-term treatment group had greater microcrack density and surface density is a significant finding because it conveys that the increase in microdamage associated with BP treatment is not present in most patients who took BPs for under 5 years. This could be attributed to the increased BMD that is associated with short-term treatments before extensive microdamage initiation occurs, effectively increasing crack density and surface density.

Since there are no differences in turnover or age between groups, the increased microdamage density observed in long-term BP treated patients may be attributed to an intrinsic effect of BP promoting microcrack initiation instead of only the suppression of turnover. One possibility is the accumulation of AGEs (advanced glycation end-products) that result from non-enzymatic collagen cross-linking induced by BP treatment ^(55, 56). AGEs have been shown to increase the brittleness of bone tissue ⁽⁵⁷⁾ and may contribute to the reported reduced bone toughness associated with BP treatment ^(42, 58). Increased microcrack density may result from this AGE-accumulation and subsequent induced bone embrittlement since it increases the potential for microcracks to initiate ⁽⁵⁵⁾.

This study is limited to BP treatment duration related changes in cancellous bone microdamage; microdamage measurements were not made in cortical bone due to the inability of the stain to penetrate to the same degree. Also, not all biopsy specimens contained sufficient quantities of cortical bone. Furthermore, this study did not focus on a particular type of BP since the specific BP drug used varied among treated patients. It is worthy to note, however, that 79% of the treated study subjects used Fosamax (alendronate). More work is needed to determine if the results reported are equally applicable for each particular BP on the market.

In conclusion, compared to untreated controls or those treated for short durations (< 5 years) with BP, long-term (≥ 5 years) BP treatment was associated with increased microcrack density in age- and turnover-matched groups of postmenopausal osteoporotic women. No evidence was obtained linking significant increases in microcrack length to bisphosphonate treatment duration.

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Authors' Roles

Study design: HHM, DP

Study conduct: WAC

Data collection: WAC

Data interpretation: all authors

Integrity of data analysis: all authors

Drafting manuscript: WAC

Revision of manuscript content: all authors

Approving final version of manuscript: all authors

Table 2.1: Subject Data and Microdamage Parameters

	Control (No BP) (n = 11)	Short-term BP (<5 yr) (n = 14)	Long-term BP (≥5 yr) (n = 15)
Age	53 (49 – 70)	60 (41 – 74)	62 (54 – 73)
Ac.f (cycles/yr)	0.09 (0.02 – 0.26)	0.11 (0.04 – 0.48) ^a	0.14 (0.03 – 0.37) ^b
BP treatment duration (yr)	0 (±0)	2.63 (±1.2)*	8.57 (±2.6)*†
Microdamage Parameters			
Mean Cr.L (μm)	67.9 (±11.0)	68.2 (±12.9)	69.2 (±9.9)
Cr.D (#/ mm ²)	3.74 (1.78 – 5.38)	2.22 (1.23 – 7.38)	4.94 (2.81 – 7.06)*†
Cr. S.D (total Cr.L μm/ mm ²)	263 (101 – 372)	140 (76.5 – 607)	355 (184 – 528)*†

Parametric data expressed as *mean(±SD)*.

Non-parametric data expressed as *median(min-max)*.

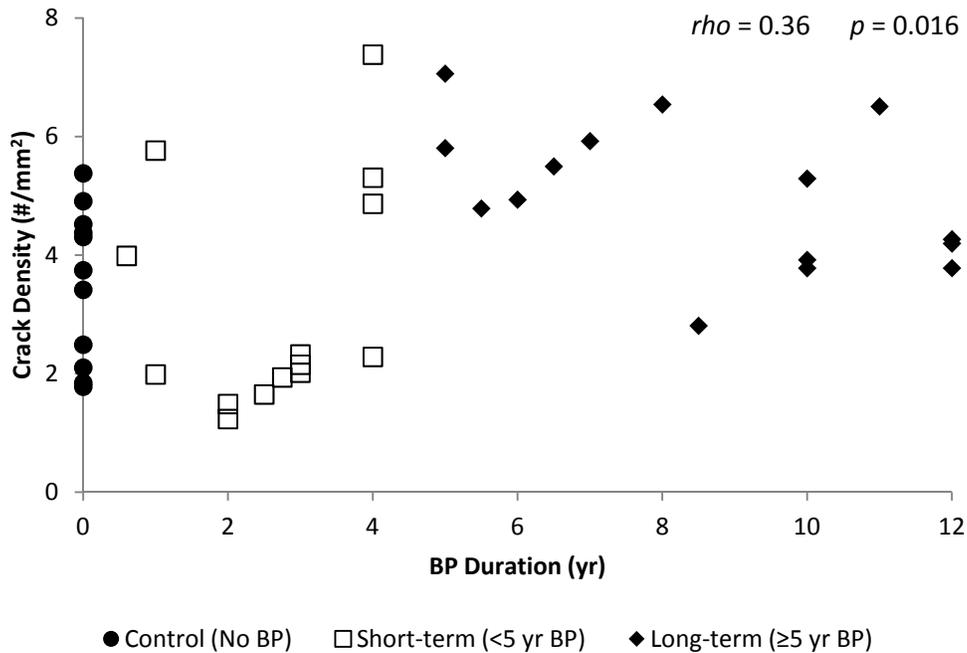
**p* < 0.05, compared to control group.

†*p* < 0.05, compared to short-term group.

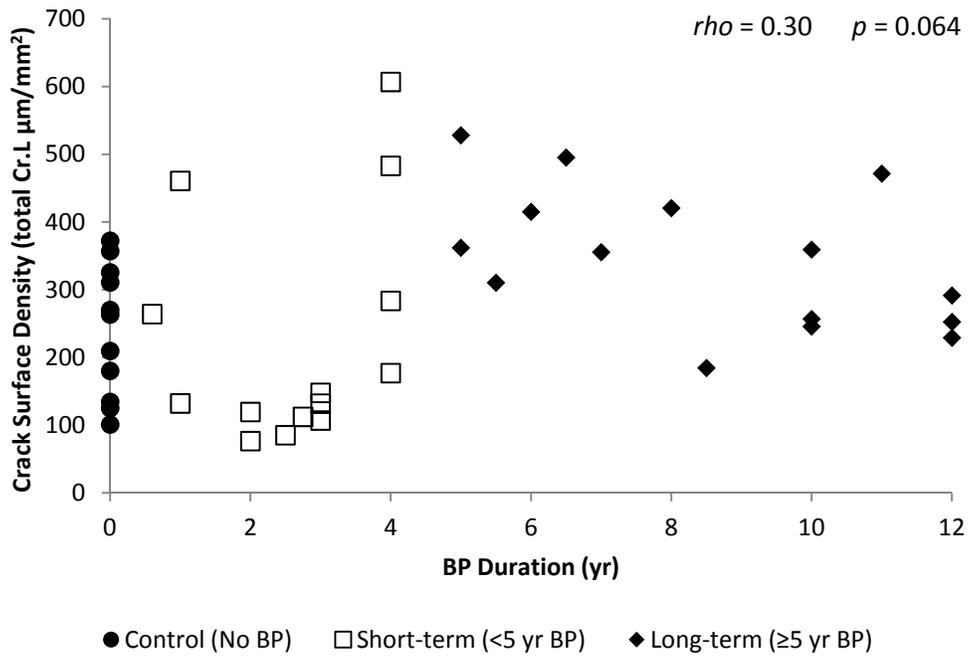
^a n = 10; ^b n = 11

Figures 2.1 – 2.3: Correlations between microdamage parameters and BP duration show a significant relationship between Cr.D and BP duration but not between Cr.S.D or Cr.L and BP duration.

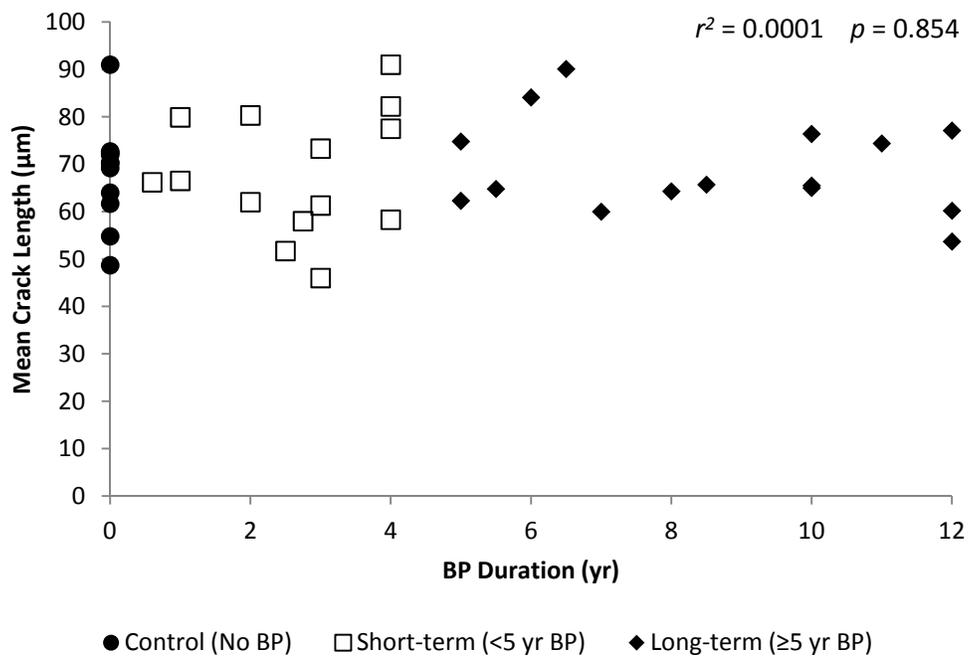
2.1



2.2

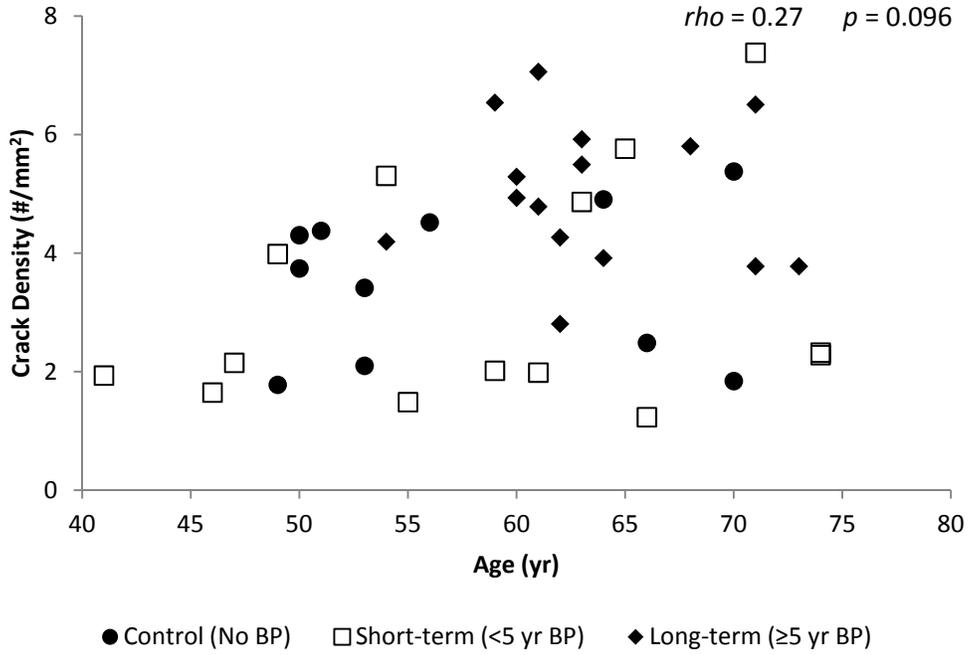


2.3

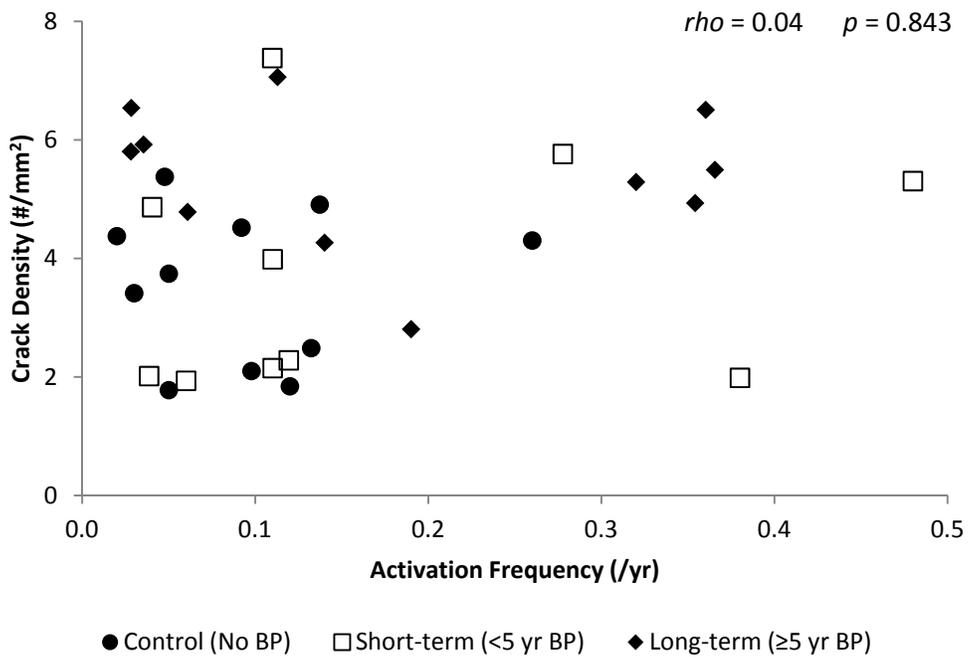


Figures 2.4 – 2.5: Neither age nor Ac.F show a strong relationship with Cr.D.

2.4



2.5



CHAPTER III: CONCLUDING REMARKS

Importance

The present study is important due to its novel assessment of bone microdamage in human bone from women treated with bisphosphonates for short and long durations (up to 12 years), and the age- and turnover-matching between groups, including untreated controls. Age and turnover matching was significant because it allowed their effects on microdamage accumulation to be eliminated and the effects of bisphosphonate treatment duration to be isolated. Significant changes in microdamage accumulation following 5 or more years of treatment imply a distinct change in bone quality associated with prolonged bisphosphonate exposure that allows for easier microcrack initiation.

Although the present study shows long-term bisphosphonate therapy is associated with greater microdamage accumulation in postmenopausal women, there is no evidence that such microdamage is associated with the reported atypical fractures. However, studies show microdamage can reduce the mechanical properties in bone, although the exact quantity of microdamage that induces clinically-relevant amounts of bone fragility is still unknown and requires further work to establish.

Limitations

This research focused only on microdamage in trabecular bone instead of cortical bone, which could be a possible limitation since atypical femur fractures occur within primarily cortical bone tissue. On the other hand, bisphosphonate-induced microdamage accumulation in trabecular bone may not be dissimilar to microdamage accumulation in cortical bone. For example, previous studies have shown cortical microdamage accumulated in dogs when treated with bisphosphonate ^(36, 61). Trabecular microdamage has actually been shown to initiate before cortical microdamage. In a study that loaded rat vertebrae in axial fatigue tests, trabecular microdamage initiated and propagated before microdamage in the cortical shell ⁽⁶²⁾. If atypical fractures are indeed influenced

by cortical microdamage, perhaps they are preceded by trabecular microdamage accumulation that contributes to diminishing the structural integrity of bone.

Future Directions of Research

The future of microdamage research might focus on the mechanisms behind microcrack initiation, or lack of propagation as influenced by long-term bisphosphonate use, for the purpose of revealing details about its possible direct or indirect causality. In order to investigate the effect of bisphosphonate on microcrack initiation, bone specimens from animals treated with alendronate were fatigue loaded and compared to untreated specimens. Alendronate-treated bone contained significantly more microdamage than the untreated bone after identical fatigue loading ⁽⁶³⁾. This agrees with the current study's finding that bisphosphonate treatment is associated with the presence of increased microdamage. Similarly, it has been shown that one year of alendronate in dogs reduced trabecular bone's ability to resist loading-induced severe and linear microcrack formation ⁽⁶⁴⁾. Although these studies show bisphosphonate therapy alters bone's ability to resist microdamage formation, the mechanism behind the interaction of bisphosphonate and bone that permits microcracks to form is not yet understood.

It has already been shown that the propagation of microcracks can be tracked using multiple stains between mechanical fatigue loading sessions ⁽⁴⁸⁾, but no studies have monitored crack propagation in bisphosphonate-treated bone with this method. Work has been done using finite element methods to assess crack growth in cortical bone microstructures and learn what factors affect it, such as cement lines, osteon strength, and fracture toughness ⁽⁶⁵⁾. Future finite element analyses could help expand the working knowledge of crack mechanics in bone, especially in trabecular bone or specimens treated with bisphosphonate.

A more comprehensive approach to bisphosphonate-induced microdamage research would be the inclusion of material and mechanical property analyses to obtain a better understanding of the mechanisms behind the association between bisphosphonates and microdamage accumulation. In addition to histology and microdamage detection, the

same bone specimens from long-term bisphosphonate-treated, short-term, and untreated patients should be analyzed with nanoindentation and Fourier transform infrared spectroscopy (FTIR) to gain insight on their individual mechanical and material properties. Uncovering links between prolonged bisphosphonate use, microdamage, collagen cross-linking, mineralization, fracture toughness, and modulus, for example, in clinically relevant human bone would offer novel information.

Conclusion

Long-term bisphosphonate use is associated with significantly more microdamage accumulation, i.e. greater crack density and greater crack surface density, compared to short-term bisphosphonate use and untreated patients. No evidence was obtained linking significant increases in microcrack length to bisphosphonate treatment duration.

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