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Short communication

Development of an *in vivo* bone fatigue damage model using axial compression of the rabbit forelimb

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ABSTRACT

Many nontraumatic fractures seen clinically in patients with metabolic bone disorders or on anti-resorptive treatment show an increased incidence of microdamage accumulation and impaired intracortical remodeling. However, the lack of basal remodeling and Haversian bone in rodents limits their translatability in studying bone damage repair mechanisms. The work presented here demonstrates the development of the forelimb loading model in rabbits, the smallest mammal with intracortical Haversian remodeling. The forelimbs of post-mortem female New Zealand white rabbits were loaded in axial end compression to determine their basic monotonic and fatigue properties. Following time zero characterization, stress fractures were created *in vivo* and animals were allowed to recover for a period of two to five weeks. The rabbit forelimb when loaded in axial compression demonstrates a consistent mid-diaphyseal fracture location characterized by a local mixed compression-bending loading environment. Forelimb apparent stiffness, when fatigue loaded, demonstrates a progressive increase until macrocrack formation, at which time apparent stiffness rapidly declines until failure. Stress fractures in the rabbit ulna display robust periosteal expansion and woven bone formation two weeks following fracture. Subsequent healing at five weeks post-fracture is marked by woven bone densification, resorption and intracortical remodeling along the stress fracture line. The rabbit forelimb fatigue model is a promising new platform by which bone's response to damage may be studied.

1. Introduction

With habitual physiological loading, bone naturally accumulates damage (Burr et al., 1985; Schaffler et al., 1995; Wenzel et al., 1996). This fatigue induced damage causes a reduction in bone's mechanical properties such as stiffness, strength, and toughness (Schaffler et al., 1996; Burr et al., 1998). However, fatigue-induced damage can be removed through the process of intracortical remodeling (Bentolila et al., 1998; Verbogt et al., 2000; Cardoso et al., 2009). Although this process should maintain bone's structural integrity, it is theorized that inadequate removal of this damage can allow microcracks to grow and coalesce to critical size resulting in a non-displaced stress fracture (Burr et al., 1997; Schaffler, 2001). Understanding the processes by which bone acquires and repairs damage is paramount clinically, especially since aging and bone diseases such as osteoporosis show an increased incidence of microdamage accumulation and nontraumatic fracture (Schaffler, 1995; Burr et al., 1997; Norman and Wang, 1997).

Currently, the non-invasive forelimb fatigue model in the rodent represents the "state of the art" system to study the biological and mechanical factors governing bone microdamage and stress fracture repair (Bentolila et al., 1998; Uthgenannt and Silva, 2007; Sloan et al., 2010; Kidd et al., 2010; Tomlinson et al., 2013). However, the lack of Haversian bone in rodents may limit their translational relevance to humans (Turner, 2001; Allen and Burr, 2010; Shane et al., 2010). Rabbits are a possible alternative with some advantageous features: they are the smallest mammal with Haversian bone (Hirano et al., 1999; Turner, 2001), share similar forelimb anatomy with rodents and are amenable to *in vivo* loading (Baumann et al., 2015), and respond to ovariectomy and a variety of bone active drugs (Pennypacker et al., 2011). The work presented herein shows the development and utilization of a rabbit forelimb fatigue model to create controlled macrodamage in the ulna. Based on previous work with the rodent forelimb model, we hypothesized that ulnar damage in the rabbit would range in proportion to controlled loading parameters. In addition, it was expected that stress fracture initiation in the rabbit ulna would

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trigger a classical healing response characterized by periosteal woven bone formation and intracortical remodeling.

2. Materials and methods

2.1. Animals

All experiments were conducted after approval from the Washington University Animal Studies Committee. A total of 58 female New Zealand white rabbits (3.5–4.5 kg; skeletally mature (Gilsanz et al., 1988; Newman et al., 1995), 7 months; Harlan) were used. For time-zero mechanical characterization, animals were euthanized (sodium pentobarbital overdose, 150 mg/kg i.v.) and stored at −20 °C until use. After thawing bodies to room temperature, both forelimbs (while still attached to post-mortem animals and with all accompanying soft tissue structures intact) were loaded by axial end loading between the flexed carpus and olecranon in a servo hydraulic material testing machine (Dynamight, Instron; Fig. 1A). Animal and forelimb allocation for each specific experiment is described in greater detail in the Supplemental information section (Table S1).

2.2. Strength testing

To determine forelimb strength and failure mode, monotonic axial *end* compression to failure was performed. Following forelimb failure, which was defined as a sharp drop in force during loading and corresponded to ulnar fracture, forelimb lateral radiographs were obtained (Faxitron). Ulnar fracture location was defined as the distance along the forelimb axis from the base of the olecranon to the compressive cortical fracture line (Supplemental info, Fig. S1). Forelimb ultimate force (F_{ult}) was denoted as the largest force recorded on the force *vs.* displacement plots before specimen failure.

2.3. Strain analysis

In order to characterize local bone deformations, three single-element strain gauges (Micro-Measurements, Vishay Precision Group) were applied circumferentially around each ulna at a section along the longitudinal axis where peak tensile and compressive strains were expected (i.e. fracture location determined in monotonic testing). Gauges 1 and 3 were placed on the medial and lateral periosteal ridge of the ulna's cross-section, respectively (Fig. 1B), where normal strains were expected to be maximum from previous work in the rat (Kotha et al., 2004). Gauge 2 was placed halfway between the other two gauges. After successful placement of strain gauges, each forelimb was loaded in axial end compression at 2 Hz haversine from 10 N to various peak force levels ranging from 24 N to 192 N (24 N increments; 20 cycles per peak force) to determine the force *vs.* strain profile. To minimize transient soft tissue effects, only peak strain values from the last 8 cycles were averaged for each force level. Regression analysis was performed (GraphPad Prism Pro, Version 6.07) to identify the relationship between force and strain. Data from the three gauges were used to calculate the orientation of the neutral axis of bending (Supplemental info, Fig. S2; Lieberman et al., 2004).

2.4. Fatigue to failure

To determine fatigue properties, rabbit forelimbs were cyclically loaded in compression between 10 N (minimum actuator position) and 155 − 185 N (maximum actuator position; 62–74% of F_{ult}) until failure (loss of load) at 2 Hz haversine. Early pilot experiments determined that this was an appropriate force range to cause failure after ~3600 cycles. The maximum actuator displacement from the tenth cycle (D_10) to failure (D_Fail) was calculated as a measure of displacement history (Supplemental info, Fig. S5).

2.5. Sub-failure fatigue loading

Next, rabbit forelimbs were cyclically loaded in compression from 10 N to 175 N at 2 Hz haversine to create fatigue damage but not complete fracture. The stopping point for each forelimb was selected *a priori* to achieve a specified change in forelimb apparent stiffness ($K=(F_{max}-F_{min})/(D_{max}-D_{min})$) compared to the tenth cycle of loading (K_10) as shown in Supplemental Table S4. Macrodamage assessment was performed by scanning excized rabbit forelimbs *via* microCT (17.5 μm voxel size, 300 ms integration time, Scanco Medical, Switzerland). 3D reconstructions of ulnae were viewed using Osirix Imaging Software (© Pixmeo SARL, 2003–2014). Microdamage was visualized by *en bloc* fuchsin (JT Baker Chemical) stained sections

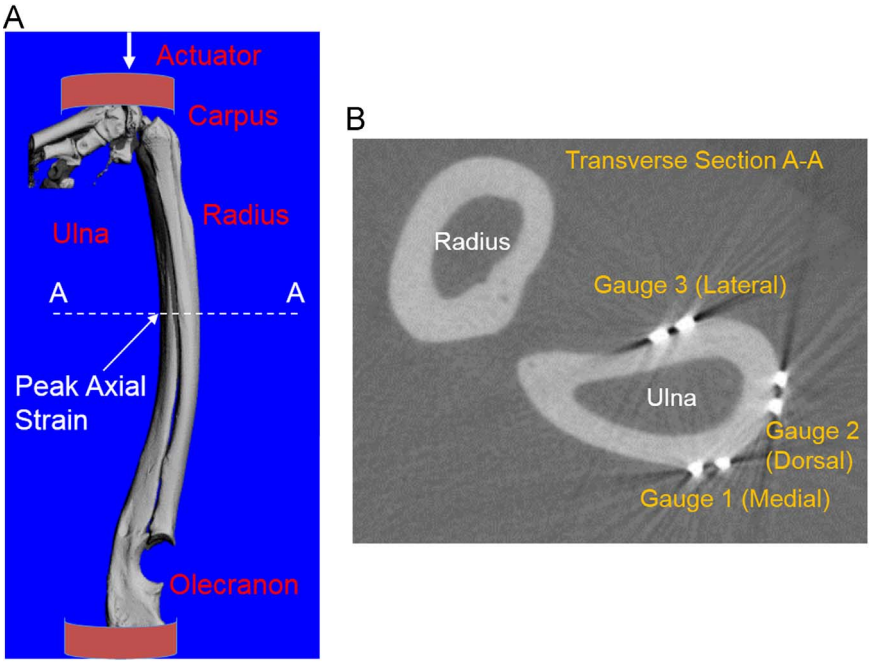

Fig. 1. A: microCT image of an isolated forelimb depicts our axial end compression loading setup and the structure of the forelimb bones. However, all loading was performed with the forelimb still attached to the body and with soft tissue structures intact. B: representative microCT image of the forelimb cross section at the longitudinal site of peak axial strain demonstrating the location of the three gauges used for ulnar strain analysis. For strain gauge testing only, soft tissues, including the periosteum, was excised around the central third of the forelimb to allow for proper strain gauge adherence.

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