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# Alendronate treatment alters bone tissues at multiple structural levels in healthy canine cortical bone

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ABSTRACT

Bisphosphonates are widely used to treat osteoporosis, but have been associated with atypical femoral fractures (AFFs) in the long term, which raises a critical health problem for the aging population. Several clinical studies have suggested that the occurrence of AFFs may be related to the bisphosphonate-induced changes of bone turnover, but large discrepancies in the results of these studies indicate that the salient mechanisms responsible for any loss in fracture resistance are still unclear. Here the role of bisphosphonates is examined in terms of the potential deterioration in fracture resistance resulting from both intrinsic (plasticity) and extrinsic (shielding) toughening mechanisms, which operate over a wide range of length-scales. Specifically, we compare the mechanical properties of two groups of humeri from healthy beagles, one control group comprising eight females (oral doses of saline vehicle, 1 mL/kg/day, 3 years) and one treated group comprising nine females (oral doses of alendronate used to treat osteoporosis, 0.2 mg/kg/day, 3 years). Our data demonstrate treatment-specific reorganization of bone tissue identified at multiple length-scales mainly through advanced synchrotron x-ray experiments. We confirm that bisphosphonate treatments can increase non-enzymatic collagen cross-linking at molecular scales, which critically restricts plasticity associated with fibrillar sliding, and hence intrinsic toughening, at nanoscales. We also observe changes in the intracortical architecture of treated bone at microscales, with partial filling of the Haversian canals and reduction of osteon number. We hypothesize that the reduced plasticity associated with BP treatments may induce an increase in microcrack accumulation and growth under cyclic daily loadings, and potentially increase the susceptibility of cortical bone to atypical (fatigue-like) fractures.

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## 1. Introduction

More than 200 million prescriptions have been dispensed worldwide for oral bisphosphonates (BPs) [1] since the first bisphosphonate approved for treatment of osteoporosis, alendronate, was introduced into the market in 1995. Indeed, bisphosphonate treatments for osteoporosis have been definitively associated with reduced fracture risk [2]. However, long-term adverse effects of the treatment started to emerge in 2005, specifically with atypical femoral fractures [3–6], osteonecrosis of the jaw [7] and esophageal cancer [8,9] all being reported for long-term users of BPs. With a rate of atypical femoral fractures (AFFs) of about 1/1000 per year for a patient on bisphosphonate, the incidence of AFFs remains low compared to the reduction in incidence of any fracture occurring under bisphosphonate treatment, rated at 15/1000 per year [10]; however, the morbidity is high with AFFs because of the catastrophic nature of the fracture and delayed healing. These considerations potentially pose critical health problems for the aging population, which prompted the American Society for Bone and Mineral Research (ASBMR) to appoint a Task Force to conduct a major review on AFFs [6,11]. In response to this review, the Food and Drug Administration in 2010 required a warning label for bisphosphonates indicating the potential risk for AFFs and mandated further investigation into bisphosphonate-associated problems.

Reducing fracture risk and maintaining bone quality is of utmost importance to bone health in aging, osteoporosis, and treatments for bone disease. Bone derives its unique stiffness, strength, and toughness from

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its hierarchical arrangement of characteristic structural features spanning molecular to macroscopic length-scales. Indeed, fracture resistance in general originates at multiple length-scales; at nanoscale dimensions, intrinsic toughening mechanisms resist the initiation and growth of cracks primarily *via* plasticity acting ahead of a growing crack, whereas at microscale dimensions, extrinsic toughness mechanisms act to impede the crack growth primarily by crack-tip shielding from crack deflection and bridging [12].

Studies addressing the mechanical properties of bisphosphonate-treated bone suggest that suppressing bone turnover decreases fracture risk by improving bone mass, *i.e.*, bone quantity. Although the majority of this work has been performed on trabecular bone, the site-specific nature of bisphosphonates and the emergence of atypical femur fractures have changed the focus of bisphosphonate research towards cortical regions. Indeed, in cortical bone, the major concern is that suppression of remodeling associated with long-term bisphosphonate use could have detrimental effects on cortical bone quality and toughness at microscopic and submicroscopic levels [13].

Long-term bisphosphonate use could have measurable effects on cortical bone structure and mechanical properties at multiple length-scales. At microscales, where extrinsic toughening in cortical bone primarily involves crack deflection[1] at the boundaries ("cement lines") of the osteons (*i.e.*, longitudinal structures with a central Haversian canal consisting of a blood vessel and nerves), BPs have a tendency to change the mineralization of the matrix [14–16], which could affect fracture risk and correspondingly reduce the contribution to fracture resistance from extrinsic toughening leading to easier crack propagation [13,17–19].

At the nanoscale level, collagen molecules and nanocrystal platelets are the basic building blocks forming mineralized collagen fibrils of bone. Fibrils are arranged in arrays and organized in fiber patterns comprising the lamellar structure of the osteons. At this level, fibrillar sliding at the interface between mineralized collagen fibrils and the extrafibrillar matrix represents a major source of plasticity in bone; it is an intrinsic toughening mechanism that promotes energy dissipation and forms plastic zones ahead of a growing crack, thereby blunting the tip of any growing cracks. As an unintended consequence of the decrease in bone remodeling, anti-resorptive agents also increase the proportion of advanced glycation end products (AGEs), which have been shown to non-enzymatically cross-link collagen and to reduce post-yield properties and toughness of bone by altering the formation and propagation of microdamage [21,22].

A better understanding of the effects of long-term bisphosphonate use on fracture resistance could provide clues as to whether bisphosphonates are directly linked to AFFs. The incidence of AFF cases is quite small, which makes it difficult to identify whether they are primarily associated with untreated or BP-treated patients [23–26] or whether BPs increase the risk for AFFs [6,11,27,28]. Thus, the prime concern of this study is to better understand whether BPs cause changes to the bone structure that could make the bone more susceptible to atypical femoral fracture. Indeed, AFFs associated with bisphosphonate use are thought to be insufficiency stress fractures, *i.e.*, a type of fatigue fracture caused by repeated daily loading of bone tissue. AFFs present a unique pattern of transverse or short oblique fracture with a smooth fracture surface, commonly seen in fatigue fractures [29]. Recent studies suggest that more homogeneity of the bone-matrix may be a possible explanation in the case of AFFs [13,18], where less deflected crack paths would result in the reported smoother fracture surfaces.

The animals examined here were already extensively studied by Burr and co-workers [22,30–32] to document changes associated with BP treatment in bone from dogs, which present similarities with human bone in their intra-cortical remodeling rates. Mechanical properties were reported in canines following 1 or 3 years of alendronate treatment at clinical doses (for postmenopausal osteoporosis) or high doses (five time greater than the clinical dose). Long-term (3 years) alendronate treatment was shown to reduce the work-to-fracture (toughness) in ribs and vertebrae [30,31] by nearly 30% at a clinical dose without significantly affecting the elastic properties of the material. Suppression of bone turnover increases the mineral content and the collagen maturity in trabecular bone [33]. Reports on cortical bone from femurs and tibiae of these animals concluded that no significant differences were found in femoral mechanical properties even at high doses [32] whereas in tibiae, post-yield work-to-fracture was significantly reduced in cortical bone at high doses (not at clinical doses) compared to control after just one year of treatment [22]. BPs are likely to affect more significantly, and in a shorter period of time, bone properties in trabecular bone where bone turnover is higher compared to cortical bone [14].

The novelty of this study lies in the combination of multiple high-resolution mechanical and structural characterizations to assess the effect of alendronate treatment across the complex multidimensional structure of cortical bone ranging from molecular to microlevels. Humeri were chosen to perform this study because, in the absence of femurs to tests, this long bone in dogs is the most similar to the femur in terms of work to fracture and cross-sectional shape [34].

Understanding the effects of bisphosphonates on cortical bone quality and fracture risk are critical issues in bone health, which should improve our understanding of atypical fractures. As studies have yet to determine the effects of long-term bisphosphonate treatments on the structural and mechanical quality of cortical bone across multiple length-scales, our intent in this study was to isolate the effects of bisphosphonates from that of osteoporosis which is well known to decrease the resistance of bone to fracture. Here we investigate the effect of BPs on cortical bone from the humeri of skeletally mature beagle dogs that do not have osteoporosis, thus separating the effects of BPs from those of underlying skeletal disease. The goal of this paper is not to trigger AFF since we are working with bone from healthy young dogs but to understand the potential effects and participation of BPs on the deterioration of bone quality that might contribute to AFFs in BP-treated osteoporotic bone under daily fatigue loadings. We use advanced x-ray synchrotron instrumentation, specifically involving computed tomography and small-/wide-angle x-ray scattering/diffraction to examine the mechanical properties at multiple length-scales in uniform groups of control dogs and dogs treated with alendronate doses typically given to osteoporotic women. Our data reveal the reorganization of canine bone tissue following BP treatment with corresponding effects on bone toughness, principally originating from changes in the collagen environment affecting bone plasticity at different structural levels and changes in osteonal density and size of the Haversian canals.

## 2. Materials and methods

### 2.1. Study design

An analytic experimental study was used to quantify the potential effects of long-term BPs on bone quality. To this end, bone characterization is compared at multiple hierarchical levels between two parallel groups (two independent variables): a control group treated for 3 years with oral doses of saline vehicle (1 mL/kg/day) and a BP-treated group treated for 3 years with daily oral doses of alendronate corresponding to the doses used to treat osteoporotic women (0.2 mg/kg/day) (see experimental details in Ref. [31]). The sample comprises canine bones from 8

[1] Whereas microcrack formation and consequent crack deflection at the osteonal structures provide the primary mechanism of (extrinsic) toughening for cracks in bone propagating in the transverse direction, for cracking in the longitudinal (splitting) direction, the intact regions between these microcracks can act as "uncracked ligament" bridges across the crack surfaces; these features further provide extrinsic toughening by carrying load that would otherwise be used to further propagate the crack, *e.g.*, refs. [19,20].