ELSEVIER

Bone 39 (2006) 318–324

BONE

www.elsevier.com/locate/bone

Chemical makeup of microdamaged bone differs from undamaged bone

Meghan E. Ruppel [a], David B. Burr [b], Lisa M. Miller [a,*]

[a] National Synchrotron Light Source, Brookhaven National Laboratory, Bldg. 725 D, Upton, NY 11973, USA
[b] Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, IN 46202, USA

Received 10 August 2005; revised 29 January 2006; accepted 3 February 2006
Available online 11 April 2006

Abstract

Microdamage naturally occurs in bone tissue as a result of cyclic loading placed on the body from normal daily activities. While it is usually repaired through the bone turnover process, accumulation of microdamage may result in reduced bone quality and increased fracture risk. It is unclear whether certain areas of bone are more susceptible to microdamage than others due to compositional differences. This study examines whether areas of microdamaged bone are chemically different than undamaged areas of bone. Bone samples (L3 vertebrae) were harvested from 15 dogs. Samples were stained with basic fuchsin, embedded in poly-methylmethacrylate, and cut into 5-μm-thick sections. Fuchsin staining was used to identify regions of microdamage, and synchrotron infrared microspectroscopic imaging was used to determine the local bone composition. Results showed that microdamaged areas of bone were chemically different than the surrounding undamaged areas. Specifically, the mineral stoichiometry was altered in microdamaged bone, where the carbonate/protein ratio and carbonate/phosphate ratio were significantly lower in areas of microdamage, and the acid phosphate content was higher. No differences were observed in tissue mineralization (phosphate/protein ratio) or crystallinity between the microdamaged and undamaged bone, indicating that the microdamaged regions of bone were not over-mineralized. The collagen cross-linking structure was also significantly different in microdamaged areas of bone, consistent with ruptured cross-links and reduced fracture resistance. All differences in composition had well-defined boundaries in the microcrack region, strongly suggesting that they occurred after microcrack formation. Even so, because microdamage results in an altered bone composition, an accumulation of microdamage might result in a long-term reduction in bone quality.

Keywords: Microdamage; Composition; Bisphosphonates; Infrared microspectroscopy

Introduction

Microdamage occurs in bone tissue as a result of cyclic loading placed on the body during daily activities [11,32,42,67]. Normally, the bone remodeling process specifically targets these microcracks for repair [4,9,42]. Current anti-resorptive bisphosphonate treatments for osteoporosis allow the microdamage burden to increase [33] because these drugs reduce targeted bone remodeling that would normally repair the damage [32]. The increased accumulation of microdamage may also be the result of increased tissue mineralization [7,12] that occurs secondary to remodeling suppression and that may make the bone more brittle. However to date, little is known about the microscopic tissue composition where microdamage occurs and whether certain areas of bone are more susceptible to microdamage than others due to compositional differences.

Bone is made up of organic and mineral components, mainly type I collagen and nanocrystalline biological apatite [65]. The exact structure of the apatite mineral is not well defined because substitutions into the mineral apatite lattice can occur in the cationic calcium sites (e.g., Na^+, K^+, Fe^{2+}, Zn^{2+}, Sr^{2+}, Mg^{2+}, Pb^{2+}) and anionic phosphate sites (e.g., F^-, Cl^-, HPO_4^{2-}, CO_3^{2-}). These substitutions can affect bone's chemical and physical properties such as solubility, density, hardness, and growth morphology [45,65]. Bone composition has been shown to change with age [22,48] and is also affected by disease [6,14,15,24,38,39,41,47] and treatment for disease [6,7,12,23].

Infrared (IR) spectroscopy is a valuable technique for examining bone composition because it is sensitive to both the protein and mineral component of bone [56,57]. By putting

Abbreviation: IR, infrared.

* Corresponding author. Fax: +1 631 344 3238.
E-mail address: lmiller@bnl.gov (L.M. Miller).

8756-3282/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bone.2006.02.052

infrared light through a microscope, bone composition can be studied with a spatial resolution of 30–50 μm [18,48,49]. Spatial resolution can be improved to the diffraction limit (3–15 μm) using a synchrotron infrared source [23,36,37,40,41].

The goal of this study was to examine whether areas of microdamaged bone are chemically different than undamaged areas and could therefore be predisposed to sustaining damage. By using infrared microspectroscopy, the mineral and protein content and stoichiometry were examined in regions of microdamage and the surrounding undamaged areas. Because the microcracks were less than 10 μm wide, a synchrotron infrared source was necessary to improve the spatial resolution of the technique.

Materials and methods

Experimental design

A detailed description of the experimental design has been previously presented [33]. For this study, a subset of fifteen beagle dogs ranging in age from 1 to 2 years old was examined. The dogs were equally divided among those that received a daily saline injection, 0.5 mg/kg/day of oral risedronate, or 1.0 mg/kg/day of oral alendronate for 12 months. These doses were 5–6 times higher than those administered to osteoporosis patients. The dogs were fed normal dog chow and provided with water ad libitum. They were kept separate in environmentally controlled rooms for the length of the study as well as 1 month prior to the start of the study. After 12 months of treatment, the dogs were sacrificed using a high dose of sodium pentobarbital (0.22 ml/kg i.v.), and the L3 vertebrae were removed.

Tissue preparation

The L3 vertebrae were bulk stained with basic fuchsin, embedded in polymethyl methacrylate, and then cut into 5-μm sections with a sledge microtome fitted with a tungsten carbide knife. Bulk staining with basic fuchsin prior to embedding and histological sectioning enables visualization of microdamage and differentiation between microdamage and artifactual cracks induced during histological preparation [10,13,27].

Infrared microspectroscopy

The process of IR microspectroscopy performed on bone has been previously described [23,39–41]. In short, the bone sections were supported between two aluminum disks (13 mm in diameter) that each contained a narrow slit (9 mm × 3 mm). The microdamaged area of bone and the surrounding undamaged area were visible through the center of the slit. Microdamaged areas were defined as those that stained positive (i.e., purple) with basic fuchsin. Undamaged areas were defined as the surrounding unstained areas. Microcracks were approximately 3–10 μm in width and 20–100 μm in length. Regions for IR imaging were defined as a rectangular area to include the microcrack and the surrounding undamaged tissue within a radius of approximately 200 μm from the microcrack. This area of analysis is larger than the area analyzed by Verborgt et al. [63] and reflects the finding that microcracks alter fluid flow velocity around the crack for a distance of up to 180 μm [21].

IR microspectroscopy was performed using a Thermo Nicolet Continuμm infrared microscope coupled to a Nicolet Magna 860 FTIR (Thermo Nicolet Instruments, Madison, WI, USA). Infrared light from the National Synchrotron Light Source Beamline U10B, Brookhaven National Laboratory (Upton, NY) was used. An MCT-B detector, cooled to 77 K, was used for its extended infrared sensitivity from 4000 and 500 cm^{-1}, which was necessary for imaging the ν_4 phosphate band from 500–650 cm^{-1}.

The sample was mounted on a motorized microscope stage and raster scanned through the synchrotron IR beam (15 μm in diameter), collecting a grid-like pattern of IR spectra spaced in 10-μm increments. Spectra of the bone were collected in transmission mode using confocal 32× Schwarzschild objectives. The spectral resolution was 4 cm^{-1}, and 256 scans were accumulated per point using Atlμs software (Thermo Nicolet Instruments). For each IR image, 200–1000 spectra were collected in absorbance mode, where the background spectrum was collected from an empty sample holder. The resulting IR map included spectra from both the undamaged and microdamaged areas, which were separated after data collection based on fuchsin staining.

For each animal, IR microspectroscopic images were collected from 2–3 areas of microdamage in the L3 vertebrae, including the surrounding undamaged area.

Infrared data analysis

For each spectrum, a linear baseline correction was first performed to account for changes in synchrotron beam current. Protein and mineral contents were analyzed using the area under the amide II protein band (1595–1510 cm^{-1}), the ν_4 phosphate band (500–650 cm^{-1}), and the ν_2 carbonate band (905–825 cm^{-1}). The integration results were expressed as ratios (phosphate/protein, carbonate/protein, and carbonate/phosphate) to account for any variations in sample thickness. Crystallinity was determined as a peak height ratio of 603/563 cm^{-1} [41]. Acid phosphate content was calculated as a ratio of acidic phosphate (538 cm^{-1}) over the peak area of the ν_4 phosphate band [41]. Collagen cross-link structure was analyzed as a peak height ratio of 1660/1690 cm^{-1} [51].

In a small number of instances (<10%), microcrack spectra contained embedding medium, indicating a space in the tissue where the microcrack formed. These spectra were removed from the data analysis. Animals from all treatment groups were pooled for comparison of microdamaged versus undamaged areas because previous studies showed that treatment with these agents and doses did not affect tissue composition significantly [12]. A mean ± standard deviation (SD) was determined for each bone composition parameter. Independent *t* tests were performed between the microdamaged and undamaged areas for each bone parameter using Prism 3 (GraphPad). Statistical differences were considered significant for $P < 0.05$.

Results

Fuchsin-stained microcracks could be easily identified from optical images (Fig. 1A). In the IR data, the absorption intensities of both the protein and mineral peaks in the microdamaged areas were slightly decreased compared to surrounding undamaged areas. This was observed because the formation of a microcrack causes the collagenous matrix to be stretched and sometimes broken and mineral crystallites to be lost in the immediate area of the crack [43]. In fact, the exposed collagen fibers and charged mineral ions at the surface of the microcrack are the mechanism behind the binding of basic fuchsin [13,27].

All data were plotted as ratios to account for loss of material in the microdamaged regions and any other thickness variations in the sample. IR imaging easily discriminated the carbonate/phosphate (Fig. 1A), carbonate/protein (Fig. 1B), acid phosphate content (Fig. 1C), and collagen cross-linking (Fig. 1F) in the area of the microcrack from regions of bone surrounding the crack. Statistical analysis of these parameters for all samples was consistent with the IR images.

The amount of carbonate in the microdamaged areas, normalized to protein or phosphate content, was consistently lower than in the undamaged areas (Table 1; Figs. 2A and B). However, since the data are plotted as a ratio to account for sample thickness variations, it is not certain whether the decreased ratio in the microcracks is due to higher carbonate or