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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 antiresorptive 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.

Keywords

Bone Mechanics; Bone Fatigue; Bone Damage; Rabbit Ulna; In Vivo Forelimb Loading

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, 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 et al., 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 et al., 2011; Shane et al., 2013;). 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., 2010). 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 trigger a classical healing response characterized by periosteal woven bone formation and intracortical remodeling.

Materials and Methods

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 kilograms; skeletally mature, 7 months; Harlan) were used. For time-zero mechanical characterization animals were euthanized (sodium pentobarbital overdose, 150mg/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; Figure 1A). Animal and forelimb allocation for each specific experiment is described in greater detail in the Supplemental Information Section (Table S1).

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, Figure S1). Forelimb ultimate force (F_{ult}) was denoted as the largest force recorded on the force vs. displacement plots before specimen failure.

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 (Figure 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 10N 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, Figure S2).

Fatigue to Failure

To determine fatigue properties, rabbit forelimbs were cyclically loaded in compression between 10N (minimum actuator position) and 155–185N (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, Figure S5).

Sub-Failure Fatigue Loading

Next, rabbit forelimbs were cyclically loaded in compression from 10N to 175N 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 excised rabbit forelimbs via microCT (17.5 μ m 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 located at the fracture site by previously described methods (Burr and Hooser, 1995; Supplemental Info, Figure S6).

In Vivo Loading – Pilot Study

Following completion of time zero characterization, stress fractures were created in a small subset of live animals ($n = 1$ per time point) to provide “proof of concept” and further rationale for development of the loading model. For these survival studies, animals were

anesthetized (30 mg/kg ketamine and 6 mg/kg xylazine i.m; maintained on 1–3% isoflurane inhalation) and the right forelimb was loaded from 10N to 170N cyclically at 2 Hz haversine until forelimb apparent stiffness increased by 10% from cycle 10. ($K/K_{10} = 1.1$; See Figure 4) Left forelimbs served as contralateral controls. After *in vivo* loading, analgesia was provided by a fentanyl transdermal patch (25 ug/hr). Animals were given calcein (10mg/kg s.c., 10 & 9 days before sacrifice, Sigma-Aldrich) and alizarin (25mg/kg s.c., 3 & 2 days before sacrifice, Sigma-Aldrich) to label bone formation and euthanized two or five weeks following fracture. These time points were chosen to coincide with peak woven bone formation (2 weeks) and intracortical remodeling (5 weeks) based on previous work in rats (Uthgenannt et al., 2007; Kidd et al., 2010).

Results

Strength Testing

Monotonic to failure testing revealed an ultimate compressive force of the forelimb to be 250 ± 22 N. In addition, on average, the ulna fractured 55 ± 5 mm from its proximal end, which when normalized corresponded to 62% of each ulna's total length. (Supplemental Info, Table S2).

Strain Analysis

Low cycle (non-fatigue) loading yielded compressive strain values at the medial and dorsal gauge sites (gauge 1 and 2), whereas tensile strain values were recorded at the lateral gauge site (gauge 3). Strain-force relationships were well fit to by simple linear regression ($R^2 > 0.99$ for gauges 2, 3; $R^2 = 0.90$ for gauge 1), although the relationships clearly deviated from linearity at > 144 N (Figure 2). Calculation of the neutral axis of bending demonstrated a mixed compression-bending loading mode of the rabbit ulna (Supplemental Info, Figure S2).

Fatigue to Failure

Fatigue life testing demonstrated a classic three-phase increase in displacement with increasing cycle number (Supplemental Info, Figure S5). The average increase in maximum actuator displacement until failure was variable at 2.92 ± 0.98 mm (Supplemental Info, Table S3). Therefore, forelimb apparent stiffness ($K = (F_{\max} - F_{\min}) / (D_{\max} - D_{\min})$) was calculated for each cycle and used to track specimen fatigue progression. Specimen apparent stiffness on average demonstrated a biphasic trend where it increased from baseline (cycle 10) before ultimately decreasing by $30 \pm 7\%$ until failure (Figure 3).

Sub-Failure Fatigue Loading

Ulnar damage increased with a decrease in forelimb apparent stiffness, our controlled loading parameter. For example, macrodamage burden was only visible via microCT when specimens were loaded beyond “Event1” (defined by a sharp drop in stiffness near the end of fatigue life; Figure 3). Furthermore, as demonstrated by 3D reconstruction of sub-failure loaded limbs (Figure 4), ulnar damage increased with progressive loss in forelimb apparent stiffness. En bloc fuchsin stained ulnar sections demonstrated macrodamage as well as other forms of reported microdamage in bone (Supplemental Info, Figure S6; Boyce et al., 1998).

In Vivo Loading – Pilot Study

Stress fractures in the rabbit ulna displayed robust periosteal expansion and woven bone formation 2 weeks following loading. In addition, remodeling of woven bone and along the stress fracture line was evident 5 weeks following loading (Figure 5).

Discussion

The primary goal of the proposed work was to characterize the basic mechanical and fatigue behavior of the rabbit forelimb under compressive axial end loading, in order to determine the feasibility of creating a large animal model that allows titration of bone damage. Our results are encouraging and warrant further development of the rabbit fatigue model.

From basic monotonic loading tests, the finding of compressive fracture at 62% of the total ulnar length (Supplemental Info, Table S2) is in agreement with previous rabbit work by Baumann et al. (Baumann et al., 2015). On the other hand, low-cycle (non-fatigue) loading revealed a nonlinear force-strain behavior (Fig. 2) that contrasts findings from the rat forelimb loading model (Uthgenannt et al., 2007). This force-dependent nonlinearity is not likely due to plastic deformation, since the ulna's force-strain behavior was nearly identical upon reloading from 24–192N (Supplemental Info, Figure S3). In addition, after loading forelimbs at each force level for 20 cycles (10 seconds), they were subsequently unloaded and allowed to recover for 250 seconds before the next cycle of loading. Therefore, there is a low likelihood that creep (viscoelastic effects) would be a major contributor to the nonlinear force-strain behavior. Instead, as the load is increased on the forelimb, the stiffness of the entire forelimb structure also increases (Supplemental Info, Figure S4); which may explain why the slope of the force vs. ulnar strain curve decreases with increasing load. This suggests that the radius carries an increasing fraction of the load as the total force increases, which may reflect a geometric realignment between the two bones. Thus, we propose that a geometric nonlinearity is the basis for the non-linear force vs. ulnar strain behavior and the force-dependent increase in forelimb stiffness observed during low-cycle (non-fatigue) loading. Future experiments are needed to test this proposal.

Fatigue loading revealed additional information about the mechanical behavior of the forelimb and confirmed our hypothesis that decreases in forelimb apparent stiffness during cyclic loading correspond to increasing ulnar damage. Forelimb apparent stiffness increased until late in fatigue life, at which point macrocracks formed in the ulna (K_Event1), and forelimb apparent stiffness rapidly declined. This initial increase in forelimb apparent stiffness before macrocrack formation in the ulna may seem counterintuitive, however we note that the forelimb is a complex structure made up of multiple long bones, joints, and soft tissue components. The cycle-dependent increase in forelimb apparent stiffness up until macrocrack formation is consistent with findings from the rat (Uthgenannt et al., 2007), and is likely due to soft tissue compaction near load contacts that occurs over time with cyclic compression. This is distinct from the force-dependent increase in forelimb apparent stiffness during non-fatigue loading (discussed in the previous paragraph), since our fatigue testing protocol is carried out at a constant peak force. Instead, soft tissue compaction is likely prominent early in fatigue and may drive the cycle-dependent increase in forelimb apparent stiffness before macrodamage formation. Following the appearance of

macrodamage (Event1; Figure 3), ulnar deformations increase enough to overcome soft tissue effects and dictate the decrease seen in forelimb apparent stiffness.

Finally, *in vivo* loading established the feasibility of the model for survival studies. Animals tolerated the loading procedure well and recovered without incident. Two and five weeks following stress fracture creation there was abundant woven bone on the periosteal ulnar surface, similar to findings in rodents (Hsieh et al., 2002; Uthgenannt et al., 2007) and indicative of a robust repair response. Moreover, at five weeks there was evidence of intracortical remodeling in the region of the ulnar stress fracture. This finding demonstrates that cortical bone damage generated by fatigue loading triggers an intracortical repair response.

In conclusion, cyclic loading of the rabbit forelimb is a feasible model to create bone fatigue damage. Changes in forelimb apparent stiffness are associated with soft tissue effects early during fatigue loading and dominated later by ulnar macrodamage creation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 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 structure 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.

Figure 2.

Strain data recorded from gauges 1, 2 and 3 were plotted as a function of loading force. Gauges 1 and 2 experienced compressive strains whereas gauge 3 experienced only tensile strains. The change in strain magnitude and sign across the ulna's cross-section demonstrate the local bending environment produced by forelimb axial compression. The nonlinear force-strain behavior at higher forces is not attributed to bone plastic deformations or viscoelastic effects, but rather load dependent alterations in forelimb apparent stiffness.

Figure 3.

A: Forelimb apparent stiffness ($F_{max} - F_{min} / D_{max} - D_{min}$) for each cycle was calculated following fatigue to failure tests and plotted versus residual cycle # (current cycle/total cycles). The forelimb apparent stiffness at cycle 10 (K_{10}) was used as a reference value. Forelimb apparent stiffness increased throughout cycling until macrocrack formation (K_{Event1}), at which time forelimb apparent stiffness rapidly declined until failure (K_{Fail}). $K_{Secondary}$ and $K_{Tertiary}$ were the recorded stiffness values halfway between K_{10} and K_{Event1} and K_{Event1} and K_{Fail} respectively. B: On average, forelimb apparent stiffness from cycle 10 progressively increased by 30% until macrocrack formation, at which time specimen apparent stiffness rapidly declined until failure. Arrows A-D demonstrate the stage of fatigue life that specimens in Figure 4 were loaded to based on forelimb apparent stiffness changes (K/K_{10}).

Figure 4.

Macrodamage was visualized by microCT in specimens loaded to sub-failure progressive changes in forelimb apparent stiffness (K/K_{10}). K_{10} was the forelimb apparent stiffness recorded at the tenth cycle of loading and K was the stiffness recorded at the last cycle before loading ceased. Samples loaded to forelimb apparent stiffness values before Event1 ($K/K_{10} \geq 1.3$) had no visible macrodamage (Panel A – 35 micron resolution). Samples loaded to progressive changes in forelimb apparent stiffness past Event1 ($K/K_{10} < 1.3$) demonstrated increasingly more visible macrodamage in the ulna (Panel B–D – 17.5 micron resolution).

Figure 5.

A: MicroCT of the rabbit ulna two weeks following stress fracture formation demonstrates robust woven bone formation at locations of peak strain found during strain analysis. B: Dynamic histomorphometry of stress fracture shows that woven bone callus formation occurs predominantly between week one and two following stress fracture. C: MicroCT of the rabbit ulna five weeks following stress fracture shows prominent callus densification and remodeling. D: Dynamic histomorphometry demonstrates areas of bone resorption and formation along the cortical fracture site and within the woven bone callus.