IBUPROFEN ADMINSTERED PRE- OR POST- SIMULATED RESISTANCE EXERCISE TRAINING DOES NOT DIMINISH GAINS IN BONE FORMATION OR BONE MASS

A Thesis

by

DAVID ARTHUR CUNNINGHAM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2011

Major Subject: Kinesiology

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Approved by:

Chair of Committee,	Susan A. Bloomfield
Committee Members,	Harry A. Hogan
	James Fluckey
Head of Department,	Richard Kreider

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ABSTRACT

Ibuprofen Administered Pre- or Post- Simulated Resistance Exercise Training Does Not Diminish Gains in Bone Formation or Bone Mass. (December 2011)

David Arthur Cunningham, B.S., Ohio University

Chair of Advisory Committee: Dr. Susan Bloomfield

Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to suppress bone formation when administered before, but not if administered after, an acute bout of mechanical load in rats. The aim of this study was to test the hypothesis that gains in bone mass and size will be diminished in adult rats given ibuprofen before, but not after, each exercise bout during 20 days of simulated resistance training (SRT). Virgin female Sprague-Dawley rats (5-mo-old, n=29) completed 9 SRT sessions using stimulated muscle contractions under anesthesia at 75% peak isometric strength on alternate days. Animals were blocked assigned by body weight to one of three groups: 1) ibuprofen (30mg/kg) before exercise, placebo after (I:P)(n=9), 2) placebo before, ibuprofen after (P:I)(n=10) and 3) placebo before and after (P:P)(n=10). *In vivo* pQCT scans measured changes in total volumetric bone mineral density (vBMD) and total bone mineral content (BMC) at the proximal tibia (cancellous), and total vBMD, total BMC and total area at midshaft tibia on days -7 and 21. Dynamic histomorphometry on both midshaft tibiae (exercised and non-exercised legs) determined mineralizing surface (MS/BS), mineral apposition rate (MAR) and bone formation rate (BFR) on the periosteal surface. There

were no differences in body weights among groups at baseline or at day 21. There were significant gains due to SRT, but not ibuprofen treatment in total BMC (+10.50 ± S.D. +8.15%) and total vBMD (+5.29 ± 3.41%) at the proximal tibia. The midshaft tibia exhibited significant gains in total vBMD (6.68 ± 3.03%), total BMC (19.18 ± 5.51%) and total area (11.68 ± 5.49%) due solely to SRT. Furthermore, there were significant increases in periosteal BFR (pre 21.89 $\mu m^3/\mu m^2/d$ ±2.63; post 717.81 $\mu m^3/\mu m^2/d$ ±100.57) at the midshaft tibia in the exercised vs. non-exercised legs in all groups but no effect of ibuprofen regimen was detected on these indices of bone formation. Within this simulated resistance protocol, we were unable to detect any impact of ibuprofen administration on the response to bone loading.

Supported by Huffines Institute of Sports Medicine and Human Performance, Texas A&M University.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Susan Bloomfield, and my committee members, Dr. Harry Hogan and Dr. James Fluckey for their time and guidance throughout the course of this research. I would also like to thank Kaleigh Camp for the many hours and hard work she assisted with this study. I also want to extend my gratitude to the Huffines Institute of Sports Medicine and Human Performance, Texas A&M University, for providing support for this study.

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CHAPTER I

INTRODUCTION

The use of non-steroidal anti-inflammatory drugs (NSAIDs) is a common over the counter drug used by millions across the globe for musculoskeletal pain and inflammation. The use of these drugs, however, has been shown to reduce the anabolic response to bone after an acute bout of exercise in animal models [1, 2, 3]. The main therapeutic action of NSAIDs is through the inhibition of cyclooxygenase-2 (COX-2), the known enzyme which synthesizes prostaglandin E_2 (PGE_2) in response to mechanical load. Ongoing studies have identified various mechanisms by which mechanically-induced PGE_2 is thought to have its effect on bone formation. Those studies were focused on an acute bout of exercise and have yet to establish the impact of NSAIDs with multiple training sessions. Multiple training sessions is idea because 1) it best translates to the lifestyle of an athlete in training and 2) it will translate to therapeutic exercise in older adults who regularly use NSAIDS. Exercise has been shown to increase inflammatory cytokines, which have a known catabolic effect on bone. Thus, the use of NSAIDs may have the ability to successfully reduce the increase in inflammatory cytokines and, therefore, cytokines' deleterious effect. However, the timing of NSAIDs administration relative to exercise is critical to ongoing research. Previous studies have shown an inhibitory effect to bone formation when NSAIDs are administered before an acute load [1, 2, 3]; however, a recent study indicates NSAIDs as a potential bone therapeutic drug when administered post- resistance training [4].

This thesis follows the style of Bone.

Gaps in knowledge are whether BFR is influenced over multiple sessions in rats when given NSAIDs and if this influences overall bone mass and size as seen when ibuprofen is given post resistance training in humans [4]? Lastly, if bone mass is influenced, then are these effects inversely related to serum inflammatory cytokine concentration?

Specific Aims and Hypotheses

Specific Aim 1: To determine the impact of oral ibuprofen administration given before or after exercise on BFR after 3 weeks of simulated resistance training in adult female rats.

Hypothesis 1: Bone formation rate will be suppressed over time in adult rats given ibuprofen *before* each training session, but enhanced if ibuprofen is given *after* each exercise bout

Specific Aim 2: To determine the impact of oral ibuprofen administration given before or after exercise on long-term adaptations in bone mass and geometry after a 3-week simulated resistance training (SRT) model in adult rats.

Hypothesis 2: Gains in bone mass and increased bone size will be diminished in adult rats given ibuprofen *before* each training session, but enhanced if ibuprofen is given *after* each exercise bout.

Specific Aim 3: To determine if the effect of ibuprofen administration given before or after exercise on the bone response to 3 weeks of chronic training is related to differences in the inflammatory cytokine response to acute exercise bouts.

Hypothesis 3: When ibuprofen is administered *after* exercise, there will be enhanced gains in BFR and bone mass due to a decrease of IL-6 following acute exercise when compared to ibuprofen administered *before* exercise and placebo control.

CHAPTER II

REVIEW OF LITERATURE

Bone Formation and Resorption

Bone is a highly dynamic tissue comprised of elastic type I collagen and hardened hydroxyapatite crystals. The combination of these two properties allows bone to be both structurally supportive for protection, as well as elastic in order to allow for bending and torsion forces on its surfaces. These forces produced through every day activities such as running and jumping, are sensed through a variety of cells within the bone matrix. The main sensing cell to external forces is known as osteocytes. Osteocytes are embedded deep within the bone matrix and provide communication to the bone surface, whether it is in cancellous, a spongy porous bone located at the end of long bones, or cortical bone, a protective compact bone located within the shaft of the long bones. Osteocytes are capable of communicating with bone forming cells known as osteoblast and bone resorbing cells known as osteoclast.

Osteoblast, the precursor to osteocytes, lay down new bone matrix on the bone surface until cell death occurs, where osteoclast located on the cell surface break down the bone matrix via acidic secretion. Bone is considered highly dynamic because it constantly undergoing turnover by a balanced rate of formation and resorptionr. Generally, bone turnover produces no net gain or loss of bone in healthy individuals. However, as individuals age there is no longer a balance between the osteoclasts and osteoblast, such that the rate of bone resorption exceeds the rate of bone formation due to increased osteoclast production and activity, thus causing a total net loss of bone. On

the contrary, as an individual becomes more active, they experience more impact on the bone, and bone formation rates exceed the rate of resorption due to increased ostoeblast production and activity, thus causing a net gain in bone. A key cytokine in the communication between osteocytes, osteoblasts and osteoclast is known as prostaglandin E_2 (PGE_2) and will be described in the preceding sections.

Prostaglandins

Prostaglandins in bone can stimulate bone formation and bone resorption depending on the local concentration. An excessive amount of prostaglandin has the ability to stimulate osteoclast for bone resorption, but small local increase in PGE_2 concentration can contribute to bone formation. Hormonal regulation of bone is possible because of an intricate network of osteocyte dendritic processes and gap junctions forming a functional syncytium, allowing osteocytes embedded in the calcified bone matrix to communicate with each other and with surface osteoblasts [5]. Mechanically-induced load incurred with weight bearing activities (i.e. resistance training, running and jumping) elicits the release of prostaglandins, which has a positive effect on bone mass. The production of prostaglandin E_2 (PGE_2), the main contributor to bone formation, is catalyzed by the enzyme cyclooxygenase (COX), which consist of two isoforms, COX-1 and COX-2 (Fig. 1). COX-1 is constantly expressed and acts more in a homeostatic or cytoprotective manner in most tissues. COX-2 isoform is absent from most tissues [6] and is the main contributor to load-induced PGE_2 signaling in bone. COX-2 is inducible, where COX-1 is constitutive with little regulation [7].

Selective and Non-Selective Non-Steroidal Anti-Inflammatory Drugs

In order to study the effects of PGE_2 after mechanical loading, research has relied heavily upon COX-1 and COX-2 inhibitors known as Non Steroidal Anti-inflammatory Drugs (NSAIDs). Commercially, NSAIDs are used as an over-the-counter drug to suppress inflammation via reduced synthesis of PGE_2 and for incidental and chronic musculoskeletal pain in the elderly and athletes. This has motivated research in order to investigate the effect of NSAIDs when taken with exercise.

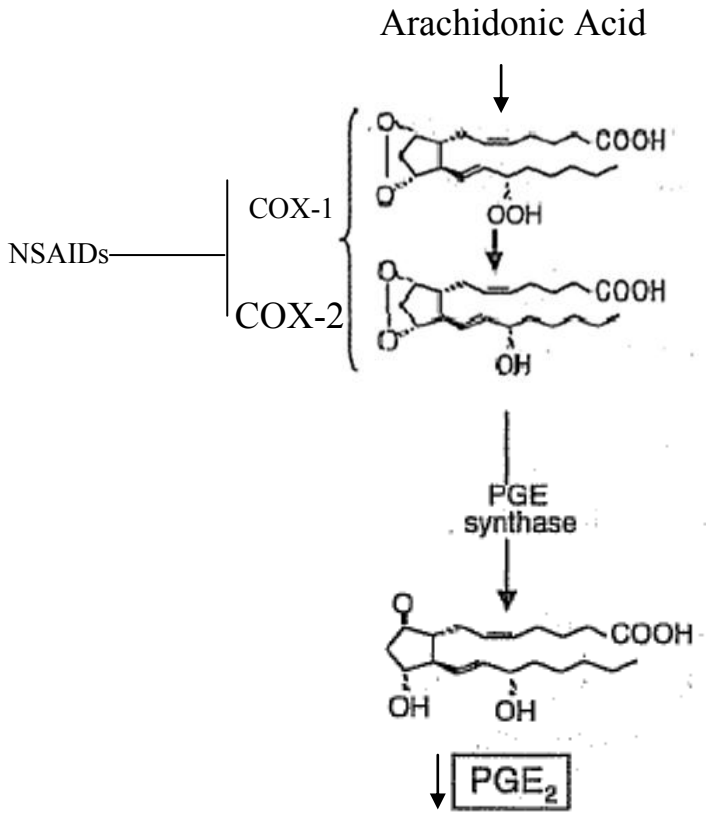

Fig. 1. Synthesis of prostaglandin E_2 and inhibition via NSAIDs. NSAIDs inhibit COX-1 & COX-2 thus decreasing the synthesis of PGE_2 (image adapted from [8]).

Fortunately, there are a variety of NSAIDs available that have various specificities to COX-1 or COX-2, which are described as non-selective, or specific COX inhibitors. COX-2 specific NSAIDs target strictly COX-2 and even at the highest does COX-1 is unaffected, where as COX-2-selective NSAIDs will have a higher affinity for inhibition of COX-2, but higher dose may affect COX-1. Non-selective NSAIDs affect preferentially COX-1; however, will affect COX-2 at the same plasma concentration as COX-1. The selectivity of a selective vs. Non-Selective NSAID is established based on the COX-1/COX-2 ratio. The COX-1/COX-2 ratio is expressed as the ratio of the 50% inhibitory concentration or inhibitory dose for CO-1 to the 50% inhibitory concentration or inhibitory dose for COX-2 (Table 1). The larger the ratio, the greater affinity of the NSAID for COX-2; this ratio will vary on whether the assay was done *in vitro* or *in vivo* [9]. Th9ese categories of NSAIDs have provided some insight into the impact of NSAIDs when delivered before or after an acute bout of mechanical load on bone formation.

Table 1
NSAIDs Selectivity.

Drug	Human recombinant enzymes[b] ($COX\text{-}1\ IC_{50}/COX\text{-}2\ IC_{50}$)	Whole blood assay[c] ($COX\text{-}1\ IC_{50}/COX\text{-}2\ IC_{50}$)	*In vivo*[d] ($COX\text{-}1\ ED_{50}/COX\text{-}2\ ED_{50}$)
Celecoxib (selective)	*375*	*7.6-9.09*	*>33*
Indomethacin (non-selective)	*.1*	*.562*	*2*
Ibuprofen (non-selective)	*.215*	*.592*	*.1*

Expressed as the ratio of the 50% inhibitory concentration or inhibitory dose for COX-1 to the 50% inhibitory concentration or inhibitory dose for COX-2. >1 COX-2 Preference : <1 COX-1 Preference (table adapted from [9]).

Non Steroidal Anti-Inflammatory Drugs and Mechanical Load

Mechanical load creates fluid shear stress within the bone lacunae, delivering a large stimulus over the entire bone lining cell, thus resulting in reorganization of the actin cytoskeleton in order to stimulate the production of PGE_2 [10]. Rawlison et al. (1991) [11] have demonstrated a significance increase in PGE_2 in less than 5 minutes after loading; the elevated levels decrease back to baseline within 15 minutes *in vitro* with osteoblast like cells. This initial production of prostaglandin during mechanical load has been suggested to be a main contributor to the end result: increased bone formation [1, 4].

Previously, it has been shown that when a non-selective COX inhibitor with a COX-1/COX-2 ratio of 2 (indomethacin) [12] is administered 3 hrs *before* ulna loading, with rats there is inhibition of new bone formation; however, if the NSAID is administered 6 hours *after* loading, there is no change in bone formation activity. This suggests there is a specific period of time in which loading induced PGE_2 production is key in bone formation [1]. It seems as if this effect is due to the rapid production of PGE_2 at the onset of mechanical load, as previously described. However, one would assume this rapid increase in PGE_2 is induced by an up-regulation of COX-2 at the onset of mechanical load, but it appears this is not the case [13]. It has been shown that fluid shear stress does not up-regulate COX-2 expression until 30-90 minutes post-mechanical load *in vitro* [14] and *in vivo* [13]. When rats are given the specific COX-2 inhibitor, NS-398, there is no significant reduction in load-induced bone formation when the inhibitor is given 30 minutes before an acute bout of load, but the expected bone

formation reduction does occur when NS-398 is given 3 hours before mechanical load [13]. NS-398 peak serum levels occurs at 30 min, so it was hypothesized that the inhibitory response to mechanical load will be the same if the NSAID is given 3 hours before or 30 min before load. The authors speculate this may be due to recent findings *in vitro* that mechanical loading affects the intracellular secretion of prostaglandin and not the new synthesis of prostaglandin [15], thus indicating the suppression of prostaglandin synthesis must occur well before the time of loading. For an overview of the previous section please refer to Fig. 2.

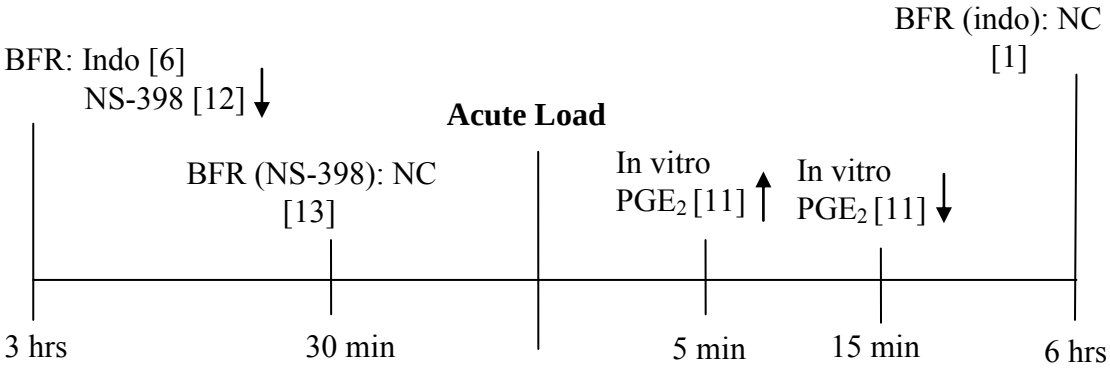

Fig. 2. Impact of the timing of NSAIDs administration relative to an acute bout of mechanical load on BFR (↑ = increase in bone formation; ↓ = decrease in bone formation; NC = no change in bone formation).

Inflammatory Cytokines

The timing at which NSAIDs are administered, pre or post induced load, in an acute bout of exercise will have a profound effect on the ability to benefit loading-induced bone formation. If NSAID administration is translated to a human model, chronic NSAID use may largely negate the benefits of weight bearing activity on bone

health. However, as mentioned, previously published studies examine responses to only an acute bout of exercise, as opposed to multiple training sessions. One notable consideration is the large increase in inflammatory cytokines post-exercise [16, 17]. Inflammatory cytokines have the ability to stimulate bone resorption activity via enhanced osteoclast differentiation and increased activity of mature osteoclasts [18, 15]. An acute bout of exercise stimulates both increased bone resorption and increased formation; increases in resorption may be induced an increase in inflammatory cytokines [4]. Whether exercised induced inflammatory cytokines have the ability to suppress bone formation with chronic training has yet to be elucidated.

The key inflammatory cytokines involved in bone resorption are the interleukins (IL-1, IL-6) and tumor necrosis factor (TNF-α) [15]. The effect of these cytokines on bone, is mediated by receptor activator for nuclear factor κ B (RANKL) and macrophage stimulating factor (M-CSF) produced by osteoblasts for osteoclastogenesis. IL-1β and TNF-α synergize to elicit a marked increase in RANKL levels, thus mediating proinflammatory regulation of osteoclast differentiation via RANKL [19, 20].

IL-6 has no effect on RANKL regulation, thus another pathway must be considered. IL-6 has been shown to indirectly affect bone resorption [20]. It affects bone resorption by activating other pro-inflammitory cytokines such as the previously mentioned IL-1 and TNF-α [21].

NSAIDs and Chronic Training

One bout of vigorous resistance exercise in humans can produce a 7-fold increase in serum levels of IL-6 by one hour post-exercise [16]. These elevated levels of cytokines are thought to be triggered by the leakage of endotoxins (lipopolysaccharides) from the intestines [22] as well as from the muscle during exercise [23]. A resistance training paradigm can produce an elevated level of inflammatory cytokines [17], thus favoring, net bone resorption over a long period of time. It is hypothesized, over weeks and months of training, the balance between bone formation and resorption will be tilted towards resorption mediated by inflammatory cytokines effect on osteoclast activity and differentiation [24]. The effects of inflammatory cytokines post-exercise are an important variable to consider. The studies of NSAIDs on bone have only looked at an acute bout of mechanical load which may not elicit the chronic effects inflammatory cytokines on bone due to training.

The use of NSAIDs post-exercise may help suppress inflammatory cytokines and thus tilt the bone formation/resorption balance back in favor of formation. Enhanced gains in bone mineral density have been observed if NSAIDs are administered post-exercise while training pre-menopausal women. This response is even greater than the typical bone response to mechanical load without NSAID administration [4] (Fig. 3).

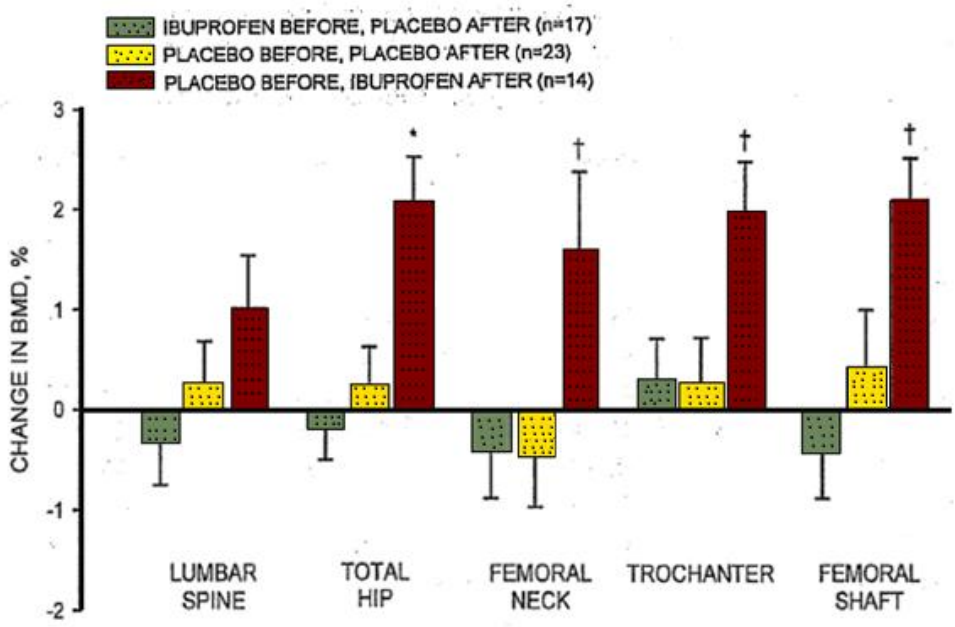

Fig. 3. Ibuprofen given post resistance training in pre-menopausal women. Ibuprofen given post resistance training in premenopausal women have enhanced bone mineral density (Image adapted from [4]).

It may be that the benefits of NSAID use when taken after an exercise session are achieved because NSAID's attenuate the elevation of inflammatory cytokines levels and therefore suppress the acute post-exercise resorption effect [4]. For example, it has been shown in male army recruits genotyped for Il-6-174 G>C polymorphism, that those recruits with Il-6 dominant polymorphism lost 6.8% of cortical area post basic training, where heterozygotes gained 5.5% and those Il-6 deficient gain 17.3% in cortical area [25] (Fig. 4). This gives evidence to any decrease in Il-6 post exercise via NSAIDs may enhance overall bone formation.

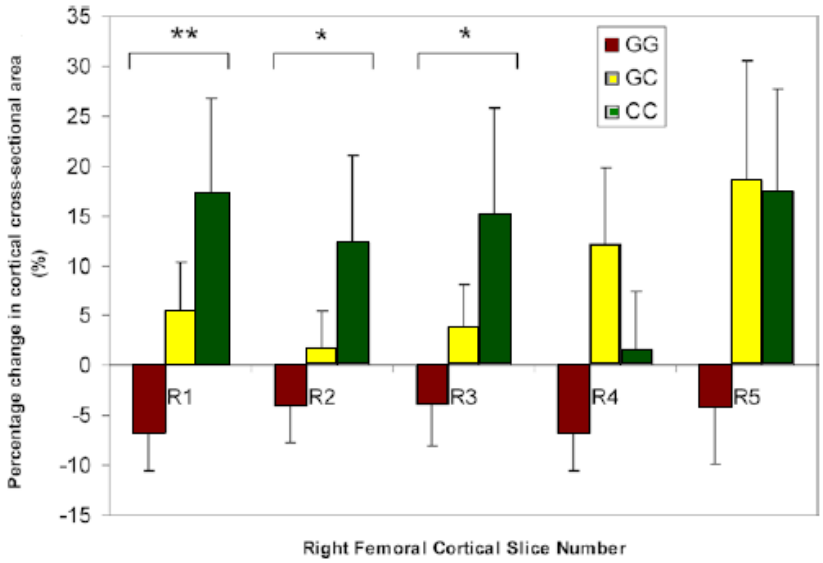

Fig. 4. Cortical resorption is IL-6 genotype dependent. Il-6-174 G>C polymorphisms in army recruits cortical area post basic training. GG Il-6 dominant, CC Il-6 deficient, GC Il-6 heterozygote (Image adapted from [25]).

In a periodontitis model, NSAIDs (specifically ibuprofen) have been shown to slow down the rate of bone loss due to this inflammatory disease [26]. Periodontitis is known to increase production of TNF-α and IL-1 via monocytes, IL-1 by oral epithelial cells, and IL-6 via many cell types. Ibuprofen has been shown to augment bone erosion via cytokines in rat fetal long bones, but ibuprofen was the least effective NSAID in achieving this effect. Ibuprofen was 700 times less potent than the most effective NSAID, ketorolac [27]. Even though NSAID's have been shown to reduce the deleterious effects of inflammatory cytokines on bone, the hypothesis that NSAID consumption post-exercise reduces exercise-induced inflammatory cytokines may be complicated by evidence of an increase in inflammatory cytokines post competition even

in those consuming ibuprofen after exercise. Ultramarathon runner [16] who consumed ibuprofen showed a 106% increase in lipopolysaccharides, an endotoxin responsible for exercise-induced inflammatory cytokines when compared to those who did not consume the NSAID. Ibuprofen has been shown to reduce glomerular filtration rate; thus, cytokine clearance from the kidney may be reduced during exercise [28]. These results do have their limitations because an ultramarathon is not a typical activity and the event is meant to push athletes beyond their limits. These results do, however, raise questions on the effects of chronic use of NSAIDs during training.

Long Term Effects of NSAIDs

Even though there has been demonstration of enhanced bone mineral density response to exercise training with NSAID given after exercise [4], it is important to consider the effects of chronic NSAID use in a non-exercise model. In a 24 week study on ovariectomized (OVX) rats treated with indomethacin, a non-specific NSAID, a decrease in cancellous bone volume was observed due to a suppression of both bone formation and bone resorption. These results were further confirmed due a decrease in BMD and compressive strength of the vertebral body [29]. It has also been demonstrated that after 3 weeks of chronic ibuprofen use, a reduction in radial bone growth and cancellous bone area of the tibial metaphysis was observed in both ovary intact and OVX rats [30]. Despite the potential benefits of NSAIDs use with chronic training, further research will have to consider the long term effects of NSAIDs if used over a significant period of time.

Gaps in Knowledge

The chronic use of NSAIDs by athletes and everyday Americans in order to treat incidental and chronic musculoskeletal pain justifies the need to identify the impact of NSAIDs and the timing of administration relative to exercise. Previous studies have identified key time points in which the effect of PGE_2 on bone formation is most active during and after an exercise bout [1, 12, 13, 14]. The use of NSAIDs may have an enhanced effect on bone formation if ingested by chronically trained individuals post-exercise, perhaps due to NSAIDs ability to suppress inflammatory cytokines induced by exercise. These cytokines are potent molecules which activate bone resorption via activation and differentiation of osteoclasts. Future studies need to identify the effect of inflammatory cytokines produced during exercise and their potential inhibitory effect on bone.

Currently, the gaps in knowledge are whether timing of NSAIDs influence bone formation rate (BFR) over multiple sessions in rats and if this can be translated to the overall bone mass and size as seen when ibuprofen is given post resistance training in humans [4]? Lastly, if bone mass is influenced, then are these effects inversely related to serum inflammatory cytokine concentration? The aims of this study are to determine the impact of ibuprofen on bone response to simulated resistance training and to explore altered changes in serum Il-6 as a potential mechanism has on bone health during resistance training.

CHAPTER III

METHODS

General Overview

Study Design**:** Female (n =29) Sprague-Dawley rats, 4-mo-old at arrival, were housed at the Agronomy Road animal facility, two to a cage, on a 12:12 light:dark cycle. After a week of acclimation, rats were be rank-ordered by body weight and then block assigned to one of 3 groups; PP: Placebo before exercise, Placebo after exercise; IP: Ibuprofen before exercise, Placebo after exercise; PI: Placebo before exercise, Ibuprofen after exercise (Table 2). This block randomization strategy helps assure groups with roughly equal mean body weight, important since bone size scales to total body weight.

Table 2
Experimental groups (total n = 29**).**

Group	**Before Ex**	**After Ex**
P:P (n=10)	Placebo	Placebo
I:P (n=9)	Ibuprofen	Placebo
P:I (n=10)	Placeb	Ibuprofen

Starting at roughly 5 months of age, rats underwent 9 SRT sessions performed every other day at 75% peak isometric strength for 4 sets of 5 repetitions. All animals received about .1 ml of vehicle (0.09% saline) with or without ibuprofen (30mg/kg) by oral gavage 2 hours before and immediately after each training session, as detailed below. Blood draws were taken pre exercise training in order to obtain baseline IL-6 cytokine levels and then 30 min and 90 min post exercise 2 days after baseline was drawn. For full timeline refer to Fig. 5.

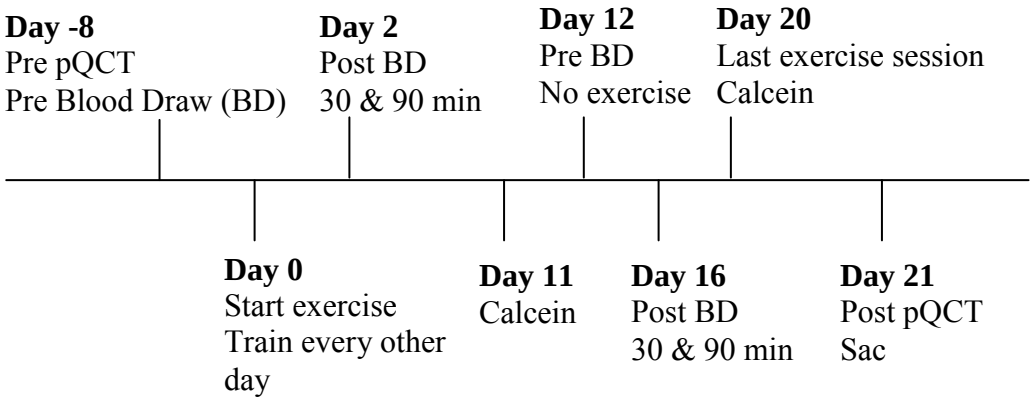

Fig. 5. Timeline of study.

***In vivo* Muscle Stimulation**

This was accomplished using an adapted version of the miniature isokinetic dynamometer originally developed for use in mice by Drs. Gordon Warren and Robert Armstrong [31]. We used a miniature isokinetic dynamometer to simulate resistance training every other day for 20 days, for a total of 9 training sessions. After the rat was anesthetized with isoflurane, it was placed in right lateral recumbency on the dynamometer platform, its foot secured to the servomotor shoe (Fig. 6). The knee was positioned so that the foot was perpendicular to the lower leg and secured with a ball-jointed clamp system. Sterilized fine wire (10 cm long; STABLOHM 800B H-ML Size 003 ---California Fine Wire Co) was stripped of insulation at about 4 mm at both ends and inserted thru a 27ga ½" needle and 1.5 mm was bent over at the end to form a

"barb." The leg was extended and the origin of the gastrocnemius muscle on the femur was palpated. The needle containing the wire was inserted straight down to the right of the femur and then the needle was withdrawn, leaving the thin wire implanted near the nerve with barb on the end. The second wire was implanted @ 4 – 6 mm dorsal to the first in the same manner. The stimulation wires were then attached to the output poles of a Grass stimulus isolation unit interfaced with a Grass S8 stimulator.

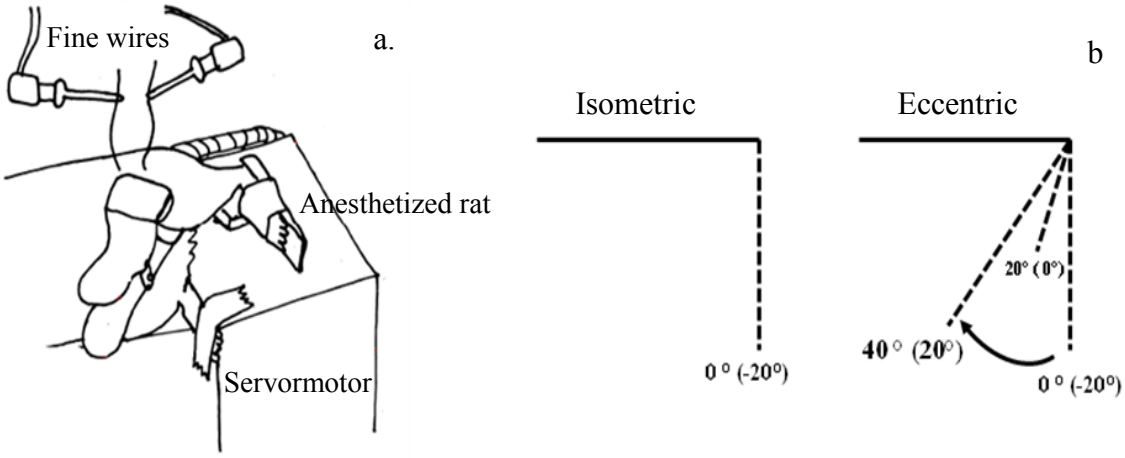

Fig. 6. Simulated resistance training device. a.) Anesthetized rat resting on simulated resistance training device b.) Isometric and eccentric foot angle throughout lower leg muscle contractions.

The first step in each training session was to optimize the isometric contraction torque production by the posterior crural muscles, yielding a peak isometric torque value specific to the rat that day. This was done with 4-5 contractions while adjusting stimulation voltage. The stimulus is 200 ms long using a 175 Hz frequency and 0.1 ms biphasic pulses with 45s in between contractions. A period of 2 minutes rest was given once the peak isometric torque was recorded. Next, 4 sets of 5 eccentric contractions

were performed at 75% of the rats' peak isometric torque. Peak isometric torque was determined to be the maximal isometric torque at the lowest voltage necessary. Voltage frequency was adjusted during the eccentric contraction in order to determine 75% isometric torque. A period of 12 seconds rest was given in between contractions and 120 seconds rest between each set. This took approximately 25 minutes. Contractions were performed over a 40° arc using an angular velocity of 200°/s at 180 Hz. The stimulus duration was 2000 ms; 1000ms isometric contraction and 1000ms eccentric contraction (Fig . 7).

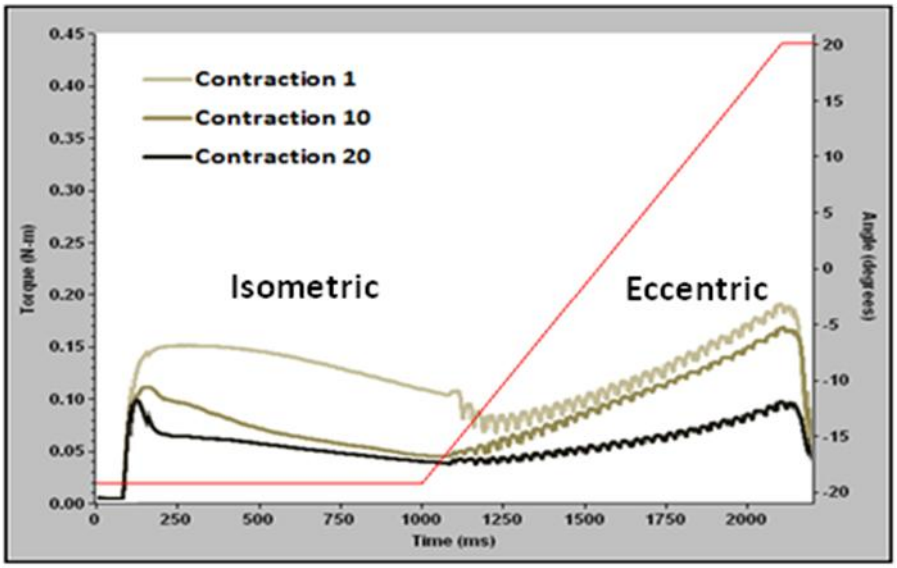

Fig. 7. Isometric and eccentric torque outputs.

Ibuprofen Administration

All animals received 0.1 ml of vehicle (.09% Saline) with or without ibuprofen by oral gavage before and immediately after each training session. Originally, the oral

gavage was performed on a concious rat; however, by the third training session all of the rats were put under light isoflourene in order to administer the ibuprofen. Anesthesia became necessary because of an unexpected death of a rat due to the rats' movement during oral gavaging. Rats received a 30 mg/kg dose of ibuprofen. The literature states that a dose between 10mg/kg and 30mg/kg is safe [32]. Previous resarch has effectively used 30 mg/kg for fracture healing studies [33, 34] and it has been stated that the ulcerogenic dose in rats is 455 mg/kg (4 daily doses) [35]. We gave the rats 30 mg/kg 3 days a weeks which is well below the 455 mg/kg ulcerogenic dose. Furthermore, fracture healing studies utilize 30mg/kg because Rissing and Buxton (1986) [36] showed an effect on the PGE levels assessed by radioimmunoassay in animals treated with 30 mg/kg/day of ibuprofen in Sprague Dawley rats.

Serum Collection

Rats were anesthetized with inhaled isoflurane (to effect) and we tied a tourniquet around the upper leg to apply intermediate pressure; the lower leg was shaved to aid in visualizing the saphenous vein, and venous blood was collected with a 25 ga needle into a microfuge tube. Blood was obtained from animals pre exercise training and 30 and 90 minutes post exercise training during week one and week 3 (Fig. 8). Once the blood clotted, the tubes were centrifuged for about 5 minutes and serum was collected and stored at -80º C for further analysis.

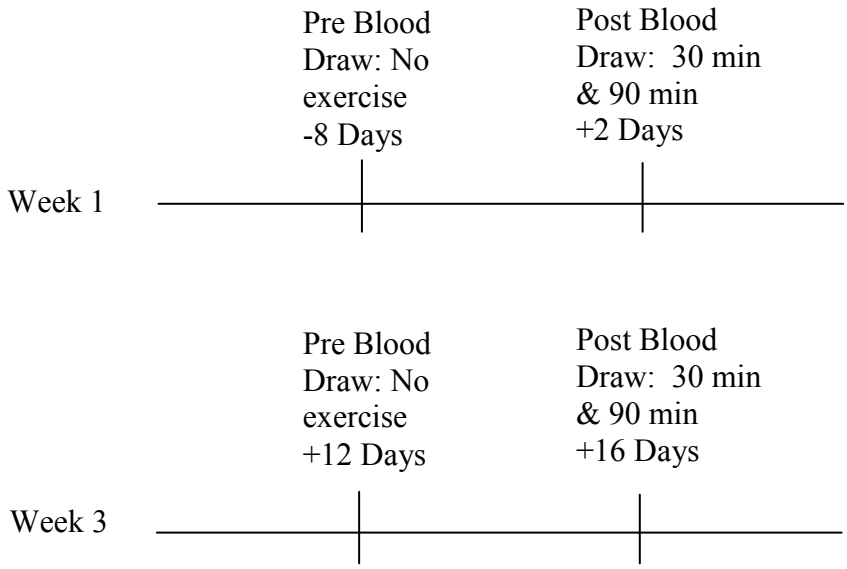

Fig. 8. Blood draw timeline

Calcein Injection

On days 2 and 9 before the scheduled sacrifice of each animal, we gave an intraperitoneal injection of calcein (using a 25 mg/kg BW solution, which amounts to 0.3 mL per injection). Calcein is a relatively inert tetracycline derivative which binds to circulating free calcium in the blood. Any bone surface that is actively mineralizing in the 48 hours following each injection incorporates this calcein-labeled calcium, producing a fluorescent label along that bone surface when histological sections are later viewed under epifluorescent light. This allows us to quantify the relative bone surface that is actively mineralizing and, by measuring the distance between double labels (when they occur), to calculate bone formed over that 7-day interval.

Outcome Measures

On days -7 and 21, animals were anesthetized and their tibiae were scanned at both the proximal and mid-shaft tibial sites using peripheral quantitative computed tomography (pQCT; Stratec XCT-M, Norland Corporation) in order to quantitate bone mineral content, density and geometry. About 100-200 uL of serum was collected from the saphenous leg vein on Day -7 before the pre-training pQCT scan (to represent basal or pre-exercise values) and *after* a training session in the first and fifth weeks to assess serum levels of inflammatory cytokine, Interleukin-6. Forty-eight hours after the final training session (day 21), rats were anesthetized and euthanized by decapitation; cardiac serum was collected, proximal and distal femurs were cleaned of soft tissue, wrapped in PBS-soaked gauze and frozen at -80º C until further analysis. Midshaft femur was snap frozen and stored at -80º C for future PCR analysis. We stored the tibia in paraformaldehyde for 24 hours at 4°C and then 70% ethanol at 4°C to enable later histomorphometric analysis of bone formation rate and immunohistochemistry analysis.

Histomorphometry Analysis

The left distal tibia were sectioned 2.5 mm proximal to the tibial-fibular junction with an Isomet diamond wafer low-speed saw (Buehler, Lake Bluff, Il) after the bones were embedded in methylmethacrylate (Sigma-Aldrich M5, 590-9). The distal tibia was sectioned to 100μm slices and then mounted on glass slides. The histomorphometric analyses were performed using the OsteoMeasure Analysis System, Version 1.3 (OsteoMetrics, Atlanta, GA) and a Bx-56 Olympus light microscope with epifluorescent

light. Labeled surfaces and interlable widths were obtained at 200x magnification of1 slide/section. Periosteal mineral apposition rates (MAR, μm/d) were calculated by dividing the average interlabel width by the time between labels (7 days), and mineralizing surface (MS) for both the periosteal and endocortical bone surfaces was calculated using the formula:

%MS/BS = {[(single labeled surface/2) + double labeled surface]/surface perimeter} x 100.

Bone formation rate (BFR) was calculated as:

BFR= (MAR x %MS/BS)

Slides were "blinded" so the measurer was not aware to which group each slide corresponded.

Peripheral Quantitative Computed Tomography

Measures of tibia bone density and geometry were taken in-vivo using our peripheral quantitative computed tomography (pQCT) device (XCT Research M Stratec; Norland Corp., Fort Atkinson, WI). The XCT-M pQCT device is interfaced with a dedicated PC with a Jazz drive for data archiving. Animals were anesthetized with inhaled isoflurane (to effect) and scan slices were taken at both the metaphyseal (5, 5.5, 6, 6.5mm from reference) and diaphyseal (50% total bone length) regions, with voxel size of 70um (Fig. 9). Total scan time was approximately 30 minutes from time of scout view until scanning was complete. Data from the three contiguous metaphyseal region slices are averaged to get a single value for each variable. Key outcome variable include

Stratec XCT-Research M (Norland Corporation):
Scan speed: 2.5 mm/sec
Voxel resolution: 70 microns
Slice thickness: 0.5 mm

Midshaft tibia site location
Two serial scans taken in vivo at 50% of the length of the tibia

Proximal tibia site location
Serial scans taken in vivo at 5, 5.5, 6 & 6.5 mm distal tibial plateau values averaged from all 4 sites

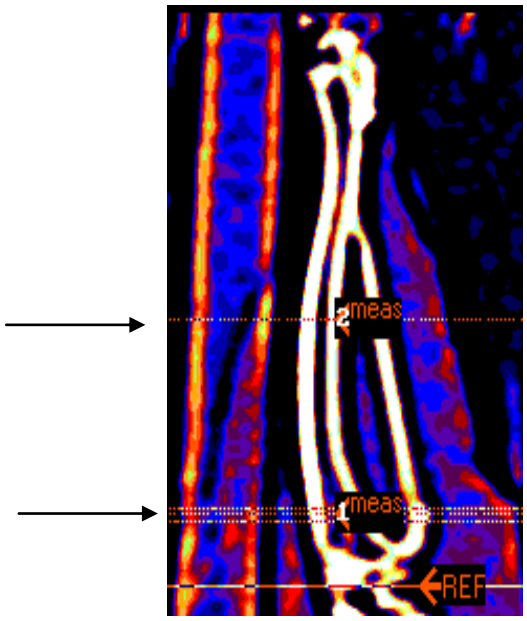

Fig. 9. Peripheral quantitative computed tomography (pQCT).

total and cortical volumetric bone mineral density (vBMD); total area, cortical area and marrow space area, as well as total bone mineral content (BMC). Machine precision (based on manufacturer data) is ± 3 mg/cm^3 for cancellous bone and ± 9 mg/cm^3 for cortical bone. In-vivo CV's from our laboratory using this method at the rat proximal tibia (with repositioning between scans) are 2.13% for cancellous vBMD, 0.23% for cortical vBMD, 1.95% for total area. Corresponding CV's at mid-diapyhisis for the tibia are 0.86% for cortical vBMD, 1.09% for cortical area, and 2.42% for marrow area.

Interleukin-6 Assay

Standard ELISA procedures were used from ImmunoDiagnostics Quantikine (for IL-6). All samples were organized so that any one rat's samples were assayed with one kit.

Statistical Analysis

To determine treatment effects and interactions, a two way (Ibuprofen Group x time) ANOVA with repeated measures on time was used on variables collected longitudinally (pQCT scan parameters, serum values). Two-way ANOVA were also performed on variables determined at sacrifice (e.g. histomorphometry analysis) to study comparisons among groups of animals. Appropriate Tukey post-hoc comparisons were used to determine pair-wise differences. All data are reported as means ± S.D.

CHAPTER IV

RESULTS

Animal Response to Training

Over the course of the 21 days of exercise training there were no-significant differences in mean body weight over the course of 21 days and among the 3 groups ($p > 0.05$) (Table 3). The animals began training at 5 months of age and the non significant changes in body mass suggest all animals were mature adult with limited growth over the course of the training period. All animals were fed *ad libitum*.

Animals responded well to the training protocol and isoflurane. Animals woke up ~5 min post training and showed no visible signs of injury post training. Some animals developed wheezing due to the ibuprofen entering the trachea while still under anesthesia; however, this problem was resolved after we started hold the animals vertically immediately after oral gavage in order to keep the ibuprofen contained within the stomach. Rats were held vertically until they showed signs of waking up.

Table 3
Adult rats over the course of 21 days did not gain or lose body

	Days				
Groups	**-7**	**0**	**7**	**14**	**21**
PI	**280.74 g**	**285.52 g**	**279.37 g**	**280.67 g**	**275.59 g**
	±11.57	±12.12	±15.56	±10.97	±12.09
PP	**281.75 g**	**274.48 g**	**271.78 g**	**284.98 g**	**279 g**
	±18.08	±15.41	±9.97	±18.75	±19.79
IP	**279.97 g**	**282.48 g**	**276.62 g**	**283.83 g**	**281.66 g**
	±13.62	±12.77	±11.71	±16.26	±19.74

Values are means ± SD. There were no significant changes in bodyweights over course of 21 days, nor among groups within time points.

Mid-Shaft Cortical Bone Mineral Content

Simulated resistance training had an anabolic effect on the cortical bone mineral content of the tibial midshaft (Fig. 10). Bone mineral content is a measure of the total bone mineral present and is an important measure in order to identify any changes in bone size and level of mineralization combined. Simulated resistance training increased cortical BMC by 19% (pre: 6.26 mg ± .4; post: 7.45 mg ±.42) ($p < 0.05$) in the control (PP); however, there is no significant difference in gain of BMC among groups (PP, IP & PI).

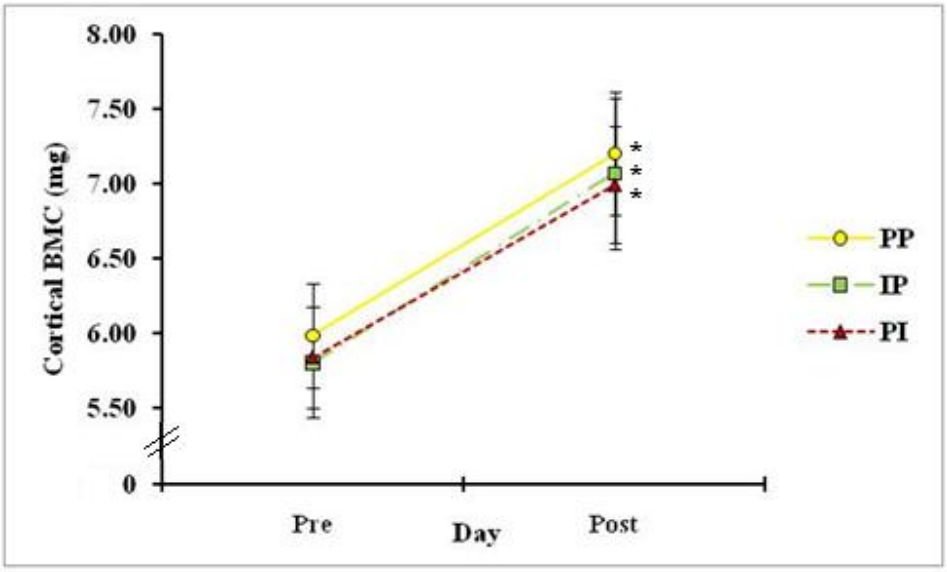

Fig. 10. Mid-shaft cortical bone mineral content. Cortical BMC of the midshaft tibia before and after 21 days of simulated resistance training.

* $p<0.05$ vs. pre value

Mid-Shaft Total Area

Simulated resistance training had a positive effect on the total area of the midshaft tibia cortical bone (Fig. 11). Although SRT increased total area by 11.4% ($p<0.05$) in the control (PP) there were no significant differences among the IP, PI and PP groups' means.

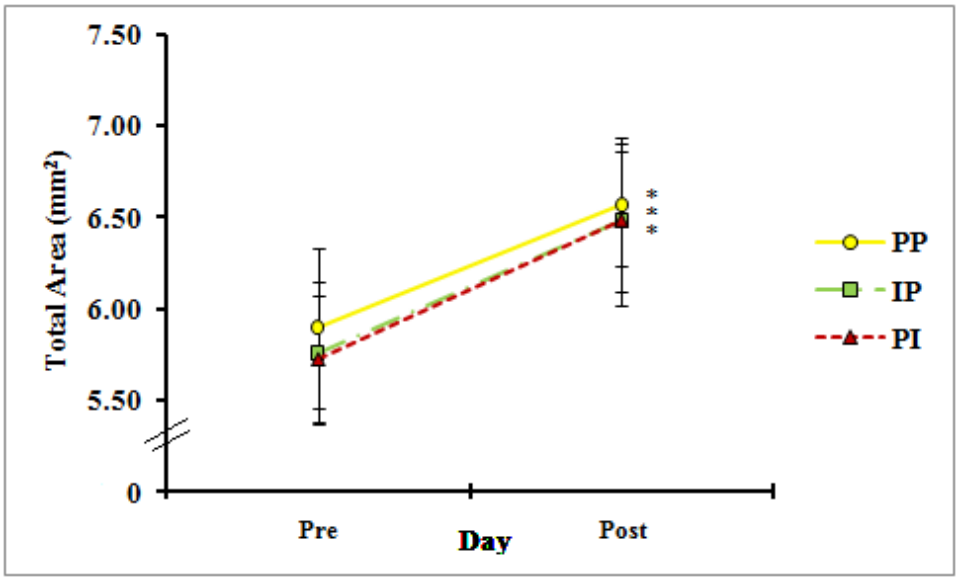

Fig. 11. Mid-shaft total area. Total area of the midshaft tibia after 21 days of simulated resistance training. The left leg received muscle contraction exercise.
* $p<0.05$ vs. pre values

Mid-Shaft Marrow Space Area

Simulated resistance training significantly decreased the marrow space area by 8.5% at the midshaft tibia in the PP and IP groups (Fig. 12) ($p<0.05$). A Tukey post-hoc test shows that PI marrow space was not significant, but trending towards significance with a p value of .06. A decrease in the marrow space is indicative of an increase in endocortical bone formation. Although there was an overall decrease in marrow space

area, there were no significant differences among groups with respect to ibuprofen treatment

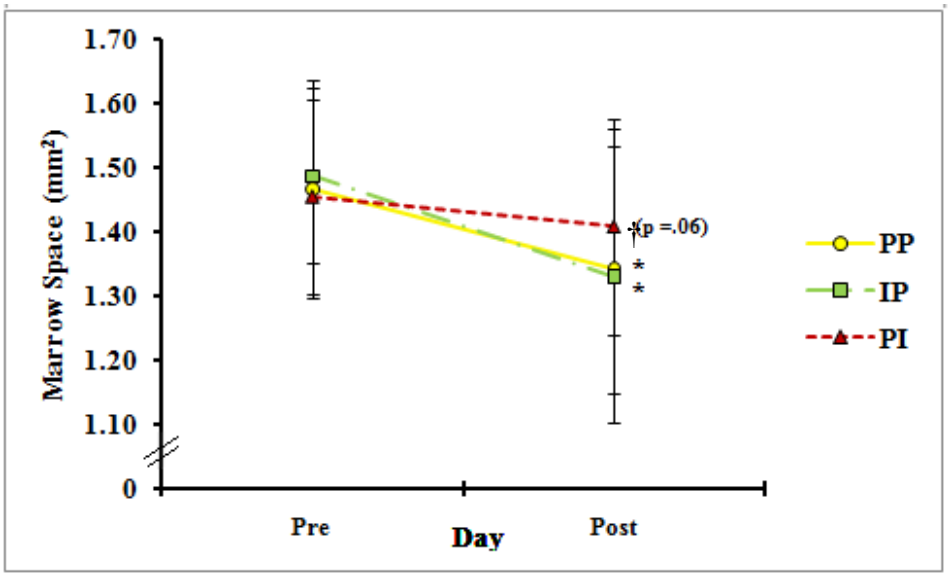

Fig. 12. Mid-shaft marrow space. Marrow space area of the midshaft tibia after 21 days of simulated resistance training. The marrow space significantly decreased with SRT in the PP and IP groups and trended in the PI group (p=.06). There were no significant differences among groups at either time point.

* p<0.05 vs. pre values

† p<0.06 vs. pre value

Proximal Tibia

The following graphs represent data collected from the proximal tibia for exercised leg. Although the proximal tibia is a mixed bone site (cancellous & cortical), the proximal region is largely cancellous bone; hence, alterations in BMC and vBMD yield more insight into the cancellous bone rather than the cortical bone presented in the previous section.

Proximal Total Bone Mineral Content

A two-way ANOVA analysis shows that, although there was a significant increase in BMC due to SRT, there are no significant differences among groups at either time point. Total BMC increased by 10.22% at the proximal tibia within the PP group ($p<0.05$) (Fig. 13).

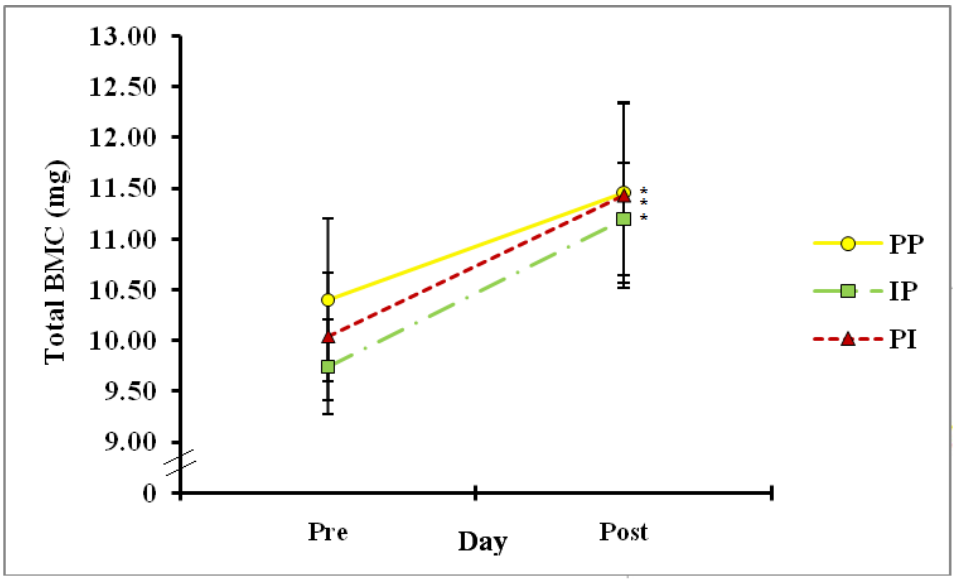

Fig. 13. Proximal total bone mineral content. Total BMC of the proximal tibia after 21 days of simulated resistance training.
* $p<0.05$ vs. pre values

Proximal Total Volumetric Bone Mineral Density

A two-way ANOVA shows that there was a significant increase in vBMD at the proximal tibia; however, there are no significant differences in post training values among groups (PP, IP & PI). Total vBMD increases by 5.25% at the proximal tibia in the PP group ($p<0.05$) (Fig. 14).

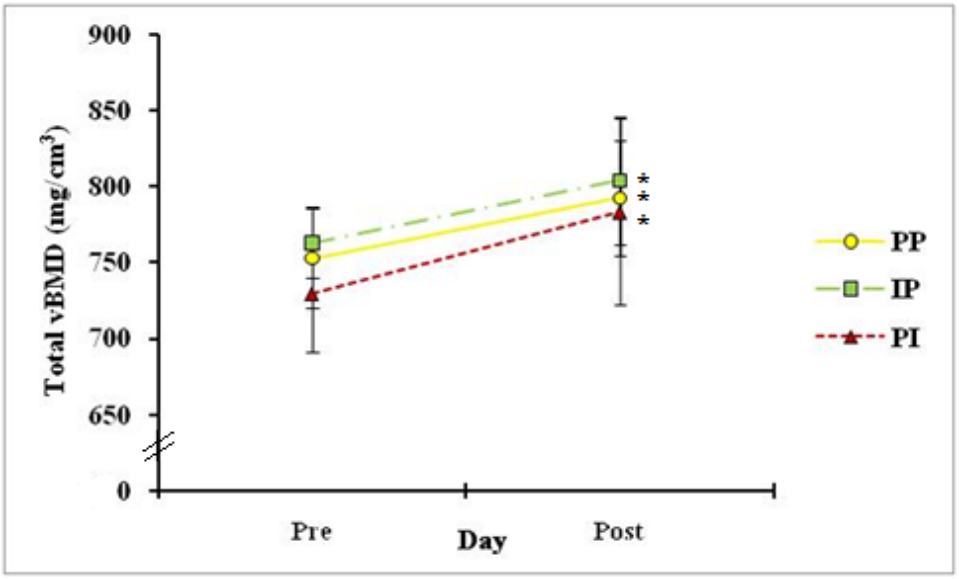

Fig. 14. Proximal total volumetric bone mineral density. Total vBMD of the proximal tibia after 21 days of simulated resistance training.

* $p<0.05$ vs. pre value

Bone Formation Rate

This analysis is performed with excised bone, which means the following data represents bone formation rate of the final week of our experiment. Since pre and post values were unable to be obtained all exercised leg data (left leg) is compared to the non-exercised right leg at day 21. Simulated resistance training results in a significantly greater periosteal %MS/BS, MAR and BFR in the exercised leg compared to the non-exercised leg. The PP group mean %MS/BS was 9.85% ± 7.89 in the non-exercised leg compared to 78.67% ± 6.75 in the exercised leg (Fig. 15a). The PP groups mean MAR was .57 μg/d ± .54 in the non-exercised leg vs. 2.33 μg/d ± .37 in the exercised leg (Fig. 15b). Bone formation rate, which is derived from %MS/BS and MAR, was .07 $\mu m^3/\mu m^2/d$ ± .08 in the non-exercised leg compared to 1.83 $\mu m^3/\mu m^2/d$ ± .29 in the

exercised leg (Fig. 15c) in the PP group. This large increase in BFR pertains only to those surfaces that exhibited lamellar bone growth. Fig. 15d I, II, III & IV is a visual representation of the fluorochrome label observed on the non exercise bone (I & III) and the double label observed on the exercised bone (II & IV). Woven bone, which was an unexpected finding detailed below, was not included in these measurements due to the difficulty in determining MAR. Fluorescent labeling of woven bone is smeared and does not exhibit distinguishable single or double label.

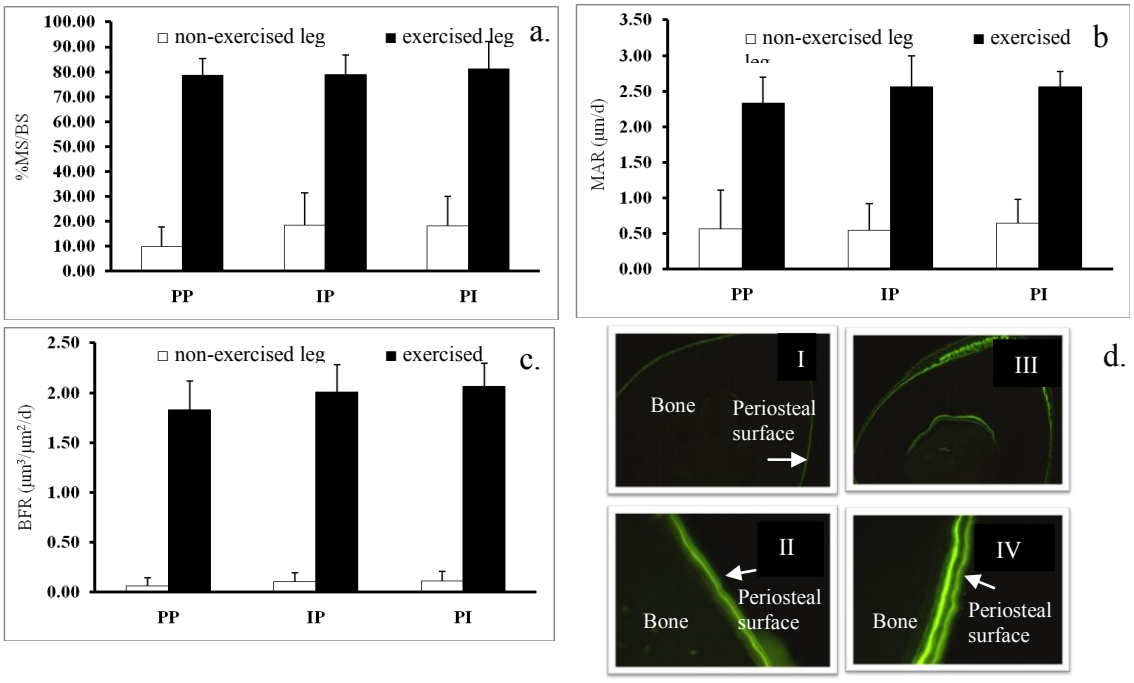

Fig. 15. Bone formation rate. Dynamic histomorphometry measures for the non exercised leg vs. exercised led ■ exercised leg, □ non-exercised leg. a.) %MS/BS of the exercised leg is significantly greater than non-exercised leg b.) MAR of the exercised leg is significantly greater than non-exercised leg. c.) BFR of the exercised leg is significantly greater than the non-exercised leg. d.) I (100X magnification) & II (200X) is a visual representation the of the non-exercised leg fluorochrome label. d.) III (100X) & IV (200X) is a visual representation of the exercised leg fluorochrome label.
% Mineralized Surface (%MS/BS) Mineral Apposition Rate (MAR, µg/d) Bone Formation Rate (BFR, $\mu m^3/\mu m^2/d$) * $p<0.05$ vs. non-exercise.

Woven Bone

Bone formation rate was measured only on lamellar bone surfaces. Unexpectedly, a significant amount of woven bone formation was observed in 100% of the exercised leg samples on the periosteal surface. The woven bone is rapidly formed bone due to hyperphysiological loads or uncommon load angles on the bone. Although woven bone contains mineralized bone, much of its composition is collagen and it is unorganized compared to lamellar bone. The simulated resistance training apparently produced a unique load on the bone which contributed to the formation of woven bone (Fig. 16). Woven bone compromised as much 14% ± 4 (PP 12± 2%, PI 14 ± 4% & PI 11± 3) of the total bone area of the tiba cross sections from the exercised legs. Despite this significant gain in woven bone with SRT, there were no significant differences in woven bone formation across groups using a two-way ANOVA .

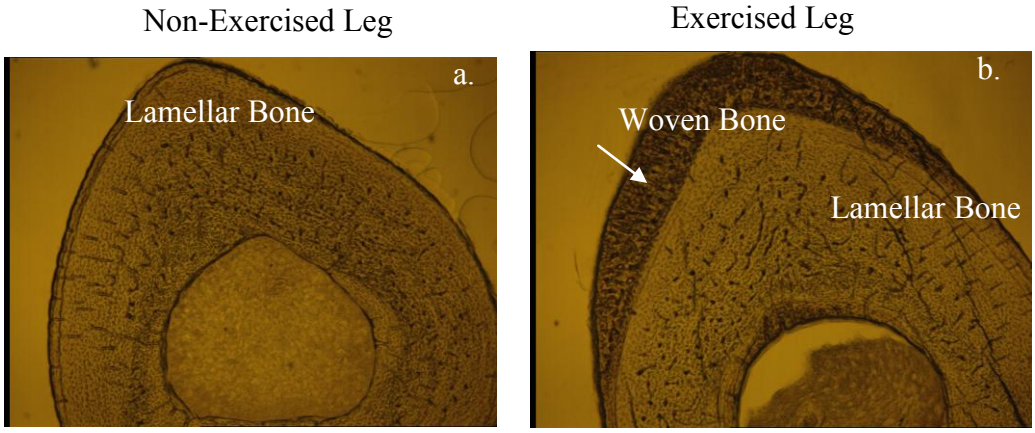

Fig. 16. Woven bone formation. a.) non-exercised leg b.) exercised leg. Woven bone was observed in the exercised leg due to the simulated resistance training.

Alterations in Serum Interleukin-6

Interleukin-6 measurements were attempted via an Il-6 ELISA rat assay; however, technical limitations of the assay kit disallowed a full analysis. The standard curve (Fig. 17) gives an O.D value of .00561 that corresponds to 0 pg/ml and the O.D. that corresponds to 62.5 pg/ml is .03737. The O.D. values for duplicate samples of 8 different animal's serum range from -.00607 to .038325 (Table 4). However, after plugging these values into the linear equation .0007x-0.0578=y, where y= .O.D., we obtained values ranging from 147 pg/ml to 274 pg/ml, after accounting for a dilution factor of 2 which is required for all rat serum. At first glance these may seem to be reasonable values; however, the animal serum O.D. values fall within range of 0 pg/ml to 62.5 pg/ml within the standard curve. This poses a problem when analyzing the data, because results falling below the lowest concentration data point on the standard curve are considered undetectable in the rat serum. This may be because there is no detectible systemic Il-6 in rats; however, this is unlikely due to previously published results indicating otherwise utilizing the same ELISA Il-6 kit. An ideal kit would allow us to choose from a range of standards spanning the lower concentration range in order to detect any changes in Il-6 due to simulated resistance training.

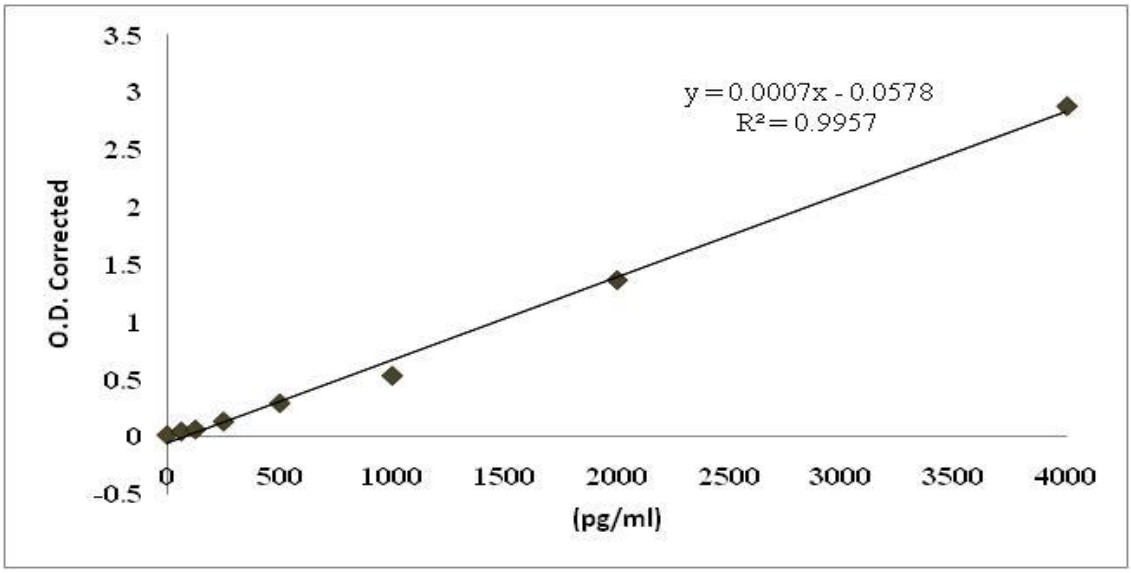

Fig. 17. ELISA Il-6 rat serum standard curve. R^2 0.9957.

Table 4
Il-6 rat serum standard curve and O.D. values.

Standard Curve pg/ml	Corrected O.D.	Animal O.D.
0	0.00561	-0.00607
62.5	0.03737	0.004905
125	0.0542	0.00724
250	0.12325	0.038325
500	0.28324	-0.005625
1000	0.52361	0.0025
2000	1.35719	0.00551
4000	2.87421	0.001035

Standard curve O.D. was obtained through creating a standard curve with known pg/ml samples. The corresponding animal O.D. indicates that all of the obtained animal sample values are < 62.5 pg/ml and therefore undetectable.

CHAPTER V

DISCUSSION

Ongoing studies have identified various windows in which mechanically-induced PGE_2 is thought to have its effect on bone formation. These animal studies are limited to an acute bout of exercise and have yet to establish the impact of NSAID use with repeated bouts of exercise. To our knowledge, there is only one published study in which researchers expanded the acute response studies to a long-term training study, testing the impact of ibuprofen in exercising pre-menopausal females [4]. Kohrt et al. (2010) demonstrated that if ibuprofen is taken after exercise there is an enhanced gain in bone mass over a period of 9 months. The aim of this thesis study was to bridge the gap between the original acute loading animal studies and the more recent human training model using simulated resistance training in rats.

Our first hypothesis, that bone formation rate will be suppressed over time in rats given ibuprofen before each training session but enhanced if ibuprofen is given after each exercise bout, is not supported by our results. Previous acute loading studies show that when NSAIDs are administered 3 hours before loading there is a suppression of the usual increase in bone formation rate [1]; however, this inhibitory effect of NSAIDs is not reflected in our results after repeated bouts of loading provided by the simulated resistance training. Bone formation rate significantly increased over the course of three weeks due to simulated resistance training regardless of when or if ibuprofen was given with no significant differences among groups. These results may be confounded by the

unexpected find of woven bone on the periosteal and endosteal surfaces of tibiae in the trained legs.

Woven bone is structurally unorganized, low in mineral density and is formed rapidly. It can result due to bone damage, hyperphysiological loading or unique loading angles [6]. In the past, woven bone was primarily associated with trauma to the bone, as suggested by Frost (1988) [37]. However, Lanyon et al. (1982) [38] demonstrated that hyperphysiological (but not pathological) loads imposed on a turkey ulna will produce woven bone on the periosteal surface that eventually consolidates into lamellar bone if loading continues for at least 6 weeks. Our loading protocol using moderate intensity muscle contractions (similar to 75% 1 RM) produced robust formation of woven bone. Since 75% of 1 RM is not considered "high intensity" by humans performing resistance training, this leads us to speculate that the stimulated muscle contractions may be imposing unique directional forces on the tibia, providing a unique strain distribution on the bone. Visual analysis of the tibiae cross sections at the tibular-fibular junction indicated woven bone production in every sample analyzed of the exercised leg consistently on the anterior periosteal surface, but not the posterior periosteal surface suggesting an isolated load where the woven bone production was observed. The robust response to our loading protocol, evidence by woven bone formation and large gains in BFR at lamellar surfaces may overshadow more subtle effects of ibuprofen on bone formation rate. Thus, we are unable to make any definitive conclusions on the timing of ibuprofen and its impact on bone formation rate.

Bone mass did significantly increase in every group over the course of 3 weeks; however, much like bone area and density gain, there were no significant differences in bone formation rate among ibuprofen or placebo-treated groups. Kohrt et al. (2010) demonstrated in adult female subjects magnified gains in bone mineral density if ibuprofen was administered after exercise training over the course of 9 months. Although our results do not reflect those previous results in humans, we cannot ignore the woven bone response observed at the tibular-fibular junction described previously. Any effect that may have been exerted by ibuprofen administration may have been washed out by the unique physiological load placed on our animals.

One key limitation of our study is the lack of selectivity of ibuprofen to the COX-2 enzyme. COX-2 isoform is the main contributor to mechanical-induced PGE_2 in bone. As previously described, ibuprofen is less selective to COX-2 than the originally used indomethacin and NS-398. Because ibuprofen actually has a greater affinity towards COX-1 than COX2, it may not adequately suppress PGE_2 in order to provide the inhibition of bone formation response as observed with indomethacin and NS-398.

Another key limitation to our results is our inability to confirm in these animals that ibuprofen had an effect on PGE_2 signaling in bone. This was because samples were saved as serum and not plasma which is necessary for PGE_2 analysis. Furthermore, PGE_2 analysis requires indomethacin immediately after blood is drawn in order to inhibit an ex-vivo synthesis. Our dose of 30 mg/kg has effectively been used in fracture healing studies [33, 34]. Also, Rissing and Buxton (1986) showed an effect on the PGE_2 levels assessed by radioimmunoassay in animals treated with 30 mg/kg/day of ibuprofen in

Sprague Dawley rats. Unfortunately, we do not have any data on altered PGE_2 levels in bone or serum to confirm that our administration of ibuprofen effectively modulated PGE_2 levels before or after our loading regimen.

Originally, we attempted to determine IL-6 serum levels after an acute exercise bout in order to suggest a mechanism for ibuprofen's effect on bone if given after exercise; however, our attempts at quantifying serum IL-6 were unsuccessful, due to the limitation of the assay kit purchased. Since we could not reproduce the enhanced bone gain demonstrated by Kohrt et al. (2010), with ibuprofen given post-exercise, determining mechanisms became a moot issue. The attempt to measure IL-6 would have, however, served to test the efficacy of our delivery method with the animals; because of ibuprofen's ant-inflammatory properties, we would have expected to see suppressed serum IL-6 levels in animals that received ibuprofen after SRT. Previously published data have demonstrated an increase in IL-6 mRNA levels, with simulated resistance training in rats [24] and in serum levels in humans after vigorous exercise [17].

In order to successfully bridge the gap between previous animal studies and recent human studies, it will be necessary to develop an adequate phenotype in order to test any future hypotheses. Although mechanical loading studies have already established that bone formation rate is suppressed when NSAIDs are administered before exercise, our mechanical loading device provides a loading stimulus that differs from previous studies. We are stimulating the surrounding muscles to actively contract rather than bending the bone directly without muscle contraction, as described in ulna loading

studies [1]. It would be desirable to perform a dose response study varying intensity of muscle contractions, in order to test the effectiveness of intensities ranging from 25% 1RM to 75% 1RM in stimulating formation of the more organized lamellar bone. Turner et al. (1994) [39] has demonstrated that the adaptive response of woven bone to hyperphysiological loads is dependent on a threshold, showing that woven bone response was either absent with lower mechanical stimulus or responded at a maximal rate if the stimulus threshold was surpassed. A dose response study would hopefully suppress the woven bone production and thus reveal any effect of ibuprofen on that bone formation response. Once an adequate phenotype is established, then a more appropriate training model can be tested.

CHAPTER VI

CONCLUSION

Non-steroidal anti-inflammatory drugs (NSAIDs) are used by millions across the globe to treat for musculoskeletal pain and inflammation. The use of these drugs, however, has been shown to reduce the anabolic response to bone after an acute bout of exercise in animal models. These studies are thus far focused on an acute bout of exercise and have yet to establish the impact of NSAIDs with chronic exposure to exercise (training). One study in humans has successfully demonstrated an enhanced response of bone to training if ibuprofen is administered after each exercise bout [4]. The aim of this thesis study was to bridge the gap between the original acute loading animal studies and the more recent human training model by using repeated bouts of simulated resistance training in rats.

We were able to produce a robust gain in bone formation rate and bone mass, but were unable to detect any change in bone response to the simulated resistance training with ibuprofen. The robust increase in bone formation indicated by new woven bon, have overshadowed more subtle effects of ibuprofen on bone formation rate. Thus, we are unable to make any definitive conclusions on the timing of ibuprofen and its impact on exercise-induced bone formation rate or bone mass. Future studies are worth pursuing based on the potential therapeutic effects of ibuprofen when taking after exercise [4]. It is still necessary to understand the mechanism of her findings via animal studies in order to optimize any potential benefits in a therapeutic setting.

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