

Brief communication

Measurement of fracture callus material properties via nanoindentation

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Abstract

In bone fracture healing, the extent to which the injured bone regains stability and strength depends on the mechanical properties of the tissues that are formed during healing. While many techniques have been used to quantify the overall mechanical behavior of fracture calluses, few data exist on the material properties of individual callus tissues. The overall goal of this study was to quantify these material properties. Nanoindentation was performed at multiple locations across thin (200 μm), longitudinal sections of rat fracture callus at 35 days post fracture. Following indentation, sections were stained with alizarin red S and alcian blue to obtain semi-quantitative estimates of tissue mineral content and proteoglycan content, respectively. Indentation moduli varied over three orders of magnitude (0.61–1010 MPa) throughout the callus. Much of this variation was due to the presence of multiple tissue types: the indentation moduli of granulation tissue, chondroid tissue and woven bone ranged 0.61–1.27 MPa (median = 0.99 MPa), 1.39–4.42 MPa (median = 2.89 MPa) and 26.92–1010.00 MPa (median = 132.00 MPa), respectively. In regions of alizarin red staining, the indentation modulus was correlated ($r = 0.62$, $P = 0.04$) with stain intensity, suggesting a positive correlation between modulus and mineral content in woven bone. In addition, the indentation modulus of woven bone along the periosteal aspect of the cortex increased with distance from the fracture gap ($P = 0.004$). These results demonstrate the usefulness of nanoindentation in characterizing the elastic properties of the heterogeneous mixture of tissues present in bone fracture callus.

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1. Introduction

In bone fracture healing, the extent to which the injured bone regains stability and strength depends on the mechanical properties of the tissues that are formed during healing. Fracture healing is a regenerative process that consists of several phases, each involving the formation of a different type of tissue [1]. In the initial phase, the inflammatory response results in the formation of a hematoma and granulation tissue. The second phase is characterized by the formation of a soft callus consisting of cartilaginous or

chondroid tissue, while the third phase involves ossification of the soft callus to form a hard or bony callus consisting primarily of woven bone tissue. Finally, the callus enters the remodeling phase, during which the woven bone is gradually replaced by lamellar bone tissue. Although these four phases are temporally sequential, the healing process is not spatially uniform. Thus, at any given time during healing, the fracture callus consists of a highly heterogeneous mixture of tissues. Further, factors such as disease [2,3] and drug treatment [4–8] can alter one or more of the phases of healing, leading to differences in composition and distribution of tissues throughout the callus. Quantifying callus tissue material properties and the relationships between these properties and the tissue composition, therefore provides a means of linking the underlying biology of the repair process to the gradual regaining of mechanical function experienced by the healing bone.

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In contrast to the wealth of literature on the mechanical behavior of fracture calluses, few data are available on the material properties of individual callus tissues [9]. Indeed, callus tissue material properties are often estimated based on information regarding tissue composition or on the material properties of similar tissues [10,11]. Nanoindentation is a promising tool for probing callus tissue properties, because of the high spatial resolution that this technique affords. With typical indent depths $<5\ \mu\text{m}$ and contact areas on the order of $\sim 10\text{--}100\ \mu\text{m}^2$, nanoindentation is well suited for studying fracture healing in small animals such as rats and mice. Moreover, the length scale of the indents is comparable with that of individual cells, suggesting that the resulting measurements reflect aspects of the local, cellular mechanical environment. Many studies to date have demonstrated the use of nanoindentation on skeletal tissues, including bone [12–17] and cartilage [18–22]. Together, these studies have indicated that nanoindentation can be used to detect changes or differences in tissue properties due to disease or genetic variations [16,23–28], to measure regional variations in tissue properties [12,18,29], and to relate tissue stiffness to mineral content [21,30].

The high spatial resolution of nanoindentation and the potential for relating indentation measurements to tissue composition suggest strongly that this testing method can be used to track the spatial and temporal progression of fracture healing. This progression has been well characterized through histological analyses with respect to the distribution of tissues present in the callus at various time points during healing. For example, intramembranous ossification, or direct bone formation, occurs along the periosteal surface of the cortex, progressing along this surface from points remote to the injury towards the fracture gap. Within and just outside the fracture gap, however, endochondral ossification—bone formation that occurs via a cartilage intermediary—predominates. The extent to which these patterns of bone formation are reflected in regional variations in callus tissue properties is not known and yet is highly relevant for defining the mechanisms by which the stiffness and strength of the bone are restored.

The overall goal of this study was to characterize the material properties of fracture callus tissues. Thin serial sections of rat fracture callus were prepared, and nanoindentation was performed at multiple locations across each section. The specific objectives were (1) to quantify the elastic properties of various tissues present in the callus; and (2) to compare the measured elastic properties with histological assessments of the types and composition of tissues present.

2. Materials and method

2.1. Harvest of callus and sample preparation

All animal care and experimental procedures were in compliance with NIH guidelines and the authors' institu-

tion's Animal Care and Use Committee. A closed, un stabilized fracture was created in the mid-diaphysis of the right femur in a retired breeder, male Sprague–Dawley rat (weight $\sim 500\ \text{g}$; age ~ 5 months) according to an established protocol [31]. The animal was killed 35 days after the fracture was created, and the harvested femur was stored frozen at $-20\ ^\circ\text{C}$ in PBS-soaked gauze until preparation for nanoindentation.

To prevent damage or alterations to the tissue properties, no polishing, formalin fixation, decalcification or paraffin embedding was involved in any of the sample preparation phases. The entire length of the portion of the femur containing the callus was excised using a dental saw. The sample was mounted on the freeze-stage of a sliding microtome (HM 450 Richard Allan, Kalamazoo, MI) and embedded in freezing medium (Histo Prep, Fisher Scientific, Pittsburgh, PA). A technique was developed in the laboratory to provide additional support to the callus tissues during sectioning. Briefly, a thin polyester membrane was coated with freezing medium and placed on the exposed surface of the callus. The membrane supported the entire face of the section during cutting, thus allowing the overall morphology of the callus to be kept intact in each $200\ \mu\text{m}$ thick section. Each section was mounted on a microscope slide using a small amount of cyanoacrylate glue, and the membrane was removed easily from the top surface of the section once the freezing medium had melted. Excess freezing medium was washed away with abundant distilled water.

2.2. Nanoindentation

All nanoindentation experiments were performed at room temperature using a Hysitron Triboindenter (Minneapolis, MN, USA) and a $50\ \mu\text{m}$ conospherical tip. During indentation, tissue sections were kept moist with water containing protease inhibitors and penicillin–streptomycin. Saline was not used, in order to avoid salt crystallization on the surface of sections, which would interfere with the contact between the tip and the sample. The reduced modulus was calculated using the Oliver–Pharr method [32], as

$$E_r = \frac{\sqrt{\pi}S}{2\sqrt{A_c}} \quad (1)$$

where S is the unloading stiffness, and A_c is the contact area. S was calculated as the initial slope (slope at 95%) of a polynomial function fit over 95–20% of the unloading curve. The elastic modulus of the tissue, E_s , is related to the reduced modulus by

$$\frac{1}{E_r} = \frac{1 - \nu_s^2}{E_s} + \frac{1 - \nu_t^2}{E_t} \quad (2)$$

where ν_s and ν_t are Poisson's ratio of the tissue and indenter tip (diamond-coated stainless titanium), respectively. The elastic modulus and Poisson's ratio of the indenter tip were 1140 GPa and 0.07, respectively, as given by the manufacturer. Because of the difficulties in measuring Poisson's ra-

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