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Skeletal Self-Repair: Stress Fracture Healing by Rapid Formation and Densification of Woven Bone

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Abstract

Stress fractures of varying severity were created using a rat model of skeletal fatigue loading. Periosteal woven bone formed in proportion to the level of bone damage, resulting in the rapid recovery of whole-bone strength independent of stress fracture severity.

Introduction—A hard periosteal callus is a hallmark of stress fracture healing. The factors that regulate the formation of this woven bone callus are poorly understood. Our objective was to produce stress fractures of varying severity and to assess the woven bone response and recovery of bone strength.

Materials and Methods—We used the forelimb compression model to create stress fractures of varying severity in 192 adult rats. Forelimbs were loaded in fatigue until the displacement reached 30, 45, 65 or 85% of fracture. The osteogenic responses of loaded and contralateral control ulnae were assessed 7 and 14 days after loading using pQCT, microCT, mechanical testing, histomorphometry, and Raman spectroscopy.

Results—Loading stimulated the formation of periosteal woven bone that was maximal near the ulnar mid-shaft and transitioned to lamellar bone away from the mid-shaft. Woven bone area increased in a dose-response manner with increasing fatigue displacement. Whole-bone strength was partially recovered at 7 days and fully recovered at 14 days, regardless of initial stress fracture severity. The density of the woven bone increased by 80% from 7 to 14 days, due in part to a 30% increase in the mineral:collagen ratio of the woven bone tissue.

Conclusion—Functional healing of a stress fracture, as evidenced by recovery of whole-bone strength, occurred within 2 weeks, regardless of stress fracture severity. Partial recovery of strength in the first week was attributed to the rapid formation of a collar of woven bone that was localized to the site of bone damage and whose size depended on the level of initial damage. Complete recovery of strength in the second week was due to woven bone densification. For the first time we have shown that woven bone formation occurs as a dose-dependent response following damaging mechanical loading of bone.

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Introduction

The mammalian skeleton has a remarkable capacity for self-repair of injuries ranging from complete fractures to partial “stress” fractures. Complete fractures typically heal through a process of endochondral bone formation whereby rapid formation of granulation tissue gives way to a fibrocartilaginous “soft callus” that is gradually replaced by a woven bone “hard callus”. The process by which a stress fracture heals is less well understood.

Stress fractures are common in athletes and military recruits. Distance runners in the United States suffer more than 400,000 stress fractures annually, and incidence rates in U.S. military recruits are as high as 4–7%⁽¹⁾. These injuries have a mechanical etiology involving repetitive skeletal loading and bone fatigue⁽²⁾. Bone, like all solid materials, is susceptible to fatigue, a process by which repetitive loading at sub-fracture stress levels leads to the formation and propagation of cracks and progressive loss of stiffness and strength^(3,4). Fatigue-induced microdamage can trigger localized bone remodeling that will repair the damage^(5,6). Current theories suggest that if the rate of damage accrual outpaces the rate of repair, microcracks will lengthen and coalesce to form a stress fracture^(2,7).

Less attention has been given to the other tissue response that occurs in a healing stress fracture – the direct formation of a periosteal callus of woven bone^(8,9). Periosteal woven bone formation increases bone cross-sectional area and thereby should reduce mechanical strains engendered by skeletal loading, which in theory will diminish or prevent the accrual of further fatigue damage⁽⁷⁾. Another consequence of woven bone formation is that the acute loss of strength attributed to the stress fracture can be reversed, effectively repairing the structure⁽¹⁰⁾.

In addition to its presence at the site of healing stress fractures, woven bone forms during skeletal development, fracture healing and after high-strain mechanical loading. Woven bone is characterized by a lack of organization⁽¹¹⁾ and is believed to form under conditions where a rapid rate of matrix deposition is needed⁽¹²⁾. When a slower rate of deposition suffices, bone formation occurs as ordered lamellar bone. Studies of bone adaptation to non-damaging, dynamic loading indicate that lamellar bone formation is stimulated in a mechanical strain-dependent manner, i.e., the rate of formation increases with increasing strain magnitude^(13,14). In contrast, the limited available evidence suggests that woven bone formation after loading occurs as an “all or none” response when a threshold value of strain is exceeded⁽¹⁴⁾. It is unknown if the same “all or none” behavior characterizes woven bone formation at the site of a healing stress fracture.

Experimental approaches have been described for creating stress fractures under controlled loading conditions. By applying cyclic compression to the rat forelimb (“fatigue loading”), an ulnar stress fracture can be created that triggers the two main tissue-level responses seen in humans – intracortical resorption and periosteal woven bone formation^(10,15). Moreover, by monitoring the progressive increase in applied displacement that occurs during fatigue loading, the level of ulnar damage can be controlled^(16,17). Our objectives were to use the rat forelimb loading model to produce ulnar stress fractures of varying severity, and to assess the woven bone response and the recovery of bone strength. Based on a recent study wherein skeletal fluoride uptake increased with stress fracture severity⁽¹⁸⁾, we hypothesized that the magnitude of woven bone formation increases in proportion to stress fracture severity, leading to the rapid recovery of bone strength regardless of the level of damage.

Materials and Methods

Study design and in vivo loading

A total of 192 adult male rats (Fischer 344, 4.5 – 5.5 months age; Harlan) were randomly assigned in equal number to one of four loading groups (30, 45, 65, or 85% of fracture), and one of two survival timepoints (7 or 14 days). Under anesthesia (1–3% isoflurane), the right forelimb of each rat was loaded in axial compression through the carpus and olecranon using a materials testing machine (Model 1331/8500R, Instron Corp., Norwood, MA). The loading method is one we described in detail recently ⁽¹⁷⁾. Briefly, a pre-load of 0.3 N was applied followed by a single bout of fatigue loading (2 Hz haversine) with a peak compressive force adjusted for body weight (0.055 N/g; mean: 20.6 N). Loading was terminated when the increase in actuator displacement reached 30, 45, 65 or 85% of the average displacement at complete fracture (2 mm). The average numbers of loading cycles for the four loading groups were 1193 ± 1593 , 2229 ± 3380 , 2415 ± 3748 , and 2340 ± 2878 , respectively ($p = 0.20$). In our previous study ⁽¹⁷⁾, we related the increase in actuator displacement to bone damage in ulnae from rats that were euthanized immediately after loading. Ulnar stiffness and strength decreased and crack length increased progressively with increasing actuator displacement. Thus, in our loading model, the actuator displacement provides a means to modulate the level of time-zero ulnar damage.

After loading, rats were dosed with an analgesic (0.05 mg/kg buprenorphine hydrochloride i.m.) and returned to their cages. They were allowed unrestricted activities and ad libitum access to water and chow until euthanasia by CO₂ asphyxiation 7 or 14 days after loading. Ulnae designated for histology were dissected immediately post mortem and fixed in formalin; ulnae for mechanical testing were stored at –20°C until use. This study was approved by our Animal Studies Committee.

Of the 192 rats we started with, four were excluded due to problems related to in vivo loading (one sustained a forelimb fracture, one did not reach the displacement limit in the allotted 3 hr time, one died from an overdose of anesthesia, and in one case the Instron machine malfunctioned during loading). Another nine rats were excluded due to technical errors (two ulnae were fractured during dissection, seven were lost or mislabeled during histological processing). Remaining were ulnae from 179 rats.

pQCT

Prior to mechanical testing or histology, we assessed the distribution of ulnar bone formation along the bone length using peripheral quantitative computed tomography (pQCT; XCT Research, Norland/Stratec) on a subset of samples ($n = 9$ –12 per group). Eight transverse pQCT slices (0.07 mm in-plane resolution; 0.5 mm thickness) were taken at 2 mm intervals along the length of each ulna, centered near the mid-diaphysis (Fig. 1A). We determined bone area and bone mineral content (BMC) at each section using a simple threshold (500 mg/cm³) and the manufacturer's CALCBD routine. We then computed relative differences in cross-sectional bone area and BMC between loaded (right) and paired control (left) ulnae.

Histomorphometry

Bone forming surfaces were labeled in vivo by double fluorochrome injection. Rats ($n = 10$ –13 per group) received a first injection of calcein green (20 mg/kg, Sigma) and a second injection of alizarin complexone (30 mg/kg, Sigma). Rats euthanized on day 7 were injected on day 0 (immediately after loading) and day 5, while rats euthanized on day 14 were injected on day 7 and day 12. Ulnae were dissected post mortem, fixed in 10% formalin for 48 hours, and dehydrated in ethanol followed by xylene. They were infiltrated and

embedded in methylmethacrylate (Fisher). Sections (100 μm thickness) were cut on a saw microtome (Leica SP1600) at locations D1, D4, and D7 (Fig. 1A) and mounted on glass slides. Sections were visualized with an inverted microscope at 4x objective (Olympus). Analysis of the fluorescent labels and the amount of woven bone was performed using ImageJ (NIH). The length of periosteal bone surface labeled with woven bone (Wo.B.LS), double lamellar label (dLS), single lamellar label (sLS), or not labeled (nLS) was determined and expressed as a fraction of bone surface (BS) (Fig. 2). In addition, woven bone area (Wo.B.Ar) and original cortical bone area (B.Ar) were measured. All loaded ulnae ($n = 10\text{--}13$ per group) were analyzed; 8 contralateral ulnae ($n = 2\text{--}3$ per loading group) were analyzed and pooled for control data.

MicroCT

Prior to mechanical testing, ulnae from a subset of rats ($n = 4\text{--}7$ per group) were embedded in 1.5% agarose gel, positioned within a 16 mm acrylic specimen tube and scanned by microCT at 16 μm isotropic resolution (μCT 40, Scanco Medical). Scans were collected across the central 14 mm and analyzed by a single operator (M.K.) using the manufacturer's software. Periosteal woven bone was readily distinguished from the pre-existing cortical bone based on the relative differences in their CT attenuation. Woven bone area and apparent mineral density (calibrated to the manufacturer's hydroxyapatite [HA] mineral phantom) were determined at 2 mm increments.

Mechanical Testing

Ulnae were loaded to fracture in three-point bending ex vivo to determine whole-bone mechanical properties. Ulnae were thawed to room temperature and maintained in a moist state using saline. They were placed on supports spaced 15 mm apart and loaded transversely at their midpoint with a single ramp waveform at 0.5 mm/s to fracture. This bending mode produces tension laterally and compression medially and corresponds to the one that occurs during forelimb compression⁽¹⁹⁾. Force-displacement data were collected and analyzed to obtain ultimate force, stiffness, post-yield displacement and energy to fracture.

Raman Spectroscopy

After mechanical testing, the fractured ends of a subset of loaded and control ulnae ($n = 3\text{--}4$ from the 65 and 85% displacement groups at 7 and 14 days) were sectioned using a diamond wafering blade (Buehler Isomet) to create an even surface. We analyzed samples from the two highest displacement groups because they had the greatest amount of woven bone. The cut surface was analyzed using a fiber-optically coupled Raman microprobe (HoloLab Series 5000 Raman Microscope; Kaiser Optical Systems, Inc) with a frequency-doubled 532 nm Nd-YAG laser excitation source. The laser was focused on the surface at a spatial resolution of $\sim 1\text{ }\mu\text{m}$ and a power $< 500\text{ }\mu\text{W}$. The spectral range of $100\text{--}4000\text{ }\Delta\text{cm}^{-1}$ was simultaneously detected with a CCD array detector with 2048 channels and a spectral resolution of $2.5\text{ }\text{cm}^{-1}$. Spectral acquisition time was 32×4 seconds per spectrum. Raman spectra were taken at 12 pre-selected and photographically documented positions along a medial-lateral traverse of the cut surface of each loaded ulna, resulting in six woven bone spectra and six cortical bone spectra. Similarly, Raman spectra were taken at six positions along each non-loaded control ulna. Each spectrum was deconvolved in the ranges of $700\text{--}1200\text{ }\Delta\text{cm}^{-1}$ and $2750\text{--}3150\text{ }\Delta\text{cm}^{-1}$ in order to calculate the peak positions, widths and areas characteristic of mineral and collagen vibrations.

Results

pQCT analysis indicated that loading caused increases in BMC and bone area that were localized to the central region of the diaphysis, with a symmetrical response centered 1 mm distal to the mid-diaphysis (Fig. 1B). Cross-sections 6 mm proximal or 8 mm distal to the mid-diaphysis showed little or no change, while cross-sections 0 and 2 mm distal to the mid-diaphysis showed maximal increases. Based on these findings we focused our histological analysis on sections 1, 4 and 7 mm distal to the mid-diaphysis.

Periosteal woven bone was observed on 92 of 93 ulnae examined histologically, in amounts that depended strongly on fatigue displacement and anatomical location. Woven bone near the mid-diaphysis increased with increasing levels of fatigue displacement (Fig. 3), indicating clearly that woven bone formation occurred as a dose-response to the level of bone damage. Notably, there was negligible new bone labeled after day 7, and quantitative analysis indicated that woven bone area did not change from 7 to 14 days ($p > 0.05$). Total bone area (woven plus original cortical bone) showed the same relative differences between groups as the woven bone area, because the original cortical area was not different between groups (data not shown). Thus, total bone area also did not change from 7 to 14 days. In addition, consistent with the pQCT results, woven bone area depended on longitudinal location, with progressively less woven bone at sections further from the mid-diaphysis ($p < 0.05$) (Fig. 4A). Moreover, there was a consistent distribution of woven bone within the cross-sections, with more woven bone medially than laterally as we have noted previously⁽¹⁰⁾. The longitudinal and transverse distribution of woven bone corresponded to the pattern of cracking, which was consistent with previous descriptions^(15–17). Cracks were observed on histological sections from 61 of 93 ulnae, with 60 ulnae exhibiting cracks at D1, six at D4, and zero at D7. Invariably, these cracks were located on the medial half of the cross-section (Fig. 3A).

Consistent with the displacement- and location-dependent differences in woven bone area, the fraction of lamellar and woven bone labeled surfaces changed significantly with fatigue displacement and longitudinal location (Fig. 4B). For ulnae in the lowest displacement group, new woven bone covered 35–50% of the bone surface 1 mm distal to the mid-diaphysis, while lamellar bone formation occurred over 40–50% of the surface. In contrast, ulnae from the three highest damage groups had 80–90% of the periosteal surface covered with woven bone and 8–18% with new lamellar bone. Unlike the progressive displacement-dependent increase in woven bone *area*, the fraction of woven bone-labeled *surface* reached a plateau at 45% fatigue displacement. Thus, once the fraction of the periosteal surface covered with woven bone approached 90%, further increases in woven bone area between groups were achieved by the formation of a thicker layer of woven bone. Regarding the effect of longitudinal location, bone formation shifted from woven to lamellar with increasing distance from the mid-diaphysis.

We performed bending tests to determine the recovery of ulnar strength and stiffness following the creation of a stress fracture. We had previously assessed changes in ulnar mechanical properties on day 0, and reported a progressive loss of strength and stiffness with increasing fatigue displacement⁽¹⁷⁾; these time-zero data were combined with new data from ulnae collected 7 and 14 days after fatigue loading. Together, the data reveal a partial recovery of strength by day 7 and a complete recovery by day 14 (Fig. 5). Notably, in contrast to the displacement-dependent loss of strength at day 0, there were no differences in the strength of loaded ulnae between displacement groups on day 14. Moreover, the initial loss of stiffness (which ranged from 15–90%) was almost completely recovered by day 14, and post-yield displacement and energy to fracture were recovered such that they were either not different or improved versus control on day 14 (Table 1).

The large increases in mechanical properties between 7 and 14 days were in apparent contradiction to the lack of increase in woven bone area during this time. To determine the material basis for the recovery of strength and stiffness after day 7, we measured woven bone mineral density using microCT. Woven bone density increased dramatically from 7 to 14 days (average 80%, $p < 0.001$; Table 2), but did not vary greatly with the level of fatigue displacement. By contrast, and consistent with the histomorphometric findings, woven bone area did not change with time ($p = 0.11$) but did depend strongly on fatigue displacement ($p < 0.001$).

Finally, we used laser Raman spectroscopy to assess whether or not the increases in apparent mineral density of woven bone between days 7 and 14 were due in part to increased mineralization of the solid bone tissue. Analysis of Raman spectra for woven bone at day 7 revealed a mineral:collagen ratio (960/2940 and 960/1003 Δcm^{-1} peak areas) approximately 40% less than for control cortical bone ($p < 0.05$; Fig. 6; Table 3). Mineralization of woven bone increased from 7 to 14 days ($p < 0.05$), although 14-day woven bone was still ~20% less mineralized than control bone ($p < 0.05$). Other notable differences between woven bone and control spectra included greater inter-site heterogeneity within the same bone, increased width of the mineral peaks, and a downshift of ~3 Δcm^{-1} in the position of the 2940 Δcm^{-1} collagen peak.

Discussion

Periosteal hard callus is the hallmark of a healing stress fracture^(8,9), yet the factors that regulate the formation of this woven bone callus are poorly understood. We created stress fractures of varying severity using a rat model of fatigue loading to test the hypothesis that the magnitude of woven bone formation increases in proportion to stress fracture severity. Our findings support this hypothesis and demonstrate clearly that the magnitude of the woven bone response was scaled to the level of applied fatigue displacement and thus to the level of initial bone damage. To our knowledge, this is the first evidence that woven bone formation occurs in a dose-response manner to a mechanical stimulus. This result is consistent with our recent study that found increased skeletal uptake of fluoride in proportion to stress fracture severity using the same loading model⁽¹⁸⁾, although in that study we could not discriminate bone formation from damage and/or vascular responses. Our conclusion that the woven bone response is scaled to the severity of the stress fracture indicates that woven bone formation is a well regulated response to skeletal injury. This extends previous studies that have described a role for woven bone in the adaptive response to elevated mechanical strain^(14,20).

It is well documented that *lamellar* bone formation occurs in a dose-response manner following application of cyclic mechanical strain of increasing magnitude^(13,14). Few investigators have focused on quantification of a *woven* bone response, although results from one study indicated that woven bone formation occurred as an “all or none” response once a threshold value of strain was exceeded⁽¹⁴⁾. There are several differences between our study and the previous study⁽¹⁴⁾ that might explain our different conclusions. First, we used the forelimb loading model rather than the tibial four-point bending model; the “all or none” response may be unique to the tibial bending model. Second, we applied a single bout of fatigue loading that produced measurable bone damage, whereas the earlier study applied multiple bouts (36 cycles/day; 2 weeks) that did not result in detectable damage. Third, the earlier conclusion was based on a measure of woven bone surface rather than woven bone area. We observed that woven bone *surface* increased from the 30% to the 45% displacement groups, but did not increase further because it had already reached ~90% (Fig. 4B). In contrast, woven bone *area* increased progressively across all four displacement groups, suggesting that this measure may be a more sensitive indicator of a dose-response.

Taken together, the woven bone surface and area data from our study indicate that once woven bone formation was activated on nearly the entire periosteal surface, the area of woven bone could increase further by the deposition of a thicker collar of woven bone. However, regardless of the thickness of the woven bone layer, the space that is occupied by woven bone is essentially established by day 7, as evidenced by the diffuse uptake of the fluorochrome labels given on days 5 and 7, with negligible new bone labeled at day 12.

We also hypothesized that the woven bone response would lead to the rapid recovery of bone strength regardless of the level of damage. Our findings support this hypothesis and demonstrate that the damage-dependent woven bone response resulted in nearly complete functional healing within ~2 weeks for all damage levels. The recovery of whole-bone strength such that loaded ulnae from the four displacement groups were not different from control (and were not different from each other) at 14 days is particularly impressive, as the initial reductions in bone strength in loaded ulnae in these groups ranged from 10 to 60%. The recovery of other mechanical properties (stiffness, fracture energy, post-yield displacement) was less uniform across the displacement groups, with evidence that the higher displacement groups recovered at a slower rate than the lower displacement groups. Additional studies are needed to confirm this finding and determine if there is a functional significance of having different rates of recovery of these different properties.

The pattern of woven bone formation in the rat ulnar fatigue model corresponds closely with the pattern of bone damage (Fig. 7). Detailed descriptions of the damage/crack distribution has been reported previously by us ⁽¹⁷⁾ and others ^(15,16). Briefly, we observe cracks only on the medial half of the cross-section, in a region near the mid-point of the ulna. The appearance of these cracks is consistent with a shear fracture, where the fracture plane is approximately 45 degrees from the direction of loading. The association between the spatial pattern of bone damage and bone formation suggests that the cracks in some way are the stimulus for the periosteal woven bone response. In further support of this notion, we observed that the amount of bone formation increased with increasing fatigue displacement, which we have previously established is directly related to the severity of cracking both within the cross-section and along the longitudinal direction ⁽¹⁷⁾. Thus, we propose that the stress fracture itself is a primary stimulus that dictates the location and extent of the woven bone response.

Our study provides novel data on the relative contributions of bone size and mineralization to the recovery of bone strength after a stress fracture, and highlights the time-dependent interplay between size, mineralization and strength. We attribute the partial recovery of strength in the first 7 days to the rapid formation of a collar of woven bone whose size depends on the level of initial damage. However, because bone area did not increase significantly from 7 to 14 days, the increases in ulnar strength that occur in the second week cannot be attributed to changes in bone size. Moreover, we do not attribute the recovery of mechanical properties to intracortical remodeling and repair of bone damage, based on the fact that intracortical osteoclasts take ~10 days to appear in this model ^(5,10,21). Rather, we attribute the increases in bone strength in the second week to woven bone densification. The apparent mineral density of the woven bone increased by 80% from 7 to 14 days, due in part to an increase of 30% in the mineral:collagen ratio of the bone tissue. By day 14, the woven bone tissue was still relatively hypo-mineralized compared to cortical bone, but other spectroscopic features of the mineral and collagen of 14-day woven bone were equivalent or only modestly different from mature cortical bone. The rapid maturation of woven bone tissue is comparable to the rapid chemical and mechanical maturation reported following lamellar bone deposition ⁽²²⁾.

There are several limitations to our study. First, the creation of a stress fracture by a single bout of loading on the order of 1 hr does not replicate the time course of the development of a clinical stress fracture. Most stress fractures likely develop over a period of days or weeks⁽²³⁾ and the prevailing view is that they are not due solely to fatigue damage but to an interplay between fatigue damage, damage-initiated bone remodeling and localized loss of bone stiffness^(2,7). While our model did not allow for remodeling activity prior to the creation of the stress fracture, it is unclear whether or not a prior remodeling state would affect the periosteal response, which was the focus of our study. A second limitation is that we cannot separate the effects of dynamic strain from those of bone damage (the actual stress fracture). Inherent in a fatigue test is that as the displacement and damage increase, the dynamic strain at the site of damage also increases. Thus, the dose-response increase in woven bone area we observed may be due to greater dynamic strains and/or more severe stress fractures in the higher displacement groups. Nonetheless, the use of dynamic loading represents a physiologically relevant scenario in which dynamic strain and bone damage are present together at the site of a stress fracture. Regardless of the physical stimulus that triggers the woven bone response, the key discovery remains that that woven bone formation in association with a healing stress fracture is not an “all or none” response but is modulated by mechanical factors.

One clinical implication of our findings is that the size of the periosteal callus may be an indicator of stress fracture severity. While this may seem intuitive, our study provides quantitative data to support the concept. A second implication is that the severity of the stress fracture may not be indicative of the time needed for functional healing. This seems to contradict available guidelines for clinical treatment, which indicate that the severity of bone damage (as inferred by the size and intensity of scintigraphic uptake on a bone scan) dictates the prescribed rest period⁽²⁴⁾. One reason for the discrepancy between clinical recommendations and our results is that the functional healing we measured may precede the relief of pain that is the basis for clinical management. Alternatively, when a bone sustains microdamage but not a true stress fracture, it may heal only by the slower process of intracortical remodeling. Paradoxically, only when bone damage progresses to the point of a stress fracture may the woven bone “repair” process become activated and lead to rapid functional healing.

In summary, our study demonstrates the self-repair process by which whole bones regain their structural integrity within two weeks after sustaining stress fractures of varying severity. In the first week, woven bone forms at the site of the stress fracture in proportion to the level of initial damage. The new woven bone is hypo-mineralized compared to normal cortical bone but nonetheless leads to a partial recovery of strength and stiffness. During the second week, the area of woven bone does not increase further but the woven bone density nearly doubles as the voids begin to fill and the solid phase of the woven bone becomes more mineralized. Thus, we attribute the partial recovery of whole-bone strength in the first week after a stress fracture to the rapid formation of a collar of woven bone that is localized to the site of bone damage and whose size depends on the level of initial damage; full recovery of strength in the second week is due to the densification of this woven bone.

Acknowledgments

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Figure 1.

(A) Locations of cross-sections examined by pQCT (dashed lines) and histomorphometry (arrows). Ulnae were ~34 mm long from olecranon to carpus. We scanned the mid-diaphysis at 2 mm intervals over a 14 mm region for a total of eight pQCT sections. (MD denotes the mid-diaphysis. D8, D7, D6, D4, D2 and D1 denote sections 8, 7, 6, 4, 2 and 1 mm distal to MD, while P2, P4 and P6 denote 2, 4 and 6 mm proximal to MD.) (B) The longitudinal distribution of increases in bone area and BMC in loaded versus control ulnae was determined by pQCT and displayed a symmetrical response centered ~1 mm distal to the mid-diaphysis, which is the center of the damaged region of the ulna⁽¹⁷⁾. Data shown are for the 65% displacement group at day 7 (n = 9) and are representative of all groups. (p < 0.05: * loaded different from contralateral control; A, B, C – sites with the same letter are not different)

Figure 2.

Fluorescent photomicrographs of transverse ulnar cross-sections illustrating the different types of labeled bone surface. Calcein green and alizarin complexone (red) were administered 7 and 2 days before death, respectively. (A) The length of each type of label was traced on the surface of the original bone and expressed as a fraction of the total bone surface. We analyzed only the periosteal bone surface. Woven bone regions were identified wherever labels were not parallel to the original bone surface. Double-labeled lamellar regions were identified wherever parallel green and red labels were observed separated by a gap. Single-labeled lamellar regions were identified wherever a single green or red linear label, or green and red co-localized linear labels were observed. (Shown is an ulna from the 14-day, 85% displacement group, section D7.) (B) Woven bone area was calculated as the sum of the individual regions of woven bone around the ulnar periosteal surface. (Shown is an ulna from the 14 day, 65% displacement group, section D4.)

Figure 3.

(A) Fluorescent photomicrographs of transverse sections from one control and eight loaded ulnae at section D1 (1 mm distal to the mid-diaphysis), illustrating that the amount of new periosteal woven bone increased with increasing fatigue displacement. There was no woven bone on any control ulnae. A stress fracture can be seen on the medial half of some sections running left to right through the cortical bone. The negligible amount of red label in the 14-day bones indicates that little new bone was added after day 7. (B) Quantitative analysis of woven bone area (Wo.B.Ar) at section D1 indicated significant increases with increasing fatigue displacement. Importantly, there was no significant difference in Wo.B.Ar between 7 and 14 days after loading. (n = 10–13 per group; # p < 0.05 between groups).

Figure 4.

(A) The amount of woven bone was greatest 1 mm distal to the mid-diaphysis, and decreased progressively 4 and 7 mm distal to the mid-diaphysis. (Sections shown are from the 65% displacement group at day 14. Similar relative findings were noted for the other groups and at day 7. Orientation as in Figs. 2 and 3.) (B) The fraction of the ulnar periosteal surface undergoing different types of bone formation in loaded and control ulnae (average values shown, $n = 10-18$ per group). Control ulnae had ~35% unlabeled surface, 60% single-labeled surface, 5% double-labeled surface and 0% woven bone surface. Fatigue loading induced increases in both double-labeled lamellar surface and woven bone-labeled surface, with the relative proportions depending on displacement level and site. The predominant response to fatigue loading at D1 was woven bone formation in the three higher displacement groups, and an approximately equal mix of woven and lamellar formation in the lowest displacement group. There was a shift from woven to lamellar bone with increasing distance from D1. At D7, less than 10% of the surface was covered with woven bone.

Figure 5.

Recovery of ulnar mechanical properties after creation of stress fractures of increasing severity. (A) Immediately after loading (day 0), bone strength (ultimate force) was significantly reduced in loaded ulnae versus control in all groups, and the amount of reduction depended on fatigue displacement ($p < 0.05$). By day 7, strength was recovered to normal in the lowest displacement group and was partly recovered in the other three groups. By day 14, strength was recovered to normal in all groups and there were no differences between groups. (B) Stiffness recovered in a similar manner as strength although at a slower rate, perhaps because the magnitude of the initial stiffness loss was even greater than the strength loss. By day 14, stiffness was recovered in the two lower displacement groups and was within 15% of control for the two higher displacement groups. ($n = 9-12$; * $p < 0.05$ loaded different from paired control)

Figure 6.

(A) Representative Raman spectra obtained from cortical bone of a left (nonloaded control) ulna, and from woven bone of right ulnae 7 and 14 days after fatigue loading to 85% displacement level. Spectra show peaks due to vibrations in the nanometer-sized apatite crystals and the collagen matrix. (Intensities have been normalized to the 2940 cm^{-1} collagen band and are shifted along Y-axis for better display.). We analyzed the intensity and width of the prominent mineral peaks at ~ 960 and $\sim 1070\text{ cm}^{-1}$ (caused by P-O vibrations within the PO_4 tetrahedra of the apatite) and prominent collagen peaks at ~ 2940 and $\sim 3400\text{ cm}^{-1}$ (caused by C-H and N-H stretches, respectively) as well as a small collagen peak at 1003 cm^{-1} (caused by the C-H vibrations of the benzene ring of the amino acid phenylalanine). Note that the intensities of the mineral peaks from the 7-day woven bone are greatly decreased compared to the control bone, whereas the 14-day woven bone is more similar to control bone. (B) Box plot of the mineral:collagen ratio as determined by the ratio of areas under the 960 and 2940 cm^{-1} peaks. Spectra from woven bone revealed a lower mineral:matrix ratio and exhibited greater intra- and inter-bone variability compared to cortical bone. (The height of the box depicts the 25th to 75th percentile values, while the error bars depict the 10th and 90th percentile values; the median value is depicted by the horizontal line within the box.)

Figure 7.

Fluorescent photomicrograph of longitudinal section from a rat ulna 7 days after fatigue loading. A longitudinal compressive force is applied at the distal and proximal ends of the ulna, resulting in compression plus bending due to the ulnar curvature. Stress fractures (cracks) like the one shown here (arrowheads) occur where the strain magnitude is greatest, on the medial aspect of the ulna near its midpoint. The pattern of woven bone corresponds to the location of the stress fracture, with the greatest amount of new bone near the midpoint on the medial side. We consistently observe a region of delayed mineralization at the site where the fracture intersects the periosteal surface.

Table 1

Relative values of energy to fracture and post-yield displacement from three-point bending tests of ulnae collected 0, 7 and 14 days after fatigue loading. Values are mean $\pm$ SD of the percent difference between loaded and control ulnae (n = 9–12).

Parameter	Days	Fatigue Displacement (% of fracture)			
		30 %	45 %	65 %	85 %
Fracture energy	0	-19.2 * $\pm$ 18.6	-27.9 * $\pm$ 30.2	-54.0 * $\pm$ 11.5	-58.2 * $\pm$ 30.7
	7	20.3 $\pm$ 30.2	19.5 $\pm$ 39.8	-25.1 * $\pm$ 34.9	-34.3 * $\pm$ 20.5
	14	40.3 * $\pm$ 38.9	60.9 * $\pm$ 40.7	37.0 $\pm$ 59.8	-18.0 $\pm$ 51.7
Post-yield displacement	0	-12.4 $\pm$ 29.2	-20.6 $\pm$ 51.9	-43.4 * $\pm$ 27.0	-53.9 * $\pm$ 45.3
	7	22.9 $\pm$ 35.7	21.2 $\pm$ 49.0	-35.1 * $\pm$ 30.0	-55.9 * $\pm$ 29.1
	14	31.3 $\pm$ 40.3	47.3 * $\pm$ 41.4	30.3 $\pm$ 66.1	-17.7 $\pm$ 46.8

* p < 0.05, loaded different from control, paired t-test

Area and mineral density of periosteal woven bone at the mid-shaft as determined by microCT (n = 4–7).

Table 2

Parameter	Days	Fatigue displacement (% of fracture)			
		30 %	45 %	65 %	85 %
Woven bone area (Wo.B.Ar. mm ²)	7	0.23 ± 0.20	0.67 ^a ± 0.18	0.89 ^a ± 0.22	0.88 ^a ± 0.17
	14	0.25 ± 0.30	0.52 ± 0.24	0.75 ^a ± 0.22	0.71 ^a ± 0.21
Woven bone mineral density (Wo.BMD, mg HA/cm ³)	7	413 ± 44	368 ± 39	353 ^a ± 33	374 ± 22
	14 [*]	706 ± 35	697 ± 50	641 ^{a,b} ± 40	658 ± 17

p < 0.05;
^{*} different from 7-day;
^a different from 30%;
^b different from 45%

Table 3

Raman spectra were obtained from contralateral control ulna (“Control cortical”), from the cortical region of loaded ulna (“Loaded cortical”) and from the woven bone region of loaded ulnae at 7 and 14 days. Cortical data from 7 and 14 days are pooled because they did not differ between timepoints.

Parameter	Control Cortical (n = 11)	Loaded Cortical (n = 14)	Loaded day 7 woven (n = 7)	Loaded day 14 woven (n = 7)
960 peak position (Δcm^{-1})	960.6 $\pm$ 0.1	960.7 [*] $\pm$ 0.1	960.9 [*] , [†] $\pm$ 0.2	960.7 [#] $\pm$ 0.1
960 peak width (Δcm^{-1})	15.9 $\pm$ 0.2	16.0 $\pm$ 0.1	16.4 [*] , [†] $\pm$ 0.4	16.2 [*] , [†] $\pm$ 0.3
1070 peak position (Δcm^{-1})	1072.3 $\pm$ 0.1	1072.5 $\pm$ 0.2	1072.8 [*] $\pm$ 0.7	1072.7 $\pm$ 0.5
1070 peak width (Δcm^{-1})	25.7 $\pm$ 0.8	26.3 $\pm$ 1.0	35.8 [*] , [†] $\pm$ 6.6	28.8 [*] , [#] $\pm$ 1.8
1003 peak position (Δcm^{-1})	1003.2 $\pm$ 0.1	1003.1 $\pm$ 0.1	1003.0 [*] $\pm$ 0.2	1003.1 $\pm$ 0.1
1003 peak width (Δcm^{-1})	9.8 $\pm$ 0.9	9.7 $\pm$ 0.9	9.5 $\pm$ 0.9	9.4 $\pm$ 1.5
2940 peak position (Δcm^{-1})	2939.7 $\pm$ 0.7	2939.7 $\pm$ 0.3	2936.5 [*] , [†] $\pm$ 0.7	2938.2 [*] , [†] , [#] $\pm$ 0.5
2940 peak width (Δcm^{-1})	50.2 $\pm$ 1.9	51.2 $\pm$ 0.9	49.7 [†] $\pm$ 1.2	50.1 $\pm$ 1.0
960 area/2940 area (mineral:collagen)	0.392 $\pm$ 0.029	0.378 $\pm$ 0.045	0.247 [*] , [†] $\pm$ 0.066	0.316 [*] , [†] , [#] $\pm$ 0.084
960 area/1003 area (mineral:collagen)	23.7 $\pm$ 4.9	22.7 $\pm$ 3.8	13.8 [*] , [†] $\pm$ 4.0	17.6 [*] , [†] $\pm$ 4.8
960 area/1070 area (phosphate:carbonate)	3.11 $\pm$ 0.43	2.84 $\pm$ 0.37	2.97 $\pm$ 0.62	3.23 $\pm$ 0.40

p < 0.05:

^{*} different from control cortical;

[†] different from loaded cortical;

[#] different from day 7 woven