

Bone loss from high repetitive high force loading is prevented by ibuprofen treatment

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Abstract

We examined roles of loading and inflammation on forearm bones in a rat model of upper extremity overuse. Trabecular structure in distal radius and ulna was examined in three groups of young adult rats: 1) 5% food-restricted that underwent an initial training period of 10 min/day for 5 weeks to learn the repetitive task (TRHF); 2) rats that underwent the same training before performing a high repetition high force task, 2 hours/day for 12 weeks (HRHF); and 3) food-restricted only (FRC). Subsets were treated with oral ibuprofen (IBU). TRHF rats had increased trabecular bone volume and numbers, osteoblasts, and serum osteocalcin, indicative of bone adaptation. HRHF rats had constant muscle pulling forces, showed limited signs of bone adaptation, but many signs of bone resorption, including decreased trabecular bone volume and bone mineral density, increased osteoclasts and bone inflammatory cytokines, and reduced median nerve conduction velocity (15%). HRHF+IBU rats showed no trabecular resorptive changes, no increased osteoclasts or bone inflammatory cytokines, no nerve inflammation, preserved nerve conduction, and increased muscle voluntary pulling forces. Ibuprofen treatment preserved trabecular bone quality by reducing osteoclasts and bone inflammatory cytokines, and improving muscle pulling forces on bones as a result of reduced nerve inflammation.

Keywords: Repetitive Loading, Work-related Musculoskeletal Disorders, Radius, Ulna, Inflammatory Cytokines, Carpal Tunnel Syndrome

Introduction

Work-related musculoskeletal disorders (WMSDs) are also known as occupational overuse injuries, or repetitive strain injuries. They account for 33% of all lost workday illnesses, and WMSDs of the hand and wrist are associated with the greatest work absences^{1,2}. Epidemiological studies have linked upper extremity WMSDs with occupational physical activities in-

volving repetitive arm motions and other risk factors (including force, duration, and female gender)³⁻⁵. The combined effects of force and repetition are associated with the greatest risks, via accumulated loading and gradual reduction of tissue tolerance⁶⁻⁸.

Cyclical loading and high force loads are known to affect bone quality⁹⁻¹². However, only a few studies have examined changes occurring in upper extremity bones as a consequence of prolonged performance of occupational tasks. A few studies have shown that occupational tasks increase the incidence of hand osteoarthritis¹³⁻¹⁵. Bone scan studies of patients with upper extremity MSDs show increased blood flow and pooling (suggestive of inflammation) in affected bones, although the sensitivity and accuracy of the results were variable across studies¹⁶⁻¹⁸. A loss of bone mineral density (BMD) was reported in metacarpal bones and distal radius and ulna of patients with long-term carpal tunnel syndrome¹⁹, a condition of reduced conduction velocity of the median nerve that is under the um-

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brella of WMSDs. Surgical release treatment for carpal tunnel syndrome rescues this decline in distal forearm BMD²⁰. These authors hypothesized that nerve-compression induced muscle weakness led to bone loss as a consequence of reduced muscular loading on the bone¹⁹, since the muscles involved in performing hand grip actions produce forces on forearm bones^{21,22}.

However, several factors contribute to bone density and quality, including the magnitude, frequency and duration of loading, and overload-induced increases in inflammatory cytokines^{9-12,23,24}. Increased interleukin-1 alpha (IL-1alpha), IL-1beta and tumor necrosis factor alpha (TNF-alpha), or their receptors, have been reported in serum of human subjects with upper extremity WMSDs²⁵⁻²⁸. Since these same inflammatory cytokines promote osteoclast activity and bone resorption²⁹⁻³¹, it is plausible that systemic or local inflammatory responses are contributing to pathological bone changes occurring with occupational physical activities.

We have developed a unique, voluntary rat model of repetitive reaching and pulling that permits the examination of responses of upper limb tissues to cumulative muscular loads and naturally occurring inflammation *in vivo*³²⁻³⁷. Rats are trained to voluntarily reach for and pull on a lever, with reach rate and force controlled operantly by food reward criteria. We have shown exposure-dependent declines in median nerve conduction velocity and increased forepaw hypersensitivity, establishing this as a model of carpal tunnel syndrome^{36,38-41}. We have also observed that performance of a high-repetition high-force task (HRHF; 8 reaches/min at force loads of 60% maximum isometric pulling force) initially induces increases in serum osteocalcin (in weeks 3-9 of the HRHF task), suggestive of bone formation, but cortical bone thinning, increased osteoclasts, and increased serum Trap5b (a biomarker of osteoclast activity and bone resorption) by week 12 of HRHF task performance, suggestive of bone catabolism³⁵⁻³⁷. We have yet to determine if the HRHF load-induced increases of osteoclasts and serum biomarkers of bone resorption correspond to alterations of trabecular morphology and bone loss, or if the shorter duration loading occurring during the initial training period (the TRHF period) induces to bone formation, which forms the basis for this study.

Our primary aim was to use our rat model to examine the effects of performing a reaching and grasping task for a short time period at progressively increasing force loads, versus the effects of prolonged performance (12 weeks) of a high repetition high force (HRHF) reaching and grasping task on forearm trabecular bone microarchitecture and bone mineral density. For the short time period, we examined tissues from trained-only rats (TRHF rats) that had undergone initial training for 10 min/day, 5 days/week, for 5 weeks, at progressively increasing force loads, until they had learned the HRHF task; these rats euthanized immediately after this training period. For the prolonged task, we examined tissues from rats that had performed the HRHF task for 2 hours/day, 3 days/week, for 12 weeks (HRHF rats), after the earlier training period. We hypothesized that the initial training period would induce increased osteoblasts and bone adaptive changes in the TRHF

rats due to the short-term progressive nature of this training regimen (described further in the methods), and that long-term performance of the HRHF task would induce bone resorptive changes in the HRHF rats due to an inflammatory response induced by prolonged bone loading at high cyclical rates and high force loads. To test this hypothesis, we treated subsets of rats daily with anti-inflammatory doses of oral ibuprofen with the hopes of shifting the balance of bone turnover away from inflammation-induced net bone resorption towards bone maintenance and perhaps even adaptation, despite continued bone loading at high force loads. We also examined other factors suggested in the literature as contributors to bone changes, including nerve compression and dysfunction, and muscle injury and weakness. This study will elucidate the extent of forearm trabecular bone changes occurring as a consequence of a high demand upper extremity occupational tasks, and the contributions of load versus inflammation.

Materials and methods

Animals and overview

All experiments were approved by the Temple University Institutional Animal Care and Use Committee and were in compliance with NIH guidelines for humane care and use of laboratory animals. A total of 113 young adult, female, Sprague-Dawley rats (2.5 months of age at onset of experiments) were used. All were food-restricted to body weights of 5% less than age-matched normal controls (the latter were used for weight-matching purposes only). These rats were randomly divided into groups as shown in Figure 1A: 1) food-restricted rats that underwent an initial training period for 10 min/day, 5 days/week, for 5 weeks to learn a high repetition high force reaching and pulling task (TRHF rats); 2) food restricted rats that trained to learn the task and then performed the high repetition high force task for 2 hours/day, 3 days/week, for 12 weeks (HRHF rats); and 3) food-restricted control rats that did not undergo training or task performance (FRC). Subsets were treated with oral ibuprofen: HRHF+IBU, TRHF+IBU and FRC+IBU. All rats were housed in a central animal facility in separate cages with a 12 hour light:dark cycle, and free access to water and environment enrichment toys. In addition to 45 mg food pellet rewards (a 1:1 mix of purified grain and banana flavored pellets; Bioserve, NJ, USA), all rats received Purina rat chow daily (i.e. same food reward and chow rations). Since rats were 2.5 months of age at onset, and 4 or 7 months of age at the point of tissue collection (TRHF were 4 mo, and FRC and HRHF were 7 mo of age at euthanasia), all were allowed to gain weight as a consequence of normal growth (Figure 1B).

Adult female rats were used in this study for several reasons: (1) Human females have a higher incidence of work-related musculoskeletal disorders than males^{4,5,42}; (2) human females are three times more likely to have carpal tunnel syndrome than males⁴³, although the Bureau of Labor Statistics has determined that carpal tunnel syndrome is less likely to be captured on OSHA logs and reported in the Survey of Occupational Injuries and Illnesses (SOII) than other work place injuries and is there-

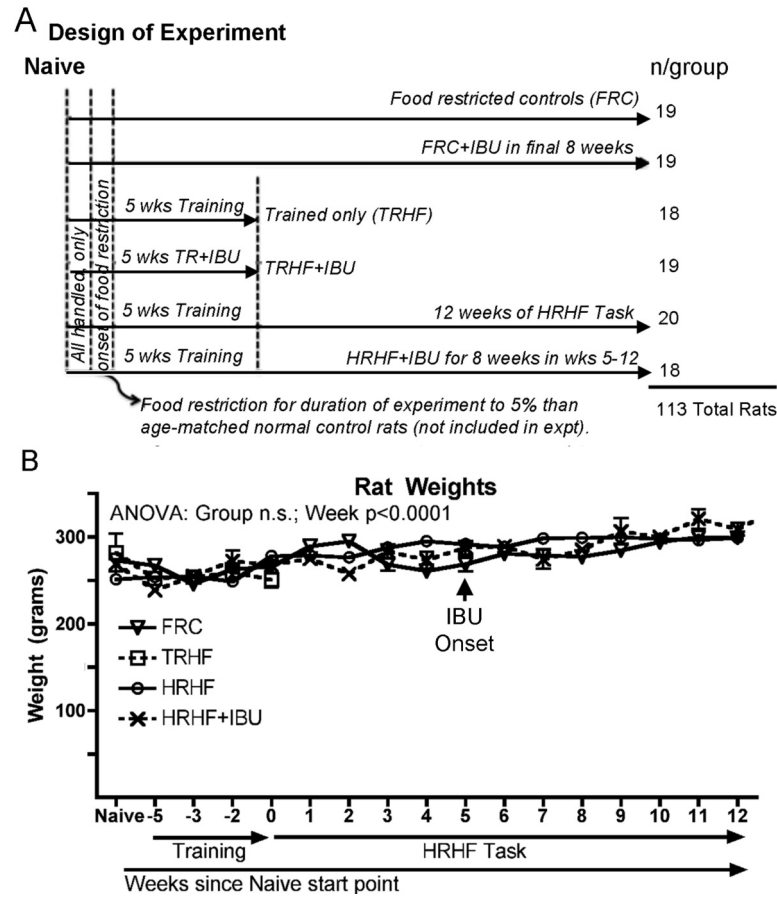


Figure 1. Experimental design and rat body weights over time. (A). Experimental design showing onset of food restriction following a 1 week period of daily handling. All rats were food restricted to 5% less than weights of age-matched normal control rats. Food-restricted control (FRC) rats rested until euthanasia at matched time points as HRHF rats. Trained and task rats underwent a 5 week training period (rats reached the HRHF level by last week of training). Trained only rats (TRHF) were euthanized after training. Task rats performed a high repetition high force (HRHF) task for 12 weeks. FRC+IBU rats received daily ibuprofen (IBU) treatment in final 8 weeks, as did HRHF+IBU rats (arrow indicates onset of ibuprofen treatment). TRHF+IBU rats received ibuprofen treatment prophylactically during training. Number/group shown at far right. (B) Rats were weighed weekly from the naive time point to euthanasia, and showed an increase in weight across weeks of experiment. No differences in weight were observed between groups at matched time points (n.s.= not significant).

fore under-estimated⁴⁴; and (3) for comparison to data from our past studies on female rats using this model.

Behavioral apparatuses, training and task regimens

The custom-designed behavioral apparatuses used were as previously described and depicted^{38,45}. The rats reached through a shoulder height portal and then isometrically pulled on a 1.5 mm metal bar, termed a force lever, that is attached to a load cell (Futek Advanced Sensor Technology, Irvine, CA) positioned 2.5 cm outside of the chamber wall. The bar was oriented vertically. The load cell output was interfaced with a signal conditioner (Analog Devices, Norwood, MA), which amplified and filtered the signal before it was sampled digitally at 100 Hz with Force Lever software (Med Associates, St. Albans, VT). The load cell was interfaced with custom written Force-Lever software that allowed us to choose a set force

level before a food reward was provided (version 1.03.02, Med Associates, St. Albans, VT). Every 15 seconds, a series of auditory indicators (Stimulus Clicker; Med Associates, St. Albans, VT) lasting 5 seconds cued the animal to attempt a reach. During this period, the animal was trained to grasp the force lever bar and pull toward the chamber wall with a graded effort of a percentage of the maximum grip strength of control rats (rats not included in this study) for at least 50 milliseconds^{38,46}. If reach and force criteria (defined below) were met within a 5 second cueing period, a 45 mg food pellet was dispensed into a trough located at floor height for the animal to lick up.

Prior to the initiation of the experiments, all rats were handled for 10 minutes/day for 1 week. All 113 rats were initially food-restricted for 7 days to no more than 10-15% less than their naive weight to initiate interest in the food reward pellets (a 1:1 mix of grain-based and banana-flavored pellets). After

that week, they were given extra rat chow to gain weight quickly back to only 5% less than age-matched normal control rats. Rats were weighed weekly, maintained at 5% less than age-matched normal controls until euthanasia, and allowed to gain weight during the study since they were young adult rats. Thirty-eight of the food-restricted rats were randomly chosen as food-restricted controls (FRC) that rested until euthanasia at time points matched to the HRHF rats (Figure 1A). The remaining food-restricted rats were randomly chosen to be TRHF or HRHF rats.

With regard to the initial training period, seventy-five food-restricted rats were trained to learn the HRHF reaching and lever-pulling task during a 5-week period of 10 min/day, 5 days/wk. During this period, the rats moved through several stages of training. First, in week 1 of training, they were placed in a plastic box outfitted with a piece of Plexiglas with a portal located at shoulder height connected to a small plastic trough. In this chamber, the rats were introduced to the 45 mg food pellets that served as food reward (a 1:1 mix of grain-based and banana-flavored food pellets). Using calibrated force transducers, we estimate that retrieval of the 45 mg food pellets required a negligible amount of force (<5% of the rats' maximum grasping force). When the rats learned to reach (without a specified reach rate) into a trough for the food pellets, a time period of typically 3 days, they were moved to the custom-designed operant conditioning chambers (Med Associates, St. Albans, VT), depicted previously⁴⁵. In the operant chambers, rats learned with the aid of auditory and light cueing to reach through a shoulder-height portal within the apparatus to isometrically pull the force lever attached to a force transducer. Using a magazine style training, in which the force lever started within the chamber for the remainder of week 1, the rats learned to grasp the force lever bar and exert an isometric pull of approximately 1% of their maximum voluntary force, without any specified repetition rate for a food reward. In week 2, the force lever bar was moved to 2.5 cm outside of the chamber and the rats were required to grasp the force lever and exert an isometric pull of 0.10 Newton's (11 grams; approximately 5% of their maximum voluntary pulling force) without any specified repetition rate for a food reward. In week 3, they were required to pull at 0.29 Newton's (30g; approximately 15% of their maximum voluntary pulling force), without any specified repetition rate for a food reward, and in week 4, at 0.58 Newton's (60g; approximately 30% of their maximum voluntary pulling force). By the end of the 5 week training period, the rats were able to perform the HRHF task of four reaches/min at 1.93 Newton's (11g; approximately 60% of their maximum voluntary force). The TRHF rats reached this HRHF level only during the last days of their 5th week of training. After training, rats were randomly divided into TRHF only rats (n=37) and HRHF (n=38). TRHF rats were euthanized at this point (Figures 1A,B).

The remaining trained rats went on to perform the HRHF task regimen (the 38 HRHF rats) for 2 hrs/day, 3 days/wk for up to 12 weeks. The daily task was divided into four 30-minute sessions separated by 1.5 hrs each in order to avoid satiation. HRHF rats were cued to reach at a rate of 8 reaches/min and

to grasp the force lever bar at a target force effort of $60\% \pm 5\%$ of the mean maximum pulling force (which equals 120 to 128 grams, or 1.17 to 1.25 Newton's). HRHF rats had to grasp the force lever bar and exert an isometric pull at the target level for at least 50 milliseconds to receive a food reward.

Limb dominance was not evident until after the training period, but was evident during the task regimen, and was recorded thereafter. Rats were allowed to use their preferred limb to reach (the "reach" limb), and their contralateral limb as support against the operant chamber wall while pulling (the "support" limb), as described and depicted previously⁴⁵. For the purpose of this study, tissues were collected and assayed from only the dominant, preferred, reach limb, since our purpose was to assess the effects of reaching and grasping repetitively on bones.

Ibuprofen treatment

At the end of the 4th week of task performance, 18 HRHF rats were administered ibuprofen (Children's Motrin Grape Flavored, Johnson & Johnson) in drinking water daily (45 mg/kg body weight), and are termed HRHF+IBU rats, as described previously³⁵. Nineteen FRC rats received oral ibuprofen in their final 8 weeks (FRC+IBU); 19 TRHF rats received ibuprofen beginning at the onset of training (TRHF+IBU). Ibuprofen dosing was tracked as 48.8 ± 6.3 mg/kg body weight for each rat by measuring the difference between the initial and final volume of suspended solution daily, and by assaying serum levels of ibuprofen (National Medical Services, Willow Grove, PA). The dose used was lower than the maximum limit for gastrointestinal toxicity in rats, yet was a dose shown to be effective in reducing chronic inflammation^{35,47}. No adverse changes in behavior suggestive of abdominal discomfort or pain were observed in the ibuprofen-treated rats (such as hunching or licking of their abdomen, or reduced food intake). Stomachs were collected from five of the HRHF+IBU rats, and examined histologically. No evidence of thinning of the stomach lining was observed.

Voluntary pulling force

Force lever data were recorded continuously during each task session for later calculation of the pulling force by the custom written Force-Lever software (version 1.03.02, Med Associates) during each task session for later calculation of voluntary pulling force via an automated script (MatLab; Mathworks, Natick, MA). Mean voluntary grasp force was the average force (in grams) applied to the force lever bar during a reach. Voluntary grasp force data were obtained from: 26 HRHF animals at week 1, 18 rats in weeks 3 and 6, and 10 rats in week 12; and 10 HRHF+IBU animals in weeks 1, 3, 6 and 12. Week 1 of the HRHF task was used as the baseline for grasp force since that was the first week that task rats actually performed the task regimens.

MicroCT analysis of the distal radius and ulna

Animals were euthanized by lethal overdose (Nembutal, 120 mg/kg body weight) at 18 hours after the last task session in

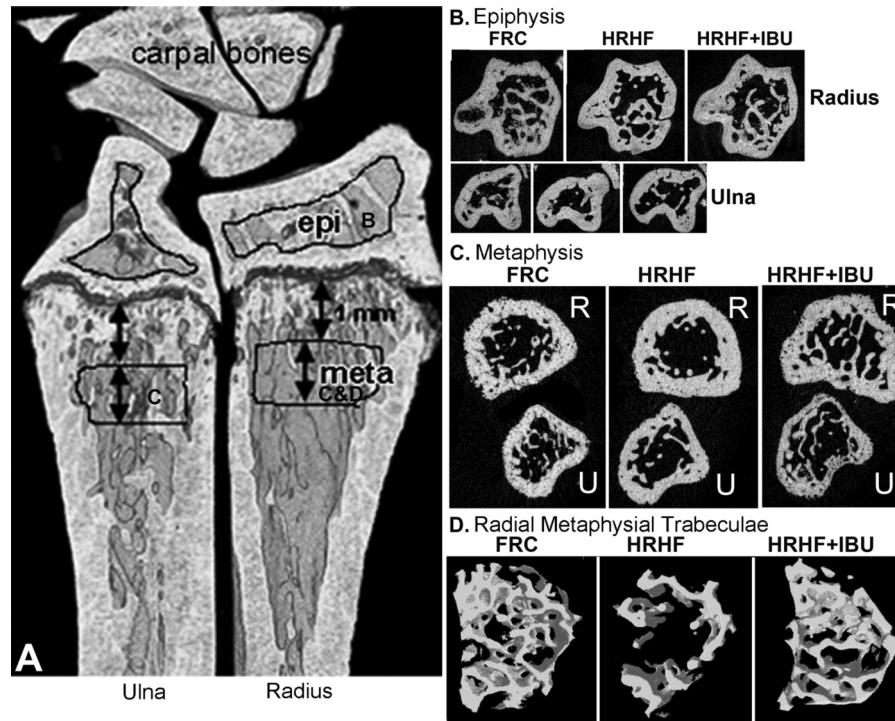


Figure 2. MicroCT images of distal radius and ulna. (A) A representative 3D model of the distal radius and ulna, cut sagittally, showing the site of the epiphyseal (epi) and metaphyseal (meta) analyses (encircled by black lines), and the sites of the images shown in panels B-D, as indicated by the letters B, C and D. (B) Representative transaxial microCT slices of the epiphysis of the radius and ulna (at 27 slices, 243 micrometers from the proximal edge of their respective growth plates) from a food restricted control (FRC), a 12-week high repetition high force (HRHF), and 12-week HRHF rat treated with ibuprofen for 8 weeks (HRHF+IBU). (C) Representative transaxial microCT slices of the metaphysis of the radius and ulna (at 166 slices, 1.5 millimeters from the distal edge of their respective growth plate) from a FRC, HRHF, and HRHF+IBU rat. (D) Representative 3D models of transaxial microCT slices through the metaphyseal region of the left radius (R) of a FRC, HRHF, and HRHF+IBU rat, with the surrounding cortical bone segmented away from the cortical bone in order to show the trabecular architecture. The transaxial reconstructions are located from 1.0 to 2.0 mm proximal to the respective growth plates, and are viewed from the bottom looking towards the growth plate. Increased separation and loss of trabeculae are seen in the HRHF rats, changes not present in FRC or HRHF+IBU rats (panel D).

order to avoid exercise-induced changes in serum cytokines. Serum collected for biochemical assays (described more below), then perfused transcardially with 4% paraformaldehyde in 0.1M PO₄ buffer (pH 7.4). For this assay, forelimb bones were collected, cleaned of soft tissues, and washed in phosphate buffered saline, from the reach limbs of TRHF (n=6), TRHF+IBU (n=7), 12 wk HRHF (n=8), and 12 wk HRHF+IBU (n=5) rats; as well as limbs of FRC (n=7) and FRC+IBU (n=6) rats. A Skyscan 1172, 12 MPix model, microCT scanner (Bruker-microCT, Kontich, Belgium) was used to image a 6 mm length of the distal radial and ulna bones (1000 slices), using a modification of previously described methods⁴⁸⁻⁵⁰: an isotropic voxel resolution size of 9 μm, source voltage of 59 kV, source current of 167 μA, Al 0.5 mm filter, rotation step of 0.40°, frame averaging of 4, ring artifact correction of 10, and beam hardening correction of 60%. After scanning, 3D image data were reconstructed using Skyscan NRecon software. Using Skyscan CTAn software, the volume of interest for trabecular microarchitectural variables was bounded to the endocortical margin. For the epiphyseal trabeculae, morphologi-

cal traits were assessed starting 0.2 mm distal to the growth plates, and then extending from this position distally for 0.50 mm (56 slices, or until the edge of the subchondral cortical bone was reached; Figure 2A), as described in Bouxsein, 2010⁵¹. For the distal metaphyseal trabeculae, morphological traits were assessed starting 1 mm proximal to the growth plates, and then extending proximally from this position for 1 mm (112 slices; Figure 2A). Upper and lower thresholds of 255 (max) and 75 were used to delineate each pixel as “bone” or “nonbone”. Trabecular morphometric traits were computed from binarized images using direct 3D techniques that do not rely on prior assumptions on underlying structures, and trabecular bone volume per total volume (BV/TV), mean trabecular thickness (Tb.Th.), mean trabecular number (Tb.N.), and mean trabecular separation (Tb.Sp.) indices were computed.

For assay of bone mineral density (BMD), a set of 3 calcium hydroxyapatite phantoms of rat bones was scanned (partially in air; partially in water) using the same settings as for the bones. Hounsfield units for the phantoms, air and water were calculated and then used to compute volumetric BMD of the

segmented metaphyseal trabeculae with surrounding marrow space for the radius of animal analyzed using microCT.

Histomorphometry of the distal radius metaphysis

Subcohorts of the above bones were then used for histomorphometry: FRC (n=5), FRC+IBU (n=6), TRHF (n=6), TRHF+IBU (n=6), 12 wk HRHF (n=9), and 12 wk HRHF+IBU (n=9) rats (reach limbs were used for the TRHF and HRHF rats). Bones from all rats but 3 of the 12 wk HRHF and 3 of the HRHF+IBU were processed and embedded in methyl methacrylate (MMA), sectioned into 3 µm thick longitudinal sections and mounted onto slides, as described previously³⁵. The remaining bones (3 HRHF and 3 HRHF+IBU) were processed and embedded in paraffin, sectioned into 5 µm thick longitudinal sections and mounted onto slides, as a consequence of other experiments in which immunohistochemical probing of the bones was needed. Slides were stained with Goldner's Trichrome for counting osteoblasts, or immunohistochemically for Trap5 and ED1 for counting osteoclasts (ED1 is a marker of osteoclasts, macrophages and their progenitors; only multinucleated ED1+ cells were counted in this study), as described previously³⁴. Numbers of cells per bone surface (N.Ob./BS and N.Oc./BS) were counted in the same region as assayed using microCT using a Nikon E800 microscope interfaced with a Q-Imaging digital camera, and an image analysis system (Bioquant Osteo 2012, v12.1). The person carrying out the histomorphometry was blinded to treatment.

Muscle histomorphometry

Flexor digitorum muscles were collected from the same rats as for microCT. After transcardial fixation and then immersion fixation for at least 24 hours, a 3 mm thick cross-sectional piece of the flexor digitorum muscle was cut, with a scalpel, from the proximal end of the muscle mass. The muscles were cryosectioned into 12 µm sections, placed onto charged and coated slides (Fisher Scientific, Tissue Path Superfrost Plus Gold Slides), dried, and stained with Hematoxylin and Eosin (H&E). The cross-sectional area (CSA) of each myofiber fascicle was quantified using a Nikon microscope interfaced with a digital camera and image analysis system (Bioquant Osteo 2012, v12.1), as described previously⁵². An average of 82 fascicles were counted per muscle. The individual measuring the areas was blinded to group assignment. The muscle sections were also examined for signs of histopathological changes after staining with the ED1 antibody. Muscles were defined as having micro-damage by the presence of atrophied myofibers and the presence of ED1+ macrophages within myofibers (internal) in the same tissue section, as described previously⁵³. The number of ED1+ macrophages within atrophied myofibers (internal) were counted in the mid-belly region of the flexor digitorum longus, using previously described semi-automated quantification methods and an image analysis system (Bioquant) connected to a Nikon E800, at three microscope field locations per rat⁵⁴.

Histomorphometry of median nerve inflammation

To examine the median nerve inflammation, flexor forelimb tissues were collected from the same rats and limbs as above

as a flexor mass, postfixed "en bloc" (nerves still intact) by immersion overnight, cryosectioned, immunostained on slides for ED1+ macrophages, as described previously⁵⁵. The number of ED1+ macrophages were quantified in 3 regions and sections per nerve using a Nikon E800 microscope linked to an image analysis system, as described previously³⁹. Adjacent sections were stained with Hematoxylin and Eosin (H&E) and examined for peripheral nerve pathology, such as increased extraneural collagen deposition (indicative of extraneural nerve fibrosis), presence of axonal swelling (indicative of axonal compression), myelin degradation visible using H&E, and visible signs of nerve disruption and nerve compression.

Biochemical analyses of serum, radius and ulna

To study serum biomarkers of bone turnover, animals were euthanized with an overdose of sodium pentobarbital (Nembutal; 120 mg/kg body weight) at 18 hours after completion of the final task session to avoid exercise induced increases in inflammatory cytokines. Blood was collected by cardiac puncture using a 23-gauge needle from: FRC (n=6), FRC+IBU (n=6), TRHF (n=9), TRHF+IBU (n=9), 12 wk HRHF (n=6), and 12 wk HRHF+IBU (n=6) rats. Blood was centrifuged at 1,800 g for 20 min at 4°C, serum collected and flash-frozen, and stored at -80°C until analyzed using commercially available ELISA kits for: Trap5b (band 5 tartrate-resistant acid phosphatase; a biomarker of osteoclast activity and bone resorption; Immunodiagnostic systems, Fountain Hills, AZ; #SB-TR102), CTX1 (the C-terminal telopeptide of Collagen type I cleaved by osteoclasts during bone resorption; Immunodiagnostic systems, RatLaps, # AC-06F1), osteocalcin (a non-collagenous protein secreted by osteoblasts; Nordic Bioscience Diagnostics, Herlev, Denmark, #Rat-MIDTM Osteocalcin). Two pro-inflammatory cytokines, interleukin (IL) beta and tumor necrosis factor alpha (TNF-alpha) were assayed using ELISA kits from (Aushon, Pierce), as described previously³³. Serum samples were tested in duplicate and presented as pg of protein per ml of serum.

Radius and ulna bones were collected for biochemical assays from: FRC (n=6), FRC+IBU (n=5), TRHF (n=5), TRHF+IBU (n=4), 12 wk HRHF (n=5), and 12 wk HRHF+IBU (n=6) rats. Reach limbs were used for the TRHF and HRHF rats. Distal regions (which included the epiphysis, growth plate and metaphysis) and diaphyseal regions were combined and the bones were assessed for IL-1beta and TNF-alpha using commercially available ELISA kits (BioSourceTM, Invitrogen Life Sciences, CA), as described previously³³. ELISA assay data (pg cytokine protein) were normalized to µg total protein, determined using a bicinchoninic acid protein assay kit.

Electrophysiological testing of median nerve conduction velocity (NCV)

In order to test focal slowing of conduction, NCV was determined for the segment of the median nerve that passes beneath the transcarpal ligament in the preferred reach limbs of anesthetized rats: HRHF (n=7) and HRHF+IBU (n=6) rats. NCV was also determined bilaterally in FRC (n=6), FRC+IBU

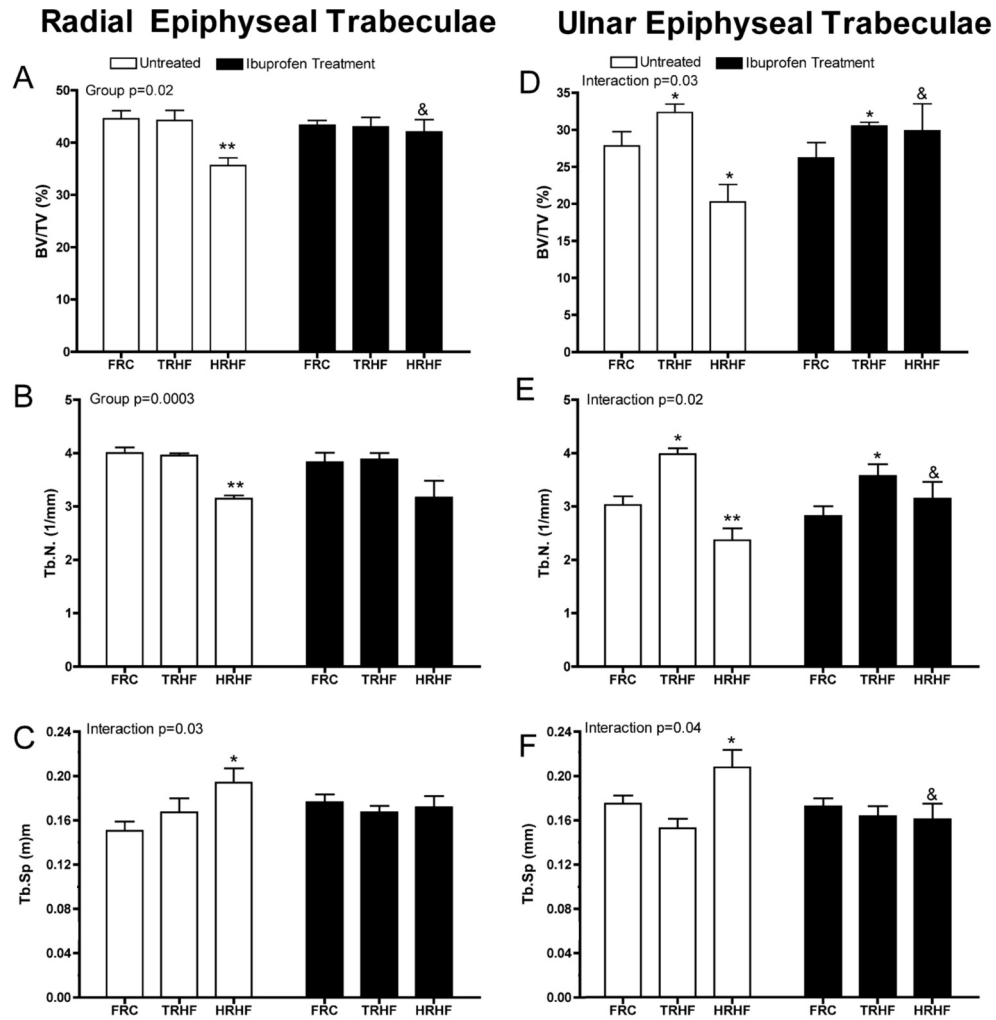


Figure 3. MicroCT analysis of trabeculae of distal radial (A-D) and ulnar epiphyses (E-H). Results for trabecular bone volume (BV/TV), trabecular number (Tb.N.), and trabecular separation (Tb.Sp.) are shown. Significant 2-way ANOVA findings shown in individual panels and hereafter. Symbols: *:p<0.05 and **:p<0.01, compared to FRC rats; &: p<0.05, compared to untreated HRHF rats.

(n=9), TR-HF (n=7) and TR+IBU (n=8) rats. The method for measurement of NCV of the median nerve in rats matched that described previously⁴¹. Both the surgeon and the person carrying out the recordings and data analysis were blinded to rat treatment. Serum was not collected from rats that underwent the NCV testing to avoid confounding interpretation of results by changes induced by this surgical procedure.

Statistical analyses

To determine differences in weight, grasp force and grip strength, a mixed-model, two-way ANOVA was used with the factors: week of task performance (weekly weights from naive to euthanasia) and group. For bone microCT variables, cell counts, cytokine data, and electrophysiological variables, two-way ANOVAs were used with the factors: group and treatment (untreated versus ibuprofen). A one-way ANOVA was used to

compare BMD changes to FRC rats. For each, the Bonferroni post-hoc method for multiple comparisons was used, with comparisons to FRC or FRC+IBU data, as appropriate for the treatment group. Adjusted p-values are reported, and after adjustment, a p value of <0.05 was considered statistically different. All data are expressed as mean ± standard error (SEM).

Results

Rat weights

A two-way ANOVA indicated that there were no significant differences in weight between the trained or task groups, compared to FRC rats (not significant). All rats gained weight in a consistent manner across the weeks of the experiment (week p<0.0001) (Figure 1B). Thus, any changes observed in bone structure were not due to changes in body weight.

Short-term loading leads to bone adaptation (TRHF rats), while long term loading at HRHF leads to bone resorption in distal epiphyseal trabeculae

Changes in trabecular microarchitecture that could be caused by increased bone adaptation was observed in the distal ulna of TRHF rats, and changes that could be caused by bone resorption were detected in the distal radial and ulnar epiphyses of 12-week HRHF rats (Figures 2A,B & Figure 3; significant 2-way ANOVA findings are indicated in figures). Specifically, increased trabecular bone volume density (BV/TV) and trabecular number (Tb.N.) were observed in the ulna epiphysis of TRHF rats (rats that underwent training only), compared to FRC rats (Figures 3D,E; post hoc findings are indicated in figures), indicative of bone adaptation. In contrast, both radial and ulnar epiphyses of 12-week HRHF rats show decreased trabecular BV/TV and Tb.N., and increased trabecular separation (Tb.Sp.), compared to FRC rats (Figures 3 A-C,F), indicative of bone resorptive changes. Ibuprofen treatment did not improve the ulnar epiphyseal adaptive changes in TRHF rats, but did prevent bone resorptive changes in the radius and ulna epiphyses of HRHF+IBU rats (Figures 3 A-F). The preventive effects of ibuprofen treatment are also indicated by the significant group x treatment interactions in radial Tb.Sp. (Figure 3C), and ulnar BV/TV, Tb.N., and Tb.Sp. (Figures 3D-F). Trabecular thickness was not affected by training, task performance or ibuprofen treatment (data not shown). Thus, bone adaptation was observed in ulnar epiphyses of untreated TRHF rats; however, resorptive changes were observed in radial and ulnar epiphyses of untreated HRHF rats, changes prevented by ibuprofen treatment.

Trabecular resorption and limited adaptation in distal metaphyseal trabeculae with HRHF loading

No significant changes were observed in the distal metaphyseal trabeculae of TRHF rats. In contrast, high force loading-induced resorptive changes were apparent in radial and ulnar metaphyseal trabeculae of HRHF rats (Figures 2A,C,D; Figures 4A-E,G,H), yet there were also limited signs of bone adaptation in the radial metaphysis in the HRHF rats (Figure 4B). Specifically, in the distal radial metaphysis, HRHF rats had decreased trabecular BV/TV and Tb.N., as well as increased Tb.Sp, compared to FRC rats (Figures 4A,C,D), indicative of bone resorptive changes. The distal ulnar metaphysis of HRHF rats had similar changes, with decreased trabecular BV/TV and Tb.N., and increased Tb.Sp., compared to FRC rats (Figures 4E,G,H). Bone adaptive changes were also present in HRHF rats, in the form of increased Tb.Th. in the distal radial metaphysis (Figure 4B). Bone resorptive changes were not present in HRHF+IBU rats (Figures 4A,C-E,G,H). Ibuprofen treatment prevented BV/TV and Tb.Sp. declines in the distal radial metaphysis (Figures 4A,D), and enhanced Tb.Th. in the distal ulna metaphysis (Figure 4F). The radius of HRHF+IBU rats showed a similar increase in Tb.Th. as that of untreated HRHF rats (Figure 4B). The preventive effects of ibuprofen treatment are further indicated by the significant effects of treatment on radial BV/TV (Figure 4A), and

on the ulnar Tb.Th. (Figure 4F). Thus, bone resorptive changes were observed in radial and ulnar metaphyses of untreated HRHF rats, changes prevented by ibuprofen treatment in HRHF+IBU rats.

Loading-induced increases in osteoclasts and osteoblasts

We next investigated the metaphyseal radius in sectioned bones for changes in osteoclast and osteoblast numbers. Osteoclast numbers were highest in HRHF rats, compared FRC (Figure 5A), an increase prevented by Ibuprofen treatment of TRHF+IBU and HRHF+IBU rats (a finding further bolstered by a significant group x treatment interaction effect on osteoblast numbers). Osteoblasts numbers were increased in TRHF and HRHF rats, compared FRC, and similarly in TRHF+IBU and HRHF+IBU rats, compared to FRC+IBU (Figure 5B), but no additional effect from ibuprofen treatment. Thus, ibuprofen treatment reduced osteoclast numbers in HRHF+IBU, but did not affect osteoblast numbers.

Bone mineral density decreased in HRHF rats

Bone mineral density (BMD) was decreased in radial metaphyseal trabeculae of HRHF rats, compared to FRC rats (Figure 5C). This decline was prevented by ibuprofen treatment (HRHF+IBU).

Serum biomarkers indicate bone formation with training, but bone resorption with HRHF task

To confirm the micro-CT results, serum biomarkers of bone turnover were analyzed (Figures 5D-F). With regard to indicators of bone resorption, Trap 5b increased significantly in serum of HRHF rats (Figure 5D), while CTX1 increased in both TRHF and HRHF rats (Figure 5E), compared to FRC rats. Ibuprofen treatment prevented the loading induced increase in serum TRAP5b in these rats (Figure 5C). The 4 week treatment of TRHF rats with ibuprofen (TRHF+IBU rats) did not alter their increase in CTX-1 (Figure 5E); in contrast, the 8 week treatment of HRHF+IBU rats prevented an increase of serum CTX-1 in these latter rats. Serum osteocalcin, a marker of bone formation, increased only in TRHF and TRHF+IBU rats, compared to FRC and FRC+IBU rats, respectively (Figure 5F). Thus, training, with or without ibuprofen, induced increased serum biomarkers of both bone formation and resorption; in contrast, HRHF-induced increases of serum biomarkers of bone resorption were prevented by ibuprofen treatment.

Voluntary lever pulling is highest in HRHF+IBU rats

Since a relationship between voluntary grip strength and radial bone mineral density has been established in humans^{21,56-60}, we next examined mean voluntary lever pulling force data collected during task performance as a proxy for muscle loading levels (Figure 6A). The untreated HRHF rats consistently pulled at a high force level of around 92 grams (92.97 ± 2.59 , mean \pm SEM), which is still a high force task as this is approximately 40% of their maximum pulling force, although lower than their target of 120 grams (Figure 6A). In contrast, vol-

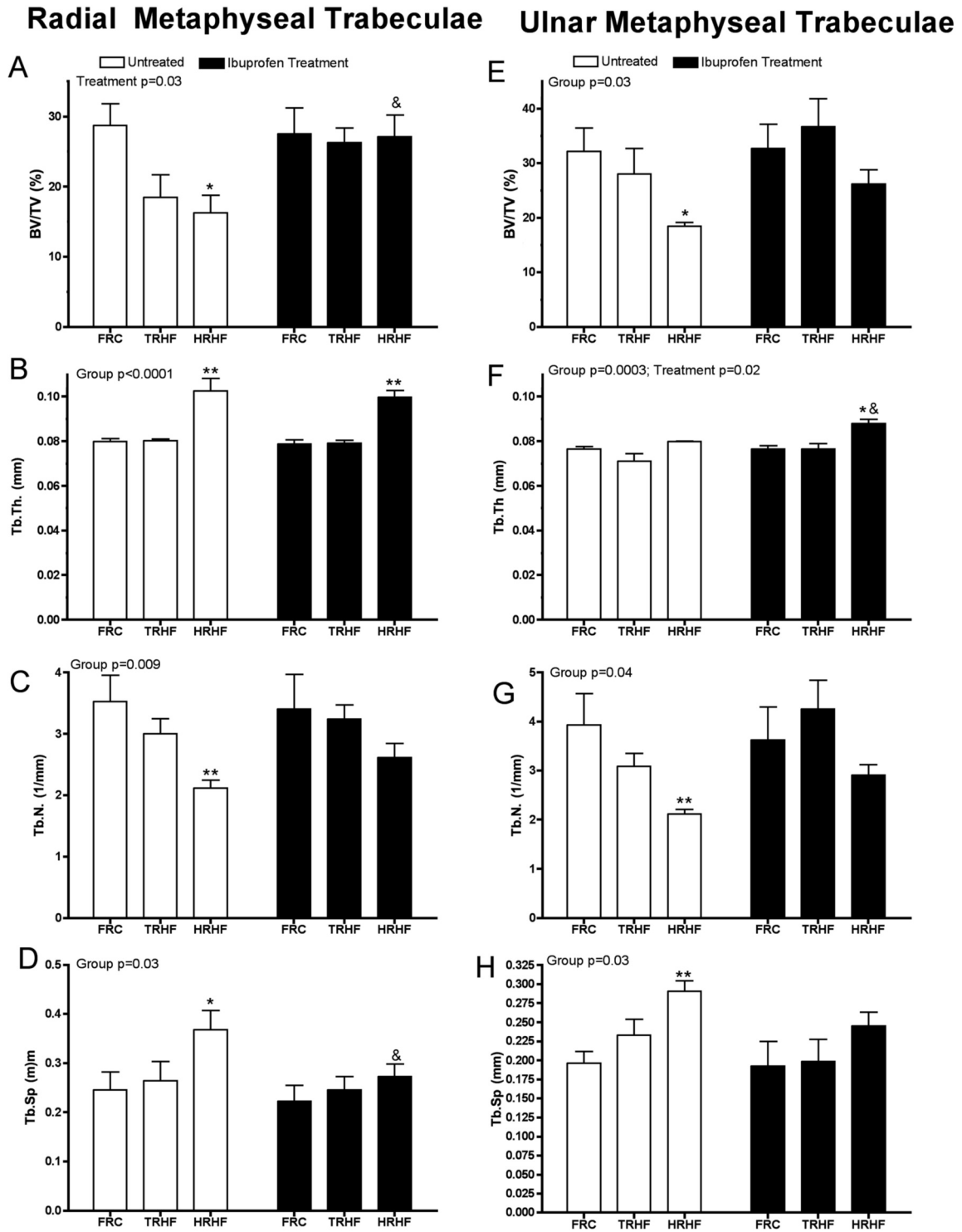


Figure 4. MicroCT analysis of trabeculae of the distal radial (A-D) and ulnar metaphysis (E-H). Results for trabecular bone volume (BV/TV), trabecular number (Tb.N.), trabecular separation (Tb.Sp.) and trabecular thickness (Tb.Th.) are shown. Symbols: *:p<0.05 and **:p<0.01, compared to FRC rats; &: p<0.05, compared to untreated HRHF rats.

untary pulling force increased to target levels in 6- and 12-week HRHF+IBU rats, significantly higher than force levels reached before this treatment. Thus, since HRHF rats had consistent pulling forces across the 12 weeks of task performance,

declines in bone structure in these rats were not the result of changes in muscle function. However, the anti-inflammatory effect by ibuprofen enhanced the rats' voluntary pulling force and likely contributed to bone homeostasis in HRHF+IBU rats.

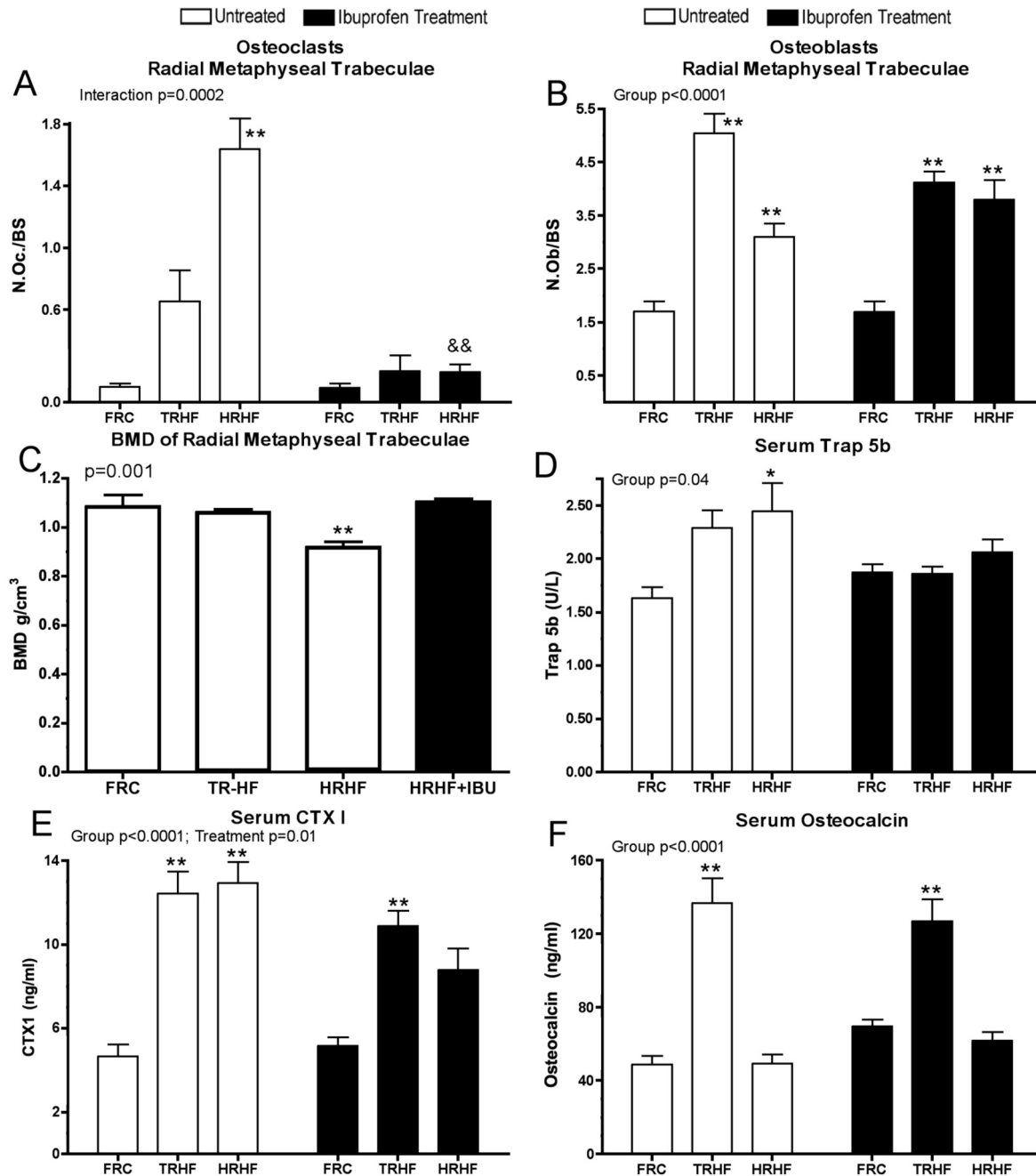


Figure 5. Bone cell histomorphometry and bone mineral density in distal radial trabeculae, and serum bone turnover markers. (A-B) Density of osteoclasts (N.Oc.) and osteoblasts (N.Ob.), normalized to bone surface (BS), of distal radial metaphyseal trabeculae. (C) Bone mineral density (BMD) of radial metaphyseal trabeculae. (D-F) Serum levels of Trap 5b, CTX1 and osteocalcin, assayed using ELISA. Symbols: *:p<0.05 and **:p<0.01, compared to FRC rats; &&:p<0.01, compared to untreated HRHF rats.

HRHF loading, with and without ibuprofen, increases in both cross-sectional myofiber area and subdegenerative myofiber numbers

Exercise-induced changes in muscle are postulated to lead to proportional changes in bone mass⁶¹. Also, repeated small amplitude contractions of muscles with incomplete recovery time between bouts of loading can lead to subdegenerative

muscle injury⁶². Therefore, we first investigated the cross sectional area of myofiber fascicles in flexor digitorum muscles (Figure 6B). Myofiber cross sectional areas were increased similarly in TRHF, HRHF and HRHF+IBU rats, compared to FRC or FRC+IBU rats (Figure 6B), although the range in fiber sizes was much greater in the HRHF and HRHF+IBU rats. Ten percent of the myofibers in the HRHF and HRHF+IBU rats cross sectional areas in the 300 micrometer² range (minimum=

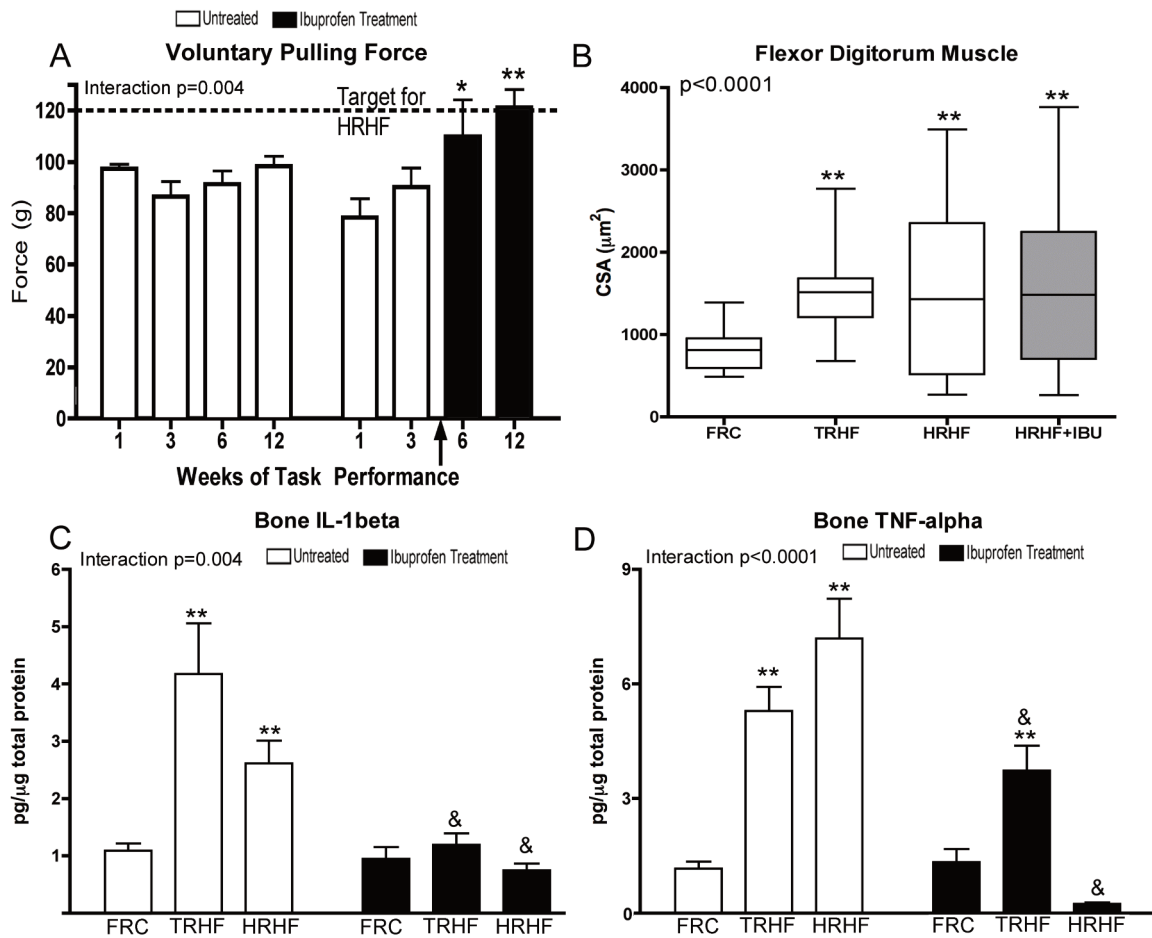


Figure 6. Mean voluntary pulling force, muscle cross sectional area, and bone cytokine levels (serum cytokine levels are provided in the text). (A) Mean voluntary pulling force on the lever bar used for the reaching and grasping task, in grams. Arrow indicates onset of ibuprofen treatment. (B) Mean cross-sectional area of myofibers in flexor digitorum muscle, quantified at mid-muscle level. (C&D) IL-1beta and TNF-alpha in forelimb bones, tested using ELISA. Symbols: *:p<0.05 and **:p<0.01, compared to FRC rats; &:p<0.05, compared to untreated HRHF rats.

264.7 micrometers² in HRHF rats; minimum= 303.4 micrometers² in HRHF+IBU rats), compared to the mean in HRHF of 1705±91.29 SEM, and the mean in HRHF+IBU rats of 1670±80.41. We next counted the number of degenerating myofibers (presence of macrophages within atrophying myofibers). HRHF and HRHF+IBU flexor digitorum muscles had 17±1.0 and 15.85±1.3, respectively, ED1 immunoreactive cells/mm² within atrophying myofibers at the mid flexor digitorum muscle belly, compared to none in the other groups, indicative of a low level of loading-induced myofiber degeneration in each group. Since similar changes in muscle morphology were observed in both the HRHF and HRHF+IBU rats, changes in muscle morphology were not contributing factors to the observed bone changes.

Bone and serum IL-1beta and TNF-alpha increased with HRHF loading; decreased with ibuprofen

Since IL-1beta and TNF-alpha are potent stimulators of bone resorption^{63,64}, and since we have previously observed increases

of both in our model in musculoskeletal tissues and serum with this HRHF task and other task demands^{33,36,40}, their levels were assayed in bone and serum. IL-1beta and TNF-alpha levels were increased in forelimb bones of TRHF and HRHF rats, compared to FRC rats (Figures 6C,D). These increases were prevented by ibuprofen treatment (Figures 6C,D), as indicated by the group x treatment interaction effects of p=0.004 and p<0.0001 respectively. Serum levels of IL-1beta were significantly increased in HRHF rats (107.5±11.04 (mean±SEM)), compared to TRHF and FRC rats (undetectable in each group), an increase prevented by the ibuprofen treatment (IL-1beta was undetectable in TRHF+IBU and HRHF+IBU rats, p<0.01 each; the group x treatment interaction effect was p<0.0001). Serum TNF-alpha was also significantly increased in HRHF rats (645.7±267.8; mean±SEM), compared to FRC rats (3.92±3.92). This increase was prevented by ibuprofen treatment (serum TNF-alpha was 22.66±7.02 in HRHF+IBU rats, p<0.01; the group x treatment interaction effect was p=0.0004). These results indicate that TRHF and HRHF task performance induces

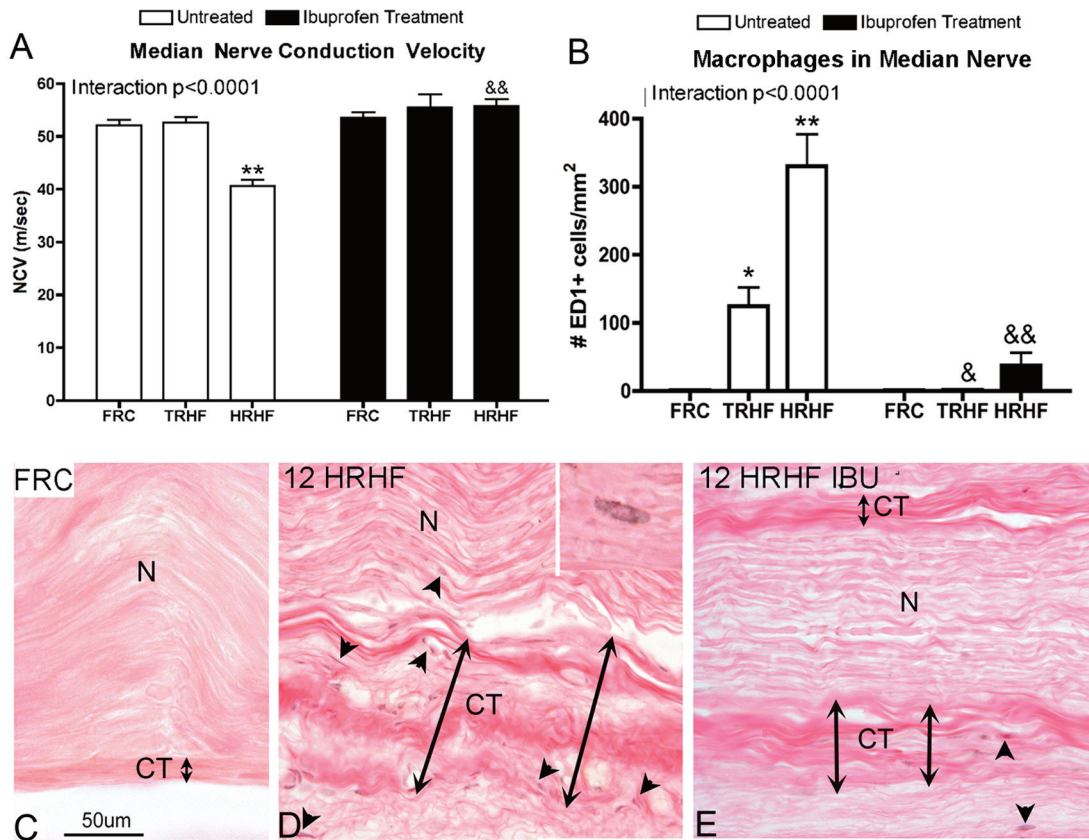


Figure 7. Changes in the median nerve in the carpal tunnel. (A) Nerve conduction velocity (NCV) in meters/second (m/sec), measured electrophysiologically. (B) Mean number of ED1+ in media nerve at the level of the wrist. (C-E) Representative microscopic photos showing width of epineurial connective tissues (CT) located around median nerve (N) at wrist level (double arrows). Arrowheads indicate ED1+ macrophages (detected immunohistochemically). Eosin counterstain. Each photo in C-E was taken at same magnification; thus, the scale bar is the same for panels C through E. Symbols: *:p<0.05 and **:p<0.01, compared to FRC rats; &;p<0.05 and &&;p<0.01, compared to untreated HRHF rats.

production of these inflammatory cytokines both locally in loaded bones, and systemically in serum; these increases were prevented by ibuprofen treatment in the HRHF rats, despite continued task performance, as supported by the significant interaction effects.

Evidence of carpal tunnel syndrome with HRHF task

Next, we examined TRHF and HRHF rats for indicators of carpal tunnel syndrome, since we have previously observed reduced conduction velocity in the median nerve with associated increases in pain behaviors³⁸⁻⁴¹. We observed a low-grade decline of 15% in median nerve conduction velocity (NCV) in HRHF rats that was attenuated by ibuprofen treatment (Figure 7A; the interaction effect was p<0.0001). Ibuprofen treatment also reduced a task-induced increase in the number of ED1+ macrophages in both TRHF+IBU and HRHF+IBU rats, compared to untreated HRHF rats (Figures 7B-E; the interaction effect was p<0.0001). A mild level of extraneural neural connective tissue was evident in HRHF rats (Figure 7D), compared to FRC rats (Figure 7C). This increase was only partially attenuated in the HRHF+IBU, (compare Figures 7E to 7D).

No axonal swellings, myelin degradation visible using H&E staining, or visible nerve disruption, were observed in the median nerves of any group (Figures 7C-D), indicating that frank axonal damage was not present at the time of tissue collection. These results combined with the muscle findings indicate that the 15% reduction in nerve conduction was not great enough to reduce muscle function in HRHF rats (Figure 6A), although the reduced nerve inflammation shown in Figures 7B and E) likely reduced discomfort, and increased voluntary muscle pulling force in HRHF+IBU rats (Figure 6A).

Discussion

In this study, several indicators of trabecular bone adaptation in forelimb bones and increased serum osteocalcin in TRHF rats were observed, indicating that short time periods of progressively increased loading to high force levels by week 5 induced net bone adaptation. In contrast, performance of a HRHF task for 12 weeks (in which voluntary muscle pulling forces remained constant in untreated HRHF rats) led to reduced trabecular bone quality and bone mineral density in dis-

tal forelimb bones, and a net loss of bone. Systemic anti-inflammatory treatment with ibuprofen prevented these bone catabolic changes. Ibuprofen treatment also prevented HRHF-induced increases in bone and serum inflammatory cytokines, osteoclast numbers, and nerve inflammation, as well as HRHF-induced declines in median nerve conduction velocity. The mean voluntary pulling force was increased in the HRHF+IBU rats, compared to FRC+IBU rats. In contrast, morphological changes in the flexor digitorum muscle were similar in both HRHF and HRHF+IBU rats, with each showing similar mean increases in myofiber cross-sectional areas and low numbers of atrophied myofibers with internal macrophages. No groups showed visible signs of median nerve injury, or differences in weight loss. The similar changes in muscle and nerve morphology, and body weight in the HRHF and HRHF+IBU rats rules out these as contributing factors to the observed changes in bone structure. Furthermore, the constant level of pulling forces in the untreated HRHF rats, rules out muscle function as a contributing factor to the observed changes in bone structure in untreated HRHF rats. Since there were many significant effects of group x treatment, these results suggest that bone catabolism in the untreated HRHF rats was the results of increased osteoclasts and inflammatory cytokines. Eight weeks of continual ibuprofen treatment prevented this catabolism, by reducing, nerve and bone inflammatory responses, and by reducing osteoclast numbers and activity, despite continued task performance. The improved muscle pulling forces and preservation of nerve conduction velocity in HRHF+IBU rats were not due to HRHF specific morphological changes in muscle or nerve, but were likely related to the prevention of median nerve inflammatory responses in HRHF+IBU rats.

It was interesting to see that short-term loading in the TRHF rats induced bone adaptation; evident as increased trabecular bone volume and number in the distal ulna epiphysis, increased osteoblasts in the distal radial metaphysis, and increased serum osteocalcin. In contrast, only a few indices of bone resorptive changes were observed in the TRHF rats (increased osteoclasts and serum CTX1). Thus, bone adaptive responses were concomitant with resorptive responses, with the former being greater, by the end of the training period in TRHF rats. During training, rats work for 10 min/day, 5 days/week, for 5 days to learn the HRHF task, which translates as ramping upwards from no reaching at all initially, to first 1% maximum pulling force by the end of week 1, 5% (0.1 Newton's) by the end of week 2, 15% (0.29 Newton's) by the end of week 3, 30% (0.58 Newton's) by the end of week 4, and 60% maximum pulling force (1.93 Newton's) by the end of week 5. This progressive level of activity was apparently enough to stimulate net bone formation, despite the increase in bone inflammatory cytokines observed in TRHF rats after the training period. These results match our prior findings of increased production of a matrix protein (periostin-like-factor) associated with tissue repair concomitant with bone adaptation in TRHF rats and with 3 to 9-weeks of HRHF loading³⁷, although this protein decreased coincident with prolonged high levels of several inflammatory cytokines and increased serum TRAP5b

in 12-week HRHF rats^{35,65}. We have also observed qualitative signs of bone adaptation in rats performing a moderate demand task of high repetition and low force for 12 weeks³⁴. Collectively, these results from our model indicate that performance of moderate demand occupational tasks for 12 weeks, or high demand tasks for short time periods, leads to bone adaptation.

In contrast, we observed only limited adaptation in the 12-week HRHF rats (only increased trabecular thickness and osteoblasts in the distal radial metaphysis), rats that were performing a high force loading task of 42% of their maximum pulling force (high force loading being defined as >30% of maximum pulling forces^{6,66}). Instead, many indicators of bone resorption were observed in the HRHF rats, including decreased trabecular bone volume density in the radial and ulnar epiphyses and metaphyses, increased osteoclasts and decreased bone mineral density in the distal radius, increased serum Trap5b and CTX1, and increased inflammatory cytokines both locally (in these bones) and systemically. Thus, indices of osteoclast-related bone resorption were greater than adaptive changes in the untreated 12-week HRHF rats, leading to net bone resorption.

Results of this study are consistent with these past studies, and support a cumulative overload hypothesis in bones leading to a reduction of tissue tolerance with continued overloading^{6,7,67-69}. Bone is known to respond to loading along a continuum ranging from anabolism to catabolism, depending on the magnitude, frequency and duration of loading^{9-11,70-72}. Repetitive loading conditions, such as in studies of rats running on treadmills, performing repetitive jumping, and performing repetitive loading at high force loads, show that increasing the intensity of weight-bearing or muscle loading exercise/activities may be associated with diminishing returns in bone morphology, such as declines in bone mass and quality^{12,37,73-76}.

Increased levels of bone IL-1beta and TNF-alpha, such as seen in this study, are known to stimulate osteoclastogenesis and activity^{23,24}. A strong role for task-induced inflammatory processes in muscle and bone tissues is supported in our model. We have previously shown that a 2-week treatment with anti-rat TNFalpha or an 8-week treatment with ibuprofen reduces a HRHF-induced increase in bone inflammatory cytokines, and enhances voluntary muscle loading (i.e., the voluntary pulling forces, as shown here)^{35,36,46}. The ibuprofen treatment also preserved articular cartilage integrity at radio-carpal joints, despite continued bone loading at high cyclical high force loads³⁵. In this study, ibuprofen treatment preserved trabecular microarchitecture and bone mineral density, and enhanced trabecular thickness in the distal ulna epiphyses. These findings match results by others showing that non-steroidal anti-inflammatory drug treatment inhibits bone resorption and aids bone healing⁷⁷⁻⁷⁹.

Declines in bone structure in untreated HRHF rats were not due to declines in muscle function, since they pulled consistently across the 12 weeks of task performance. However, the ibuprofen treatment improved voluntary pulling force in HRHF+IBU rats. In fact, the highest voluntary pulling forces were observed in week 12 HRHF+IBU rats. These latter find-

ings are suggestive of task-induced discomfort occurring as a result of increased inflammatory cytokines in the flexor digitorum muscles, findings reported previously³⁶. We have reported that ibuprofen treatment of HRHF rats improved reflexive grip strength, as did treatment of rats with an anti-TNF α antibody^{36,46,67}, further supportive of an inflammatory component to muscle function with repetitive tasks. Thus, muscular function appear to be influenced by inflammatory responses occurring in tissues and perhaps also in sera, as shown previously^{33,67}.

Myofiber size increased with training, and with HRHF task performance (in both the HRHF and HRHF+IBU groups). HRHF and HRHF+IBU rat muscles also had low increases of atrophied myofibers containing internal macrophages, indicative of subdegenerative muscle changes. Since these changes were occurring in both groups, these subdegenerative muscle changes are likely due to fatigue failure effects^{7,8}. Because muscle and bone are biomechanically linked^{70,80}, it is often assumed that greater physical strength is associated with a greater load on the bone, and corresponding increases in bone mass^{81,82}. A few studies have shown site-specific correlations between grip strength and bone mineral density in the radius of non-athletic women⁵⁸, young athletes (wrestlers and basketball players)⁸³, and males⁵⁶. However, one study showed that factors other than muscle size accounted for 12-16% of the variance in differences in bone traits, such as bone mass⁸¹. These authors concluded that other factors associated with loading that are distinct from muscle size contribute to bone adaptive responses. Since similar changes in muscle morphology were observed in the HRHF and HRHF+IBU rats, our results also indicate that other factors contribute to changes in bone mass, in our case, osteoclast activity and inflammatory changes occurring in not only bone but also in muscles and nerves.

The early onset and then eight weeks of ibuprofen treatment prevented the development of nerve inflammation and declines in conduction velocity in the HRHF+IBU, and partially attenuated nerve fibrosis in these rats. We have previously reported signs and symptoms of carpal tunnel syndrome in our model, with declines in nerve conduction velocity, and increased nerve inflammation and pain behaviors in 12-week HRHF rats³⁸. A two week treatment of ibuprofen reduced fibrogenic proteins levels and fibrosis in flexor digitorum muscles of HRHF rats⁶⁷, supportive of an inflammatory mechanism to the nerve fibrosis observed in this study. We can rule out a contribution from nerve injury, since no signs of axonal swelling, myelin degradation, or gross nerve disruption were observed in the median nerves of any group.

Lastly, the observed differences between the ulna and radius are likely due to anatomical differences, since the radius has a direct articulation with the carpal bones, whereas the forces applied to the ulna are transmitted and distributed across the interosseus membrane to the radius. We should also note that the observed increases in serum osteocalcin and CTX-1 could be occurring at any bone site involved in task performance, such as the metacarpal bones, diaphysis of forelimb bones, or humerus. Therefore, one limitation of this study is that we did

not examine other bony sites of the upper extremity for either adaptive or resorptive bone changes. This should be a topic of future studies, since prior studies by Kuiper and colleagues has shown that serum levels of biomarkers of collagen type I turnover, including CTX1, alter with prolonged occupational loading at high physical demands, and are associated with vertebral column shrinkage^{84,85}. Another limitation of this study is its length of 5 weeks of training and then 3 months of task performance. This time frame allowed us to determine the effects of inflammation early in the process of upper extremity overuse injury with nerve involvement. However, patients treated for carpal tunnel syndrome typically have had symptoms for years, and have sometimes reached the state of muscle atrophy and wasting by the time of surgical intervention. Thus, our findings of the usefulness of ibuprofen treatment should be considered in that light.

In conclusion, trabecular bone adaptation was observed in TRHF rats, despite increased levels of bone inflammatory cytokines, indicative of a muscle loading mechanism with short-term duration loading at progressively increasing high force loads. In contrast, 12 weeks of HRHF task-induced declines in trabecular bone volume and structure was linked to inflammatory mechanisms in the form of increased inflammatory cytokines and increased osteoclast numbers and activity, but not muscle loading as that was constant in the untreated HRHF rats across the 12 weeks of task performance. We were also able to rule out changes in muscle and nerve morphology, or weight loss as contributing factors to the observed changes in bone structure. Provision of a non-steroidal anti-inflammatory drug during weeks 5-12 of this 12 week task preserved trabecular bone volume and structure, and even allowed for increase in trabecular thickness in the ulnar metaphysis (although no net bone formation in the form of increased bone volume). Thus in our model, bone structure is maintained and may even exhibit some adaptation when inflammatory processes were suppressed, despite continued bone loading at high repetition high force loads in this task paradigm.

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