VASCULAR AND MECHANICAL CHANGES IN BONE IN RESPONSE TO
CHRONIC ISCHEMIA AND MECHANICAL LOADING

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Michael Govea
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COMMITTEE MEMBERSHIP

TITLE: Vascular and Mechanical Changes in Bone in
Response to Chronic Ischemia and Mechanical
Loading

AUTHOR: Michael Govea

DATE SUBMITTED: November 2011

COMMITTEE CHAIR: Dr. Trevor Cardinal, Assistant Professor

COMMITTEE MEMBER: Dr. Scott Hazelwood, Associate Professor

COMMITTEE MEMBER: Dr. Lanny Griffin, Department Chair

ABSTRACT

Vascular and Mechanical Changes in Bone in Response to Chronic Ischemia and Mechanical Loading

Michael Govea

Peripheral artery disease (PAD) and osteoporosis have recently been shown to be associated; with the parallel occurrence suggesting that PAD related ischemia may cause or enhance the onset of osteoporosis. In order to determine the mechanism linking osteoporosis and PAD this paper will examine the effects of ischemia and mechanical stimulation on bone remodeling. An immunohistochemistry protocol for vessel marking in bone was also developed. Ischemia was induced in a mouse model to determine vascular and mechanical property changes in bone in response to hypoxia, and mechanical loading-induced remodeling was analyzed for vascular changes. Both ischemia and mechanical loading increased bone vessel density, with ischemic bone increases seen at day 7 and 14. Bone stiffness increased after induced ischemia at day 28. These results point to resultant hypoxia from ischemia drives bone mechanical property changes, likely through stimulation of bone remodeling. We also conclude that an increase in vessel density is seen after induced mechanical loading of bone. Establishing the vascular contribution to the remodeling process may reveal treatment opportunities for remodeling-dependent pathologies such as osteoporosis.

Keywords: Ischemia, Induced Remodeling, Angiogenesis, Osteoporosis,
Peripheral Artery Disease

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If you only do what you know you can do – you never do very much.

- Tom Krause

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CHAPTER 1: INTRODUCTION

Peripheral artery disease (PAD) and osteoporosis, although affecting separate organ systems, have recently shown associated and parallel occurrence. The diseases share common risk factors [1], including age dependent association [2], and PAD occurrence is 5 times greater in women with osteoporosis than women without the condition [3]. To understand the relationship between these diseases, this chapter provides an overview of the normal vascular and bone remodeling systems, the individual disease pathologies, and a discussion of the potential interplay between the two systems.

Normal Vascular Function

The circulatory system is the most vital component of a multi-cellular organism, because it performs the nutrient and waste exchange, including oxygen delivery, required to maintain homeostasis [4, 5]. This critical function arises from the limited distances molecules can travel via diffusion, such that in a complex multicellular organism, convective and diffusive transport effectively contribute to the exchange of nutrients and wastes in all cells. This exchange is accomplished with a highly branched network [4] that can be divided into arterial and venous sides. The arterial side of the vasculature carries oxygenated blood away from the heart (except in the case of the pulmonary circuit) and is responsible for flow and pressure regulation of the blood supply [4, 5]. The venous side of the vasculature transports deoxygenated blood back to the heart and is primarily responsible for the

inflammatory response (venules) and function as the blood reservoir [4, 5].

Interfacing the two sides of the vascular system are the capillaries, the smallest vessels that are the site for nutrient and waste exchange [4, 5].

Within the main vessel classes, arterioles and venules reside in the network as a transition between the arteries and capillaries and the capillaries and veins, respectively. Arterioles and venules are larger in diameter than the capillaries but smaller than their respective main vessels [4, 6, 7]. The network of arterioles, capillaries and venules reside within the tissues and comprise what is typically referred to as microvasculature. Acting as the functional component of the circulatory system, the microvasculature is directly responsible for the nutrient and waste exchange at the tissue and cellular level [4, 5]. Characteristics of the microvasculature, such as total vessel area, influence flow within a given region. For tissues to function properly, delivery of blood must be modulated to match nutrient and oxygen delivery with nutrient and oxygen demand [4, 8]. Individual vessel types of the microvasculature provide unique contributions to the system's function.

The rate of oxygen and nutrient exchange is grossly dependent on the amount of blood flowing through a given region. Blood flow regulation is accomplished by the arterioles in the microvascular network [4, 6, 7], due to their ability to constrict or dilate with the help of smooth muscle cells (SMC), which contract or relax, effectively modulating blood flow to a given downstream tissue [4, 5, 8]. In addition to blood flow, vessel structure greatly contributes to mass transfer, with capillaries having the thinnest walls, a lack of smooth muscle, and small diameter to facilitate maximal delivery [4, 5]. Total capillary area and variability in capillary structure

within a local microvasculature also contribute to the capacity for mass transfer.

Lastly, partial pressures dictates oxygen diffusion into tissue; the greater the oxygen pressure differential, the greater the gas exchange [4]. Interplay of these microvascular components dictates the mass transfer profile within a tissue.

Different tissues have unique vascular requirements determined by factors such as: pressure gradients, physiologic function, and spatial properties. For example, the muscle, kidney and liver have similar spatial and metabolic needs, but due to their physiologic functions, capillary structure varies dramatically. SEM images compiled in **Figure 1** illustrate this eventuality, and highlight the distinct structural differences between the vessel networks. Vascular networks in bone are physically constrained by the tissue itself, thus are primarily dendritic (stem and leaf branching pattern) [9] and the capillary number per arteriole in bone is greater than what is typically seen in muscle, as evidenced by a single arteriole feeding a large capillary network in the humeral growth plate, for example [9]. **Figure 2** illustrates the dendritic vascular networks in bone. In addition to nutrient and waste exchange, bone vasculature has a large influence on local bone deposition [9]. For example, in fracture repair, vasculature invades the tissue void and accompanying osteoclasts degrade any cartilage or matrix prior to mineralization in regions adjacent to the invading vasculature [9].

Figure 1: Tissue specific microvascular networks.
The SEM comparisons highlight the distinct morphological differences of the vasculature depending on the organ/tissue. White bar is 40 μm [10].

Figure 2: Microvasculature in bone.

Top figure depicts periosteal vasculature with the main arteriole and venule marked (Ma and Mv). The bottom figure illustrates vasculature in the subchondral bone, with the C indicating vascular cartilage and the arrows in the sub-figure indicating capillary loops. Used with permission [11]

To account for variability in microvascular needs between tissues, the artery-arteriole-capillary network configuration can exhibit different arrangements. A dendritic, or stem and leaf, network features a main feed vessel branching into two

smaller, parallel daughter vessels, followed by those daughter vessels splitting into two more smaller vessels, and so on [6, 7, 12]. Many vessel branches, or bifurcations, occur before the resulting daughter vessels reach capillary size. All vessels in the resultant network are connected in a linear and one directional manner, with each vessel pair being fed by only the 'parent' vessel upstream with no other flow influences [6, 7, 12]. Arcaded networks feature similar bifurcation characteristics, but not all vessels are connected in a strictly sequential orientation. The two network configurations are illustrated in **Figure 3**. Anastamoses, the connection between two arterioles of the same relative size, are prevalent, and result in many vessels of similar diameter running in parallel with each other, affecting flow profiles in downstream vessels [12]. These parallel running vessels are termed collaterals and function, amongst other things, to maintain a constant blood pressure in the tissue and regulate blood flow [4-7, 12]. The uniform tissue flow parameters ensure consistent oxygen and nutrient exchange within the tissue. The presence of collaterals facilitates this homogenous mass transfer profile, enabling constant fluid parameters and maintaining tissue homeostasis.

Figure 3: Vessel network arrangements.

Examples of dendritic (A) and arcaded (B) vessel network arrangements. Only illustrating the arterial side of the network fed by one main vessel, with bifurcations and anastomoses marked with red dots.

Direction of flow is also indicated by the arrow

In addition to tissue effects, blood flow is also dependent on vessel topology because flow is largely dictated by vessel diameter. Network morphology is tied to tissue anatomy and physiology, for example, the kidney requires a large network of smaller vessels to increase mass transfer. Acutely, flow can be modulated by dilation or constriction of vessels, from either a conducted or flow-mediated response [13]. Chronically flow is altered through structural remodeling, driven by environmental or flow-based alterations [12, 14]. An example of structural remodeling attributed solely to flow mediated changes is illustrated in **Figure 4**. The most common structural remodeling processes are arteriogenesis and angiogenesis and play a vital role in preserving tissue functionality.

Figure 4: Depiction of vessel network alterations.
Flow mediated vascular changes [14]. Am Physiol Soc, used with permission

Arteriogenesis

Arteriogenesis is the increase in lumen size in preexisting vessels or arterialization of capillaries [15]. An occlusion or obstruction in an artery or arteriole will result in reduction of blood flow to distal tissues. Arteriogenesis occurs to normalize the increased shear resulting from the occlusion experienced by implicated collaterals, through the manipulation of collateral diameter or number, termed collateral enlargement or capillary arterialization, respectively [12, 15, 16]. A decrease in the blood flow to the impaired tissue will lead to an increased pressure drop in the collaterals that circumvent the occlusion. An increased pressure drop leads

to an increase in blood flow in these effected collaterals, and subsequently, an increase in shear stress [16, 17].

The primary stimulating factor for both forms of arteriogenesis is an increase in fluid shear stress (FSS) along the luminal surface of the endothelium [15, 16, 18, 19]. Elevated FSS causes initial endothelial distress and vasodilation in parallel collaterals in response to acute occlusion [8, 17]. Highly arcaded vascular networks do not show appreciable loss of perfusion immediately following occlusions due to the initial dilatory response, but networks comprised primarily of dendritic vessel connections, as in bone, show much greater initial loss of perfusion [12]. The presence of collaterals appears to be tissue specific and dependent on VEGF and the VEGF-A gene expression [20].

Due to the vessel network configuration and spatial boundaries present in bone, capillary arterialization is the most likely mechanism of arteriogenesis to occur. In order for enlarged capillaries to undergo arterialization, SMCs must be recruited in one of several ways: proliferation or migration of SMC from upstream arterioles, pericyte differentiation (as determined by expression of smooth muscle markers, such as alpha actin) [12], or fibroblasts migration and subsequent differentiation into a smooth muscle phenotype [21]. Platelet derived growth factor (PDGF-B) is implicated in arterialization, impacting SMC proliferation [22].

Fluid flow alterations, associated wall stresses, and endothelial inflammation appear to regulate the arterialization process leading to capillary remodeling [12, 17]. Fibroblasts are activated through endothelial cell distress signals caused by increases in wall stresses or inflammatory conditions such as oxidative stress [17, 21]. Once

sufficient arterialization and/or collateral enlargement has occurred, blood flow within each vessel will increase. Due to the increase in blood flow, the initial increased pressure gradient subsides resulting in reduction in fluid shear stress, signaling the maturation of the vessel/vessel network and arteriogenesis is halted, as the stimulus is no longer present [19].

Although the vasculature is remodeled in response to the tissue ischemia, the original occluded artery is not replaced by a single vessel but by increases in existing vessel networks [19]. Not all vessels in the network undergo remodeling and the optimal total tissue resistance is not fully reestablished because of the increased number of vessels that compensate for the larger occluded artery [19]. Despite this, arteriogenesis is a critical response that results in vascular compensation for an occlusive event.

Angiogenesis

Insufficient blood flow, termed ischemia, will result in a diminished capacity for oxygen and nutrient delivery. The function of angiogenesis is to alleviate a hypoxic environment by network expansion in ischemic tissues [8, 15, 23, 24].

Sprouting of new capillaries and splitting of existing capillaries, termed intussusception, helps re-establish sufficient oxygen delivery to the tissues [25, 26].

Sprouting angiogenesis, defined as creation of new capillaries, commences with the activation, or distress, of the local endothelial cells [15]. Hypoxia, or lack of oxygen, is the primary stimulus for sprouting angiogenesis which initially results in increased permeability and cell proliferation in response to hypoxia [8, 15, 25, 27].

Under normal conditions, oxygen is present in the intracellular environment and binds to Hypoxia Inducible Factor (HIF), rendering it ineffective. An unbound HIF molecule (in a hypoxic environment) is one of the main perpetrators of the angiogenesis and leads to several cellular responses [8, 15, 25, 27] including cytokine release and initiation of growth factor production. Fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), among other molecules, are some of HIF's most relevant downstream transcriptional targets [8, 15].

Following increased vessel permeability driven by HIF activated VEGF, degradation of the vessel basement membrane and local extracellular matrix (ECM) occurs [15, 28]. ECM degradation, through VEGF activation of matrix metalloproteinases (MMP), provides a region for vessel growth [15]. Local VEGF also triggers endothelial cell proliferation and differentiation in proportion to and in accordance with the local VEGF gradient [15, 28]. FGFs induce mitogenic (cellular division) qualities as well, and influence chemotactic (cellular movement) properties of sprouting cells leading to endothelial migration [23]. Cell to cell contacts are established with adjacent, proliferating cells [23] facilitated by platelet endothelial cell adhesion molecule (PECAM); the main cellular adhesion and signaling protein within the endothelium [28]. With continued VEGF and FGF exposure, proliferation and migration continue to form a new capillary loop, and interaction with surrounding ECM and perivascular cells promotes lumen formation [15].

Vessel lumen development is regulated by a host of factors including VEGF and occurs through cell reorganization, with the basement membrane reformed by the endothelial cells themselves [23, 25]. Pericytes, which are structural cells, assist with

lumen formation and endothelial cell guidance during this vessel growth process [23-25]. Capillary loops form within 5 days of response initiation [24, 25, 28] and increase the mass transfer capacity within the local tissue, partially alleviating the ischemic environment.

Intussusception is the splitting of capillaries through pillar formation in the vessel lumen [26], stimulated by increases in shear stress [25, 26] that result in upregulation of VEGF receptors [28]. Consistent with common angiogenic modulators, intussusception requires VEGF and FGF [24, 26, 28]. The intussusception process initiates with a collagen pillar forming at either side of the lumen and extend from the vessel walls, eventually connecting the opposite sides of the capillary. As more pillars form and elongate, facilitated by myofibroblasts, a rift in the lumen arises, splitting the capillary into two distinct vessels [25, 26]. This angiogenic type does not require much cell proliferation [25, 26], and in adults, occurs primarily in pulmonary system, with some prevalence in myocardium and skeletal muscle [25]. With intussusception, blood vessels can be formed more rapidly than by sprouting [25, 26], by consuming less energy than sprouting [25].

Angiogenesis is vital to the restoration of blood flow to ischemic tissues, however, angiogenesis alone cannot fully restore necessary blood flow in a model of chronic ischemia [24]. Because the capillaries have little control of how much blood is delivered, a robust angiogenic response alone cannot relieve tissue ischemia without the contribution of upstream arterioles [29].

Tissue damage from vessel occlusion and ischemia is localized, affecting primarily the capillary and arteriole networks. Because of this occurrence, one can

expect that most vascular changes will arise within the arteriole and capillary domain through angiogenesis and arteriogenesis [12]. Knowledge of vessel adaptation will help reveal interplay between the vascular other physiological systems.

Normal Bone Remodeling

Bone provides mechanical structure through two main tissue types: cortical and trabecular [5, 30]. Cortical bone is comprised of compact osteons, which form the outer layer of the bone and provide the bulk of the strength [5, 30]. Trabecular bone resides inside of the cortical bone, aids in load distribution, and is referred to as spongy bone because of its porous, matrix structure [5, 30]. Because of its load bearing function, bone is regularly remodeled [5, 30] and adapts through targeted sculpting of the tissue [31]. Bone structure is optimized for strength with respect to its weight, and its trabeculae are approximately aligned with the principal directions of applied stress [31, 32], indicating that bone adapts its structure in response to local strain [30].

Bone remodeling involves osteoblasts, osteocytes, and osteoclasts, which collaborate in basic multicellular units (BMUs) [32], depicted in **Figure 5**. Osteoblasts are involved in bone formation and synthesis, osteoclasts dissolve and resorb damaged or unnecessary bone, and osteocytes are mature osteoblasts that become integrated into the mineral matrix of bone and sense mechanical loads [5, 30-32]. In normal circumstances, the main stimulus for remodeling starts with the osteocyte. These cells respond to the local environment and mechanical factors such as stretch, pressure, and flow [30]. Sensing of high strain can trigger remodeling, and

formation of microcracks will lead to osteocyte apoptosis, a potent remodeling stimulator [5, 30-32]. Fatigue loading is the main perpetrator of microcracks, and is defined as the occurrence of cracks that propagate in the tissue at lower than fracture magnitudes if the load is applied as a cyclic loading and unloading [30]. The magnitude of fatigue damage is proportional to bone structure and vascular changes that occur during the remodeling process [33].

Figure 5: Process of bone remodeling, displaying the main cell types [34]

Microdamage or apoptosis results in osteoclast recruitment to the site of the crack or damaged cell [5, 30-32]. The osteoclasts dissolve and remove the damaged tissue, which is then replaced with new tissue by osteoblasts [5, 30-32]. Upon mineral deposition, osteoblasts become integrated into the boney matrix and mature into osteocytes [5, 30-32]. Bone endothelium, in locations of damage or growth, actively recruit osteoclasts for either remodeling or vascular expansion [9]. Recruitment is tied to the RANK / RANKL complex interaction; RANK is an osteoclast membrane

receptor and RANKL is the associated ligand expressed on endothelial cells and osteoblasts [9]. Hematopoietic derived osteoclasts and osteoblasts circulate in the peripheral vasculature and exist in bone marrow, and while poorly understood, it is thought that endothelial cell adhesion relocates them from these two sources into the tissue of interest [9]. However, osteoclast extravasation is not required for bone removal, evidenced by lower bone mass in non-vascularized tissue compared to vascularized tissue [9].

Osteoclasts produce heparinase, which releases VEGF from heparin, which is bound to the ECM, allowing VEGF to activate the VEGF receptor, subsequently increasing expression of the RANKL complex on osteoblasts, and increasing expression of RANK on endothelial cells [9, 35]. Local angiogenesis is subsequently induced by the activation of RANK, for reasons unknown, contributing to the vessel remodeling as the vasculature advances [9, 35]. Only cells adjacent to capillaries give rise to bone tissue and eventual mineralization (because of the RANK / RANKL interactions between the osteoblasts, osteoclasts, and endothelial cells), therefore implicating any surrounding vasculature in the remodeling process [9, 34]. From these findings we can deduce that the invading vasculature is a primary factor for regulation of the ossification process [9], specifically, VEGF appears to regulate osteogenesis and angiogenesis, and is required for bone formation [9]. Thus, interaction between both the bone and vascular system dictate normal bone function.

Impaired Bone Function: Osteoporosis

Osteoporosis is generally described as a systemic skeletal disease, characterized by deterioration of bone micro-architecture leading to increased bone fragility [34, 36]. Specifically, osteoporotic bone contains fewer and narrower trabeculae, with a loss of connectivity between individual trabeculae, and decreased wall and osteoid (mineralization) thickness attributed to loss in osteocyte density and viability [37-39]. Osteoporosis is often an asymptomatic condition prior to a fracture event [36] and clinically, osteopenia and osteoporosis are defined by loss of bone mass measured in the lumbar spine or hip. Osteopenia and osteoporosis are 1 SD and 2.5 SD, respectively, below the average bone mass of healthy, thirty-year old and sex-matched control subject [37].

Peak bone mass in humans is seen anywhere from 25 to 30 years of age [37], but from that point in time, fracture rate and osteoporosis increase dramatically with age [40]. Three to six percent of women have osteoporosis by 50, 50-75% of women have osteoporosis by age 90 [40] and there is a 40% risk of major fracture for women over 50 [40]. For men, there is a 1% prevalence at age 50 and 15-28% prevalence at age 90 [40]. Female osteoporosis is almost exclusively related to menopause, during which greatly accelerated bone loss can account for 33% of lifetime bone loss [37]. Enhanced bone resorption is the main contributor related to estrogen deficiency, but in the years following menopause, lack of bone formation is observed and contributes significantly to the disease onset [34]. Furthermore, duration of menopause is more important than the age of onset in terms of osteoporosis onset and development, with longer menopause leading to increased osteoporosis [36]. Additional risk factors for

developing osteoporosis include alcohol use, tobacco use, drug use, inactivity, and poverty [41].

Fracture is the primary risk for patients with osteoporosis and there are 1.5 million osteoporosis related fractures per year in the US [41]. The femoral head is particularly susceptible to osteoporosis [38], and hip fracture accounts for 18% of all osteoporotic fractures [42]. Osteoporosis and related fractures in the Americas and Europe contribute to the loss of 2.8 million disability adjusted life years (DALY) [40], which accounts for 1.75% of DALYs for non communicable diseases in western society and 0.83% of worldly DALYs [42].

Osteoporosis is likely to be caused by a change in the complex interactions among regulators of bone cell function [34]. Common cells and molecules involved are the osteoclasts, osteocytes, osteoblasts, and estrogen, and in osteoporotic patients, osteoblasts have impaired function leading to inadequate bone formation over time [34, 39]. More importantly, the osteoclasts seem to be highly activated and unregulated in osteoporotic patients [34, 39] resulting in excessive bone resorption, decreased bone mass, and microarchitectural deterioration of the skeleton [34]. The specific mechanisms are not well identified, but some factors such as estrogen deficiency seem to contribute to the increase in systemic osteoclast activation [34, 36]. Other factors may be chronic glucocorticoid treatments as well as a host of cytokines, as they seem to contribute to osteoclast activation and subsequent bone resorption [34, 37]. The combination of osteoblast dysfunction along with enhanced osteoclast activity leads to the marked loss in bone mineral density [39].

Due to the prevalence, morbidity and mortality associated with osteoporosis, several treatments are currently utilized. Bisphosphonates inactivate osteoclasts [4, 34], leading to decreased resorption, but also an increase in stiffness and micro-damage buildup which can have severe long-term complications [4, 34]. Strontium ranelate (SR), although not currently FDA approved, acts as both an anti-resorption and bone forming drug concurrently [43]. SR increases cortical bone thickness in osteoporotic patients and induces a more robust trabecular bone network [43].

Treatments thus far have focused primarily on targeting the osteoclasts and osteoblasts, but other cellular mechanisms have yet to be targeted. The interdependence of the vascular system and bone remodeling process needs to be explored further so that new treatment targets can be revealed and utilized. For example, by stimulating angiogenesis and vascular remodeling, bone remodeling and formation could be stimulated.

Impaired Vascular Function: PAOD

Peripheral arterial occlusive disease (PAOD), often referred to as peripheral artery disease (PAD), is a major cause of disability and lifestyle changes worldwide [44]. The cause of PAD is atherosclerosis in peripheral vessels and is defined by ischemia in a patient's extremities [44].

PAD has about 10% prevalence in the population with increasing occurrence in elderly patients [44]. Some estimates number the individuals affected with PAD at 20 million in the United States [44]. In elderly patients there is a much higher occurrence, and onset of acute limb ischemia attributed to PAD is typically related to

increased risk of mortality and amputation in the effected limb [29]. Peripheral atherosclerosis, which leads to PAD shares common risk factors with coronary artery disease including: advanced age, female sex, diabetes, tobacco use, and hypertension [17, 44]. Increased mortality has been observed when PAD is diagnosed in conjunction with coronary artery disease [44, 45] and additional disease comorbidities include chronic arterial disease, hip fracture, lower extremity performance (measured by treadmill exercise duration) and decrease in bone marrow density (BMD) [1, 17].

Diagnosis of the disease is primarily performed by using the Ankle Brachial Index (ABI) which measures the blood pressure ratio between the ankle and arm, during both rest and exercise [1, 44, 46]. Typically, symptoms arise when the ABI is less than 0.8, with pain and tissue loss occurring at values of <0.5 and <0.3 , respectively [44].

Growth of atherosclerotic plaques can progress gradually, mainly caused by an accumulation of oxidized LDL (low density lipoprotein) in the subendothelial space [47]. Atherogenesis leading to the development of PAD is a function of turbulent flow in conduit arteries and subsequent inflammation of the endothelium [47]. Specifically, subendothelial ox-LDL activates macrophages, leading to oxidative stress, inflammatory response, plaque formation, and eventual endothelial dysfunction. The larger the plaque becomes, the greater the vessel occlusion [47]. Development of PAD leads to lower-limb pain with exercise, also known as intermittent claudication, and possible critical limb ischemia [17, 45], lower limb pain at rest. Disease progression is most often undetected due to the gradual nature of

disease onset and ordinarily has asymptomatic presentation until reaching a critical level [44].

Pathophysiology of the disease usually takes on the following characteristics. Flow induced collateral dilation occurs in the side stream collaterals near the occlusion as an acute response to a developing plaque [17]. Chronic response occurs via vascular remodeling, and appears to result in the decrease of collateral resistance over time, but distal resistance associated with the microvasculature does not change significantly in symptomatic patients [17].

Often in symptomatic patients, the endogenous vascular compensation response is impaired, including lacking the capacity for vasodilation, severely diminishing the capacity for repair [45, 48]. Depending on the tissue characteristics, remodeling activity will vary. An arcaded vascular network experiences primarily arteriogenesis, where as a dendritic network experiences more angiogenesis and some arteriogenesis. [12]. In a diseased state unable to compensate, blood flow becomes restricted in affected tissues and ischemia will result, leading to inefficient nutrient delivery, eventually resulting in a tissue death [8].

PAOD and Osteoporosis Link

A large majority of the research of PAD has been in muscle [8, 12, 15, 16, 24, 27, 29, 45, 48, 49], leaving implications in bone poorly defined. However, recent findings may bridge the gap to bone by relating chronic ischemic disease and osteoporosis. High level associations between PAD and hip fracture are explained by age, lower bone mineral density (BMD) and poorer lower extremity performance

(based on treadmill walking) [1, 35], including direct associations between osteoporosis and PAD in women [2]. Risk factors for osteoporosis and atherosclerosis overlap; including advanced age, smoking, diabetes, and physical inactivity [1]. Furthermore, PAD occurrence is 5 times greater in women with osteoporosis than women with normal BMD [3], femoral neck BMD is a predictor of PAD [3], and low BMD correlates with calcification of atherosclerotic plaques [50].

Osteoprotegerin (OPG), which inhibits RANK mediated osteoclast activation, shows increased levels in patients with PAD and may help explain initial relationships between the two diseases [34, 50]. Prior discussion revealed that the RANKL complex is implicated in local inflammatory bone loss by activation of osteoclasts [9, 34]; and that local angiogenesis triggered by activation of RANK contributing to vessel remodeling [9, 35]. In the case of osteoporosis, where there is an excess of osteoclast activity, OPG production is stimulated by tumor necrosis factor as a response to suppress the osteoclast activity, and by endothelial cells as an autocrine anti-apoptotic factor [50]. Since there is increased OPG, which disrupts the RANK / RANKL interaction, the angiogenesis may not be stimulated, resulting in diminished capacity for bone remodeling. This may explain the ischemic and disease condition based on bone physiology.

Vascular necrosis and chronic inflammation correlate to osteoporosis and increased resorption through recruitment of osteoclasts to the site of endothelial activation [9, 35]. Invading vasculature in bone is responsible for regulation of the ossification process and angiogenesis is required for bone regeneration [9]. In a patient with PAD the vasculature lacks the ability to acutely dilate or outwardly

remodel, diminishing the capacity for structural vessel adaptation [17]. This vascular impairment may lead to a diminished capacity for bone repair, thus leading to osteoporosis.

Symptoms associated with PAD, namely intermittent claudication, may also contribute to the development of osteoporosis. A patient's activity will be restricted with pain related symptoms, such that normal mechanical stimulus is diminished, leading to bone removal associated with remodeling. And if bone resorption is due to advanced age or estrogen deficiency, the mechanical integrity of the bone may be compromised, leading to osteoporosis.

Aside from previously described treatments that target osteoporosis alone, Vitamin D administration has seen positive results for patients with both PAD and osteoporosis [51]. Vitamin D increases BMD in osteoporotic patients with PAD and alleviates symptoms associated with lack of vitamin D, including bone aches, pain, and weakness [51]. It was hypothesized that due to pain associated with both diseases, the patients were less active, resulting in less vitamin D adsorption from the sun [51]. This treatment strategy would be effective if the cause of the combined disease stemmed from inactivity, as previously suggested.

While each hypothesis is plausible, determining the mechanism linking osteoporosis and PAD will stem from initial examination of the effects of ischemia on bone remodeling. Determining how parameters, such as vessel density and bone remodeling, change with ischemia is crucial to understanding the interaction between the two diseases.

Aims and Hypotheses

As the first step to understanding the disease links, the main focus of this research is to examine the physiologic responses to ischemia and fatigue loading on bone remodeling. Interplay between bone remodeling and vascular remodeling has been examined in fracture repair and in induced remodeling [52, 53], but little is known regarding the remodeling response to an ischemic environment. Based on the void in understanding, and in order to providing a foundation of initial observations, the objectives of this thesis are:

1. *Establish a protocol for use of immunohistochemistry for identification of blood vessels in bone.* The goal of this aim was to create a reliable method to quantify the vascular changes in bone in response to ischemia and fatigue loading.
2. *Determine the effects of ischemia on tibial mechanical properties and microvasculature, and examine any time dependence associated with the changes.* The purpose of this aim was to quantify and correlate changes in vasculature in response to induced ischemia.
3. *Determine if increased vasculature will occur in response to mechanical fatigue loading, consistent with prior research.* The goal of this aim was to demonstrate the vessel identification protocol's effectiveness and to determine time dependant changes in the bone vasculature during load-induced remodeling.

CHAPTER 2: IMPACT OF ISCHEMIA ON VASCULARIZATION AND MECHANICAL PROPERTIES IN BONE

Introduction

Osteoporosis is likely caused by a change in the complex interactions among regulators of bone cell function [34]. Treatments thus far have focused primarily on regulating osteoclasts and osteoblasts, with limited success, but other cellular mechanisms have yet to be targeted. Possible interdependence of the vascular system and bone remodeling process needs to be explored further so that new treatment targets can be revealed and utilized. There appears to be a correlation between male patients with PAD and lower BMD [1], and a direct association between the two diseases in elderly women [2]. This parallel disease occurrence suggests that PAD related ischemia could cause or enhance the onset of osteoporosis, so in order to test this hypothesis, it is necessary to know the direct impact of ischemia on bone. One causal explanation is that hypoxia resulting from induced ischemia will result in alteration of gene (VEGF, HIF) expression and cell (osteoclasts, endothelial cells) activation, directly impacting BMU activity through the microvasculature. This change in BMU activity could affect the bone's mechanical properties and would be directly dependent on local vasculature and blood supply.

As a starting point there are some known relationships between the vascular system and bone tissue, but interaction between the two systems is largely uncharacterized. Known associations include a linear correlation between total vessel area and bone formation [54], and angiogenesis occurring in areas of bone formation,

preceding mineralization [52]. The relationship between blood flow and bone remodeling has been only slightly defined in a mechanically loaded environment [52].

Compared to structural adaptation in muscle, little is known about the resulting mechanical or vascular changes attributed to diminished blood flow in bone. Previous studies have primarily examined subchondral bone and the immature femoral head to elucidate the pathologic alterations resulting from ischemia [55, 56]. four weeks after an ischemic event, the subchondral bone exhibited significantly increased mineralization [55], and hypoxia induced local chondrocyte death in the region of ischemia [56]. Furthermore, revascularization in the subchondral region occurs within 4 weeks of ischemic event, but revascularization of the bone requires more time [56]. Although somewhat narrow in scope, these studies have provided initial observation of an ischemic environment in bone and lead to the possibility that vascular changes precede bone formation and in areas of ischemia. This possible relationship is one of the primary aims of the research presented in this chapter.

As a possible explanation for this relationship between vascularization and bone formation, it appears that hypoxia and the hypoxia inducible factor (HIF-1) pathway are intimately involved in bone's response to ischemia [56, 57]. The HIF-1 pathway is activated in ischemic tissue [56], and consistent with muscle, the HIF and vascular endothelial growth factor (VEGF) pathways are upregulated in bone in response to hypoxia [57]. Specifically, VEGF and HIF-1 are upregulated in osteoblasts in the region of distress, fracture, or hypoxia [57] and early angiogenic response in adjacent cartilage is implicated in the revascularization process of bone

[56]. Thus, the HIF-1 pathway couples the angiogenic and osteogenic pathways during bone modeling and development [56].

Since the HIF pathway is active in ischemic regions, and HIF regulates angiogenesis, the next step is to determine the extent of angiogenesis. Angiogenesis is required for bone regeneration and osteogenesis [33, 57], but additional contributors to the angiogenic response have not been examined. PECAM may facilitate interactions between angiogenesis and osteogenesis [58], as it has been implicated in angiogenesis thru endothelial activation signaling, cellular adhesion and extravasation, and is expressed in hematopoietic-derived osteoclasts [58]. For bone remodeling PECAM mediates normal osteoclastogenesis by binding the Syk protein (which stimulates osteoclast precursor proliferation), regulating its downstream effect. The PECAM knock-out mouse lacks the ability to bind Syk, leading to unregulated osteoclast precursor proliferation and subsequently, increased osteoclastogenesis and bone resorption [58], resulting in a diminished trabecular bone volume [58]. For vascularization in the ischemic bone environment, there is an upregulation of VEGF and PECAM, indicative of angiogenesis [56]. Thus, PECAM appears to influence both bone remodeling, through osteoclast regulation, and vascularization in bone.

These observations and conclusions are not unexpected, as complement processes occur in muscle, but these conclusions help to reveal commonalities, including HIF and PECAM, between revascularization and re-ossification, indicating a need for further research. However, these studies did not examine cortical bone changes or vascular alterations in mature bone. Because PAD and osteoporosis onset

occurs in developmentally mature individuals, examination of mature bone is much more applicable to the disease states. Due to the gap in understanding, one goal of this project is to elucidate vascular necrosis and angiogenic changes in cortical bone stemming from the response to ischemia. We also examined any mechanical changes resulting from ischemia induced revascularization. If there is a vascular remodeling response to the ischemic environment, as would be expected, we should see increased in vasculature, followed by enhanced mechanical properties.

Methods

Summary

Chronic ischemia was induced in the mouse by resection of the femoral artery vein pair. Forty three C57BL/6 male mice aged 2-3 months were separated in to two groups. All animals except for day 0 underwent the ischemia surgery, with one group of mice designated to observe mechanical properties, while the second group was used for histological assessment. The day 0 animals did not undergo any procedure and served as a control time point. Each of these initial treatment groups was further sub divided into four time points, **Table 1**. Depending on the designation, the tibias from each animal were excised and prepared for either mechanical testing or histology. A 3-Point Bending apparatus was used to elucidate mechanical properties and immunohistochemistry (IHC) was employed for determining blood vessel density in the cortical bone of the tibia.

Table 1: Experimental design and animal designation.

Displayed as number of animals per experiment at a given time point and procedure.

Time Point (Days)	Histology	Mechanical Analysis
0	4	4
7	5	4
14	6	8
28	6	6

All surgical procedures and animal handling was approved by the Institutional Animal Care and Use Committee (IACUC) prior to experiment initiation.

Additionally, all surgical procedures were preformed in an aseptic environment. Mice

were maintained in micro-isolation cages with a twelve hour light/dark cycle and ad libitum access to feed and water.

Surgical Model of Chronic Ischemia – Femoral Artery Resection

Surgeries were performed under a stereomicroscope and animals were anesthetized with isoflurane (1.5-2% in O₂ at 1ml/min) throughout surgical preparation and procedure. The hair on the medial aspect of each hindlimb was removed with trimming clippers and depilatory cream, and disinfected with chlorohexidine diacetate. Next, the animal was placed supine on a heating pad with a rectal-feedback probe maintaining a 35° C temperature, with the animal's nose in the anesthetic mask. An incision was made on the medial aspect of the left hindlimb and extended from the abdominal wall to just above the ankle. Connective tissue was blunt dissected from the underlying skeletal muscle and skin, and sterile saline was used to irrigate the wound. The nerve adjacent to the isolated artery-vein pair was separated, being careful not to damage it. The femoral artery-vein (a-v) pair was ligated with 6-0 silk suture proximal to the profunda femoris, and the saphenous a-v pair was ligated halfway between the knee and ankle. The fat pad and accompanying epigastric a-v pair were removed. The profunda femoris was cauterized at the bifurcation from femoral a-v pair, and the saphenous vessel pair was cauterized just distal to the ligation. Minor vessel branches from the main a-v pair were cauterized prior to resecting the a-v pair away from the host tissue starting at the distal ligation and terminating just distal to the proximal ligation. A depiction of the operation and

region of vessel removal is displayed in **Figure 6**. The incision was closed using 7.0 polypropylene suture.

Figure 6: Depiction of resection surgery.

Region of vessel removed from the medial aspect of the left hindlimb. Dashed line corresponds to the portion of vessel excised to induce the ischemic condition.

A contralateral sham surgery involved an incision of equivalent length and location on the medial aspect of the right hindlimb, and blunt dissection of connective tissue, just as in the previous limb. This sham procedure served as an internal control for any effects of general surgical trauma. A subcutaneous injection of the analgesic buprenorphine (0.075mg/kg) was delivered following the procedure and the animal was allowed to recover until ambulatory in a heated chamber. A detailed surgical protocol is reproduced in Appendix A.

Tibial Excision and Preparation

At the assigned time point, the animals were euthanized by cervical dislocation and both tibiae were excised. Two methods were employed depending on the continued use of the tibia. For mechanical characterization, an incision was made in the medial aspect of the hindlimb and was extended the full length of the hindlimb. The skin was separated and cut away from the underlying muscle from the proximal end of the femur to the heel, exposing the distal tendons. All of the tendons were cut at the ankle; the patellar tendon was also cut. All surrounding musculature around the tibia and fibula was peeled off the bone and removed. The ankle and knee joints were severed and the fibula was removed before the now isolated tibia was wiped clean of any remaining tissue with a Kimwipe (Kimberly-Clark, Neenah, WI). Appendix A details the full protocol. The excised tibiae were then frozen at -20°C for approximately 72 hours before thawing and mechanically testing.

For histological experiments the tibia was excised in a similar manner. The skin was removed and the patellar and ankle inserting tendons were cut, however, only the gastrocnemius muscle was removed from the tibia and the fibula was left intact as well. These two modifications provided better support during sectioning and allowed for preservation of the periosteum [52]. Appendix A details the full protocol. Once isolated, the tibias were immersion fixed in Histochoice (Electron Microscopy Sciences, Hatfield, PA) for 48 hours. Samples were then decalcified for a minimum of 5 days in EDTA (Sigma-Aldrich, St. Louis, MO) prior to processing and embedding. The day 14 and 28 samples were washed and stored in phosphate

buffered solution (PBS) following decalcification in EDTA until the samples were processed and embedded.

Mechanical Property Testing

A servo controlled, 3-point bending apparatus was utilized to quantify the material and mechanical properties of the tibia. Due mainly to length constraints, a 3 point apparatus instead of a 4 point apparatus, which provides more uniform loading, was used [59]. Prior to placement into the testing tray, the tibiae were thawed and moistened with PBS. The bones were positioned with the anterior surface of the tibia face down so that when the specimen is viewed at eye level the bone is in a concave orientation; this orientation prevented movement of the tibia during the test, which was a problem with the convex orientation. The two lower supports of the tray were 10mm apart and the third ‘point’ was brought down equidistant from each of the lower two points to apply a load. The bones were tested in an anterior-posterior orientation with the former face down. A load cell (500N, Applied Measurements LTD, UK) was attached to the descending arm and force-displacement data was gathered at 685Hz. The maximum displacement allowed after contact with the bone was 1mm, with the actuator moving at 2mm/min, as per the guidelines put forth by the testing protocol. The 1mm maximum displacement sufficiently captures the fracture event, without recording superfluous data after the fracture. The apparatus and set up is displayed in Appendix B.

At the conclusion of the test the outside and inside diameters were measured at the point of fracture. The measuring apparatus is depicted in Appendix B. Along

with equations described by Jamsa [60], detailed in **Table 1**, the Ultimate load (N), Ultimate stress (Pa), Elastic modulus (Pa) and stiffness (N/mm) were obtained.

Table 2: Equations used for Mechanical Property Characterization [60]

Variable of Interest	Equation Used	Legend
Stress	$\sigma = \frac{FL(r_{outside} / 2)}{4I}$	σ = stress F = Force L = Support spacing
Elastic Modulus	$E = \frac{FL^3}{d(48)I}$	$r_{outside}$ = Outer radius I = Moment of inertia d = Displacement
Moment of Inertia	$I = \frac{\pi(D_{outside}^4 - D_{inside}^4)}{64}$	$D_{outside}$ = Outer diameter D_{inside} = Inner diameter

Immunohistochemistry

Identification of vessels was performed by staining an endothelial-specific glycoprotein with GS-1 Lectin. Following the fixation and decalcification, the bones were cut in half transversely at the midshaft, dehydrated, and impregnated with paraffin wax in an automated processor before embedding in paraffin wax. Using a RM 2255 (Leica Microsystems, Buffalo Grove, IL) microtome, 6 μ m sections were cut from the blocks starting at the midshaft. The sections were mounted on Super Up Rite – Bond Rite slides (ThermoFisher Scientific, Houston, TX) and incubated at 80°C for ten minutes. After a xylene wash and a graded ethanol rehydration series, antigen retrieval was performed using citrate buffer (RICCA Chemical Co., Arlington, TX) at 95°C for 10 minutes. Treated slides were washed in PBS for 20

minutes before 1 hour incubation of Biotinylated GS Lectin I at 10µg/ml (Vector Labs, Burlingame, CA). Vectastain AB enzyme (Vector Labs, Burlingame, CA), prepared in accordance with the manufacturer's directions, was applied for a 30 minute incubation. After a PBS wash, the slides were developed with peroxidase and DAB substrate kit (SCBT, Santa Cruz, CA) for 15 minutes. Half of the samples were counterstained with 0.5% Methyl Green (Fisher Scientific, Houston, TX). A final distilled water rinse, and ethanol and xylene dehydration were executed before coverslip application. Appendix A details the full protocol.

Finished samples were imaged using bright field microscopy (Olympus, Center Valley, PA) and a Retiga Exi camera (Q Imaging, BC, Canada). Vessel densities as a percentage of the total tibial area were determined with the use of Image J (NIH, Bethesda, MD).

SpO₂ Measurements

The induction of an ischemic environment in the hindlimb as a result of the surgical intervention was validated using a pulse oximeter (Mouse STAT, Kent Scientific, Torrington, CT). Additionally, time to reperfusion following the resection procedure was of interest, and the SpO₂ measurements quantify the degree of reperfusion. For all measurements, the 'cuff' was placed around the arch of the hindpaw. Hindlimb pulse oxygenation was recorded prior to the resection surgery, immediately after the resection surgery, and just prior to euthanizing the animal at the prescribed time point for tibial excision.

Statistical Analysis

A two way ANOVA was used to identify statistical differences. If applicable, comparisons were made between the operated and sham tibia, using a Student's t – test, and comparisons were made between time points and bone regions using one way ANOVA. Some of the figures display percent change data, which was normalized by the averaged day 0 data. Results are expressed as mean $\pm$ SE. Statistical significance was set $p \leq 0.05$, unless otherwise stated.

Results

Induction of Ischemia

Hindpaw oxygen tensions were not different between the two hindlimbs prior to surgery, but levels were significantly diminished in the operated hindlimb immediately following the procedure, **Figure 7**. Based on the significant decrease in SpO₂ in the distal hindlimb, it can be concluded that ischemia was indeed induced by the surgery. Comparison of the operated hindlimbs before and after the procedure revealed a post surgical decrease in SpO₂ levels, furthering the notion of an induced ischemic environment. Interestingly, the unoperated hindlimb showed an increase in SpO₂ levels following the surgery ($P < 0.001$).

Figure 7: %SpO₂ in the hindlimb prior to and immediately following surgery.

Black bar is contralateral sham and Grey bar is operated hindlimb.

* $p < .05$ vs. sham. 'a' $p < .001$ vs. pre-surgical

Reperfusion of the ischemic hindlimb appeared to have occurred prior to day 7, as no differences were observed between time point specific operated and non-operated hindlimbs, and there were no differences between the three time points ($p = 0.206$), **Figure 8**.

Figure 8: %SpO2 in hindlimb at each time point following induced ischemia.
Black bar is contralateral sham and Grey bar is operated hindlimb.

Mechanical Properties

In accordance with the hypothesis, the thought is that mechanical property changes will give insight into the degree of bone remodeling and modeling occurring. Quantification of mechanical properties is presented as a way to test the hypothesis.

Tibial stiffness was not significantly different from the contralateral sham at day 7 or day 14, but stiffness was increased at day 28, **Figure 10**. However, there was

no change in stiffness between time points before or following the ischemic event. Interestingly, day 0 and day 28 sham stiffness was significantly lower than day 7.

Figure 9: Force – Displacement curve from 3 point bend test.

Linear portion of trace, used for stiffness calculation, and ultimate load point is clearly visible as the peak in the trace.

Figure 10: Tibia stiffness by time point.

Day 0 is un-operated control. Black bar is contralateral sham and Grey bar is operated hindlimb. * = $p < .05$ vs. sham at Day 28. 'a' = $p < .05$ vs. Day 7 sham.

The ultimate load data showed similar findings associated with the tibial stiffness. There was not a significant increase in the resected compared to sham at any time point, but there was trending towards statistical significance for the resected data at day 28 ($p = 0.061$ with 57% Power), **Figure 11**. However, there were not any significant differences between the ischemic time points. Additionally, unlike the stiffness data, there were no differences between the sham time points.

Figure 11: Ultimate Load by time point.

Day 0 is un-operated control. Black bar is contralateral sham and Grey bar is operated hindlimb.

Ultimate stress and modulus were not different between time points or between the ischemic and non-ischemic tibiae (data in Appendix C).

The systemic changes in bone mass were quantified with cross-sectional cortical thickness measurements at the site of fracture. **Figure 12** depicts the field of view used to obtain measurements. No differences were seen in the data, and there were no differences between time points for either the resected or sham groups, **Figures 13.**

Figure 12: Microscope field of view for thickness measurement.

Figure 13: Cortical Thickness at each time point.
Black bar is contralateral sham and Grey bar is operated hindlimb.

Quantification and correlation of bone mass characteristics with the mechanical data were analyzed with cross-sectional area. There was a significant increase at day 14 compared to sham, however, the sham data was significantly

decreased at day 14 compared to day 7, **Figure 14**. No differences were observed across the resected time points using a one-way ANOVA ($P = 0.3$).

Figure 14 : Cross sectional bone tissue area, by duration after surgery.

Black bar is contralateral sham and Grey bar is operated hindlimb.

* $p < .01$ vs. sham. # $p < .05$ between Day 7 and 14 sham data points.

Histological Analysis

Analysis of tibial cross sections encompassed quantification of several parameters including regional bone area and cortical vessel number. Vessel densities were calculated as the number of positively marked vessels per square millimeter in the photomicrographs. **Figure 15** illustrates positively marked vessels in cortical bone of the tibia.

Figure 15: Positive and control vessel marking in cortical bone.
 10x region overview (A, D) with dashed box indicating region of magnification. 40x view (B, C, E, F) of specified region. Positively stained vessels are marked with white, closed, arrows (B,E). The respective serial controls (no primary antibody) are marked with black, open, arrows (C, F). The image on the left depicts vessels of a Day 14 resected tibia and the right image illustrates vessels in a Day 28 resected tibia. Grey scale bar is 150 μm and black scale bar is 50 μm .

Vessel density increased at day 14 compared to sham, and both day 14 and 28 were significantly increased compared to day 7 and day 0, **Figure 16**. There were no differences between time points for the sham data points.

Figure 16: Vessel density by time point.

Day 0 is un-operated control. Black bar is contralateral sham and Grey bar is operated hindlimb. * = $p < .05$ vs. sham at Day 28. Both 'a' points are significantly different from both 'b' points and vice versa $p < .01$.

Discussion

The majority of the conclusions will be discussed in detail in Chapter 4 with this section serving as an overview of findings. The data in **Figure 10** suggest that there may be bone modeling in the sham tibia by day 7, evidenced by the significant increase in stiffness. Furthermore, the data suggest bone removal and/or remodeling is occurring in the sham hindlimb by day 28, as the stiffness returns to a pre-surgical level.

For the ischemic hindlimb, it seems that remodeling is occurring in response to ischemia, evidenced by the increase in stiffness at day 28 and the trending of the ultimate load data. It appears that the ischemia alone is triggering the remodeling, most likely through angiogenesis, substantiated by Figure 16. In response to the induced ischemia there is a marked increase in vascular density in the bone by day 14 and that increase from the day 0 and day 7 baseline is sustained until day 28. Interestingly, the most significant increase in vasculature occurs at day 14, prior to any observed mechanical changes. Because of this, it appears that the vascular response is driving the mechanical and remodeling response, such that an increase in vasculature, resulting from the ischemia, is directly affecting bone remodeling. Although similar interpretations have been made for bone modeling in fracture repair, this is the first examination of direct ischemia effects.

The vasculature is required for bone remodeling, but the seemingly direct connection of the ischemic environment to stimulated remodeling is a novel observation. Furthermore, it appears that angiogenesis is sufficient for bone remodeling and this ischemic influence on bone remodeling could further our

understanding of the system interactions. Thus, the stimulation of angiogenesis alone could have an effect on bone, providing new treatment strategies for patients with both PAD and osteoporosis.

CHAPTER 3: IMPACT OF INDUCED BONE REMODELING ON VASCULARIZATION

Introduction

Osteoporosis is generally described as a systemic skeletal disease, characterized by deterioration of bone micro-architecture leading to increased bone fragility [34, 36]. Specifically, osteoporotic bone contains fewer and narrower trabeculae, attributed to loss in osteocyte density and viability [37-39]. Fracture is the primary risk for patients with osteoporosis and there are 1.5 million fractures per year in the US [41]. Osteoporosis and related fractures in the Americas and Europe contribute to the loss of 2.8 million disability adjusted life years (DALY) [40], which accounts for 1.75% of DALYs for non communicable diseases in western society and 0.83% of worldly DALYs [42]. Based on the disease prevalence and lack of robust treatment options, osteoporosis is an important area of research.

Osteoporosis is likely to be caused by a change in the complex interactions among the regulators of bone cell function [34]. Normally, bone experiences microdamage from physiologic loading and adapts through targeted sculpting of the bone tissue [31]. This repair and shaping of bone is executed by the basic multicellular units (BMUs), composed of osteoclasts that remove tissue and osteoblasts that deposit tissue into the tissue void [31]. A remodeling event commences when osteocytes (mature osteoblasts) experience elevated or diminished loads and undergo apoptosis, a potent bone remodeling stimulus [31]. The integration of these three cell types, along with intracellular signaling drives the remodeling of

bone. In a patient with osteoporosis, the cellular interactions are altered, resulting in an increase in bone removal that is unmatched with bone deposition. Tissue removal is the first part of the remodeling process [61], and with inadequate bone deposition, the bone loses structural integrity. From this information, we can conclude that osteoporosis is intimately related to the bone remodeling process.

Greater understanding of the pathophysiology of osteoporosis requires greater comprehension of basic bone remodeling and further knowledge regarding these processes may provide insight into new treatment targets and for osteoporosis treatment. In both fracture repair and mechanical loading experimental models, the physiologic response is heavily influenced by bone modeling, remodeling, and vascular growth processes, which is why remodeling regulation is studied in these contexts. In fracture repair models, vasculature invades the void caused by the fracture and accompanying osteoclasts degrade any local cartilage or matrix [9]. Vascular changes precede bone formation [33], thus, angiogenesis must occur and, in fact, angiogenesis is necessary for the first stage of fracture healing [33, 62]. As the vasculature advances into the region of repair, for reasons unknown, angiogenesis can be aided by osteoblasts stimulated by a load 'shock wave' that results in indirect production of VEGF and contributes to local angiogenesis [9]. Callus deposition, which is a bony formation comprised mainly of woven bone, is prevalent in these types of fracture repair models [53]. It appears that the woven bone does not undergo classical bone remodeling, and merely acts as a 'splint' while cortical bone modeling and remodeling transpires in the region of fracture [53]. Additionally, the woven bone response provides the majority of the structural integrity during the post fracture

repair [53]. Although not directly tied to osteoporosis, fracture repair research reveals basic interactions between the vascular growth and remodeling pathways, which may lead to further understanding of the physiologic deficiencies seen in osteoporotic bone.

Because osteoporosis is dependent on remodeling, examination of only the remodeling response is most closely relatable to the disease. The way to assess normal, physiologic bone modeling and remodeling is through stimulation of bone using mechanical loading. Mechanical loading is utilized to propagate bone microdamage, which induces bone remodeling, even in mice, which don't undergo regular remodeling under normal conditions. This practice is well defined [32, 33, 52, 53, 63] and alters the interstitial shear stress experienced by osteocytes, driving osteoblast and osteoclast activity based on the amplitude of stress and any resultant osteocyte apoptosis [32], and thus, mechanical stimulation is sufficient for BMU associated remodeling [32]. Additionally, the protein periostin, expressed in osteoblast precursors, becomes upregulated with increases in applied mechanical stress and regulates osteoblast differentiation and adhesion, which is necessary for new bone formation [64].

A variety of outcomes have been observed as a result of mechanical loading, including a significant increase in periosteal woven bone [53] and the role of trabecular bone in microdamage propagation. The trabecular bone is a porous structure comprised of both rod and plate-like elements [65], with an anatomical depiction of these structures shown in **Figure 17**. Longitudinal rod density appears to determine the propensity for induced microdamage, even though these structures

account for only a small fraction of the total tissue [65]. Since microdamage is a very robust stimulation for bone remodeling, induction of microdamage is a critical outcome of mechanical loading. Typical axial loading results in one directional deformation, but in transverse loading, trabeculae bending occurs [66]; which seems to be a likely method for microcrack development [65, 66]. Coupled with the finding that remodeling events transpire in the direction of loading [32] and remodeling is proportional to fatigue damage [33], the magnitude of bone formation and remodeling can be modulated based on the apparatus and associated loading parameters.

Figure 17: Trabecular bone representation.
Rod elements are shaded and plate elements are opaque [67].

Compared to fracture healing, little is known regarding the contribution of vascularization and specific cellular remodeling interactions (between osteoclasts and

endothelial cells, for example) in response to loading. Previous macro scale observations regarding the interaction of bone modeling, remodeling, and vascularization include: magnitude of fatigue damage is proportional to vascular changes [33] and vascular increases (number and area) are correlated with fatigue magnitude [52]. Vessel size and number also increase with loading and appear to occur in areas of bone formation, with a maximum occurring 2mm distal from the midshaft of the tibia [52]. From a chronological perspective, vascular alteration occurs before bone deposition, with the vasculature providing a spatial template for loading associated bone formation [52]. These findings have mostly focused on bone modeling, but research solely regarding remodeling events is novel. Since much can still be learned regarding remodeling, opportunities for research are numerous. By characterizing the normal bone remodeling response, possible osteoporosis treatment targets can be realized. Progressing from these ideas, this study hopes to visualize and quantify vascular changes in the cortical and periosteal bone in response to axial loading and subsequent bone remodeling. Spatial and loading duration considerations will also be included to further understand interplay between the vascular system and bone remodeling in hopes of better understanding the normal bone remodeling process.

Methods

Summary

Bone remodeling was induced in a mouse model by axially loading the tibia in a cyclic manner. To determine the vascular response to physiologic loading animals were subject to either 2 weeks or 5 weeks of loading. Tibiae were excised following the determined loading duration and were analyzed for vessel density in the cortical and any newly formed bone using IHC.

All procedures were approved and conducted in accordance to IACUC guidelines. Six month old mice were maintained in micro-isolation cages with a twelve hour light/dark cycle ad libitum access to feed and water.

*Mechanical Loading*¹

Animals were anesthetized with isoflurane (1.5-2% in O₂ at 1ml/min) throughout the loading procedure. Once the animal was at an appropriate plane of anesthetic, it was placed supine on a custom platform within an EnduraTEC Elf3220 (Bose, Framingham, MA), with its left heel stabilized in a fixed location. A small fixture attached to the load cell was then lowered onto the proximal head of the vertically oriented tibia, until a preload of .2N was established to prevent movement during the procedure. The cyclic loading cycle was run with the following parameters: sinusoidal waveform, 1200 cycles at 4 Hz and an applied load range of 1 to 3.2 N. The right hindlimb of the animal was left unloaded to serve as the contralateral control. Each animal was loaded for 5 consecutive days followed by two

1: Mechanical Loading Procedure was performed by Josiah Kessler

days of rest per week. The animals were loaded for either 2 weeks (n = 3) or 5 weeks (n = 6).

Tibial Excision and Preparation

At the conclusion of the prescribed loading duration, the animals were euthanized by cervical dislocation and both tibiae excised. An incision was made in the medial aspect of the hindlimb and was extended the full length of the hindlimb. The skin was separated and cut away from the underlying muscle from the proximal end of the femur to the heel, exposing the distal tendons. All of the tendons were cut at the ankle; the patellar tendon was also cut. Only the gastrocnemius muscle was removed from the tibial segment in order to provide better support upon sectioning and for preservation of the periosteum [52]. The ankle and knee joints were severed, and once isolated, the tibiae were immersion fixed in Histachoice (Electron Microscopy Sciences, Hatfield, PA) for 48 hours. Samples were then decalcified for a minimum of 5 days in EDTA (Sigma-Aldrich, St. Louis, MO) prior to processing and embedding.

Immunohistochemistry

Identification of vessels was performed by staining an endothelial-specific glycoprotein with GS-1 Lectin. Following the fixation and decalcification detailed previously, the bones were cut in half transversely at the midshaft, dehydrated, and impregnated with paraffin wax in an automated processor before embedding in paraffin wax. Using a RM 2255 (Leica Microsystems, Buffalo Grove, IL) microtome,

6 µm sections were cut from the blocks starting at the midshaft. The sections were mounted on Super Up Rite – Bond Rite slides (ThermoFisher Scientific, Houston, TX) and incubated at 80°C for ten minutes. After a xylene wash and a graded ethanol rehydration series, antigen retrieval was performed using citrate buffer (RICCA Chemical Co., Arlington, TX) at 95°C for 10 minutes. Treated slides were washed in PBS for 20 minutes before 1 hour incubation of Biotinylated GS Lectin I at 10µg/ml (Vector Labs, Burlingame, CA). Vectastain AB enzyme (Vector Labs, Burlingame, CA), prepared in accordance with the manufacturer's directions, was applied for a 30 minute incubation. After a PBS wash, the slides were developed with peroxidase and DAB substrate kit (SCBT, Santa Cruz, CA) for 15 minutes. Half of the samples were counterstained with 0.5% Methyl Green (Fisher Scientific, Houston, TX). A final distilled water rinse, and ethanol and xylene dehydration were executed before coverslip application. Appendix A details the full protocol.

Finished samples were imaged using bright field microscopy (Olympus, Center Valley, PA) and a Retiga Exi camera (Q Imaging, BC, Canada). Cross-sectional area and circumferential measurements were made for each of the zones illustrated in **Figure 18** based on sample images. Vessel densities as a percentage of the total tibial area were determined with the use of Image J (NIH, Bethesda, MD).

Figure 18: Tibia cross sections illustrating the regions of analysis.
Two examples of tissue sections divided by anatomical position for regional quantification of vessel densities

Statistical Analysis

A two way ANOVA was used to identify statistical differences. If applicable, comparisons were made between the loaded and non loaded leg, using a Student's t – test, and comparisons were made between loading duration time points and bone regions using one way ANOVA analysis. Results are expressed as mean $\pm$ SE. Statistical significance was set $p \leq 0.05$, unless otherwise stated.

Results

Quantification of parameters including: regional bone area, regional and cross sectional circumference, periosteal vessel number, and intracortical vessel number will provide insight to the degree of vascular remodeling in relation to bone structure changes. Vessel densities were calculated as the number of positively marked vessels per square millimeter in the photomicrographs. **Figure 19** illustrates positively marked vessels in both the intracortical and periosteal regions of the tibia.

Figure 19: Positive and control vessel marking, intracortical bone and periosteum.

10x region overview (A, D) with dashed box indicating region of magnification (both cortical and periosteal tissue is captured in all images). 40x view (B, C, E, F) of specified region. Positively stained cortical vessels are marked with white, closed, arrows and positively stained periosteal vessels are marked with black, open, arrows (B, E). The respective serial controls (no primary antibody) are marked in a similar fashion (C, F). The image on the left depicts vessels of a 2 week loaded tibia and the right image illustrates vessels in a 5 week loaded tibia. Black scale bar is 150 μm and white scale bar is 50 μm .

Vessel density was significantly increased in the two-week loaded tibiae compared to the contralateral control and 5-week loaded tibiae, **Figure 20**.

Figure 20: Total vessel density for both 2 and 5 week loading cycles.

Black bar is control tibia, Grey bar is loaded tibia.

* $p < .05$ vs. control, # $p < .05$ vs. experimental time points.

Analyzing only vessels within the intracortical bone, vessel density is significantly increased compared to control at 2 weeks, but not at 5 weeks. Additionally, the 5 week control vessel density is significantly greater than the 2 week control density, **Figure 21**. Furthermore, the 2-week intracortical vessel density ratio is significantly increased compared to the 5-week vessel density ratio, **Figure 22**. The ratio was calculated as the loaded tibia vessel density over the control tibia vessel density.

Figure 21: Intracortical vessel density for both 2 and 5 week loading cycle
 Black bar is control tibia, Grey bar is loaded tibia.
 * p < .01 vs. control. # p < .05 vs. 2 week control

Figure 22: Intracortical vessel density ratio for 2 and 5 week loading cycles.
 Ratio is Loaded density / Control density.
 * p < .01 vs. control.

Though differences were observed for total cortical bone vessel density, no such conclusions were supported for periosteal density, calculated as the number of vessels within the periosteal region per circumferential length, **Figure 22**,

Figure 23: Periosteal vessel density for both 2 and 5 week loading cycles.
Black bar is control tibia, Grey bar is loaded tibia.

Regional analysis of the tibial cross sections revealed only one difference; after 2 weeks of loading there was a significant increase in the total lateral vessel density, **Figure 23**. It seems that the large difference seen in the lateral region is greatly affecting the results regarding total tissue characteristics.

For a majority of the time points the standard error is quite large, due to the fact that some samples within a time point and specific area did not exhibit positive staining. Interestingly, the control, 2 week, medial data point did not have any positive staining in the data set, reflected in the figure.

Figure 24: Regional vessel density for both 2 and 5 week loading cycles.

Black bar is control tibia, Grey bar is loaded tibia.

* $p < .05$ vs. control.

Discussion

The majority of the conclusions will be discussed in detail in Chapter 4 with this section serving as an overview of findings. The histological data points to a time and location dependence for changes in vascular density in mechanically loaded tibiae. It appears that at 2 weeks of mechanical loading, there are regional, intracortical and total increases in vessel density. Additionally, observed differences compared to control at 2 weeks are no longer seen at the 5 week loading time point. These two observations can be explained simultaneously with a few key findings. As discussed previously, bone overuse leads to increased bone remodeling [61]. It has been demonstrated that the initial loading stimulus, rather than the long-term stimulus, has a greater impact on bone formation and remodeling [61]. A reasonable explanation for the dependence of bone formation on initial loading stimulus, and why changes are observed at two weeks and not at five week, are 'desensitization' of bone cells occurring with increased loading durations, evidenced by a bone formation plateau [61]. Through cytoskeletal reorganization within the osteocyte due to routine loading, the mechanosensitivity and activation threshold is altered, resulting in diminished bone formation and remodeling over time [61]. If there is a correlation between vascularization and bone formation/ remodeling, Figure 20 would support the desensitization hypothesis.

Figures 21 and 23 reveal regional and intracortical differences in vascular densities. Lateral changes are explained by high levels of local strain and previous research has shown increased matrix mineralization in the lateral region in response to mechanical loading [68]. And consistent with prior conclusions, these changes are

only observed at the 2 week loading time point. Interpretation of the intracortical vessel density changes may prove to be much more meaningful. Bone modeling occurs in the periosteal region with mechanical loading [53], so one can hypothesize that remodeling is the primary process occurring in the intracortical bone, as there are few modeling events in mature bones in the intracortical region. With this assumption, and the fact that vasculature is required for bone remodeling, the increase in intracortical vessel density at 2 weeks appears to be indicative of remodeling events. If that is indeed the case, it seems that the apparent remodeling profile is time and somewhat location dependent. Extrapolating to osteoporosis, if the vasculature is intimately involved with the normal remodeling response, this interaction and time dependence could be targeted as a possible treatment target or diagnostic.

CHAPTER 4: DISCUSSION

The governing objective of this study was to determine the role of the vascular system in the context of bone remodeling induced by mechanical loading and ischemia. Understanding the vascular role in the bone remodeling and modeling processes may reveal treatment strategies for patients with osteoporosis and concurrent PAD. The aims of the thesis listed below will guide the discussion.

- 1 Establish an immunohistochemistry protocol to identify blood vessels in bone.
- 2 Determine the effects of ischemia on tibial mechanical properties and microvasculature, and examine any time course of the changes.
- 3 Determine if vascularization will occur in response to mechanical fatigue loading, consistent with prior research.

Aim 1: Immunohistochemistry Protocol Development

Overview

Most conventional vascular quantification methods used in muscle (ink fill, polymer casting, side stream imaging, whole mount imaging, and laser Doppler) are not feasible in bone due to tissue location and/or structure, namely, the anatomically deep and calcified nature of the tissue. Because of this, little work has been done regarding blood vessel quantification in bone in the context of remodeling.

Immunohistochemistry (IHC) was used to circumvent some of the bone specific imaging restrictions present with whole mount and in vivo procedures. The IHC

protocol aimed to provide replicable and consistent vessel quantification utilizing basic histological techniques.

Of the few prior studies that used IHC in bone, all but one examined staining bone marrow, and in one instance, a VEGF-A antibody was used as a spatial quantification stain in trabecular bone. Other studies have also done some ink fill/polymer casting, but the published procedures were not well replicated in the local lab setting. Based on the apparent lack of publications using IHC, its use for cortical bone staining is uncommon, and this appears to be the first time that Lectin has been used in this specific application.

Biotinylated GS Lectin I was utilized in the protocol after a PECAM antibody failed to yield any positive staining. The Lectin used is highly targeted, attaching to an endothelial-specific glycoprotein.

Some challenges arose due to the lack of prior information regarding Lectin IHC in cortical bone. Tissue section integrity reduced sample efficiency due to the heat antigen retrieval and bone-tissue slide adhesion. Additionally, some bone tissue degradation may have occurred in the day 14 and 28 samples that underwent an extra PBS rinse prior to tissue processing. This possibility may help explain the results observed in Figure 14, where the bone crosssectional area was diminished at day 14 compared to other time points. Peroxidase incubation times were highly sensitive and were subsequently optimized to provide sufficient positive staining without sacrificing image clarity and positive contrast. Because the protocol using Lectin was new, the resolution of the stain was not very high, most likely staining fewer vessels than were actually present in the tissue, compared to other published vascular data

[52]. Furthermore, counterstaining of the sections using Methyl Green, Hematoxylin, and Eosin did not provide improved contrast and identification of positively marked vessels.

Despite these impingements, the protocol adequately met its goals and provided quality stains for imaging and analysis. The success of the protocol is best illustrated by Figures 15 and 19, which highlight the contrast between positively stained vessels and their serial controls (no Lectin applied).

Future Work and Improvements

Although the protocol was repeatable and fairly efficient, additional troubleshooting and optimization could be performed to increase the efficiency. Aside from increasing the percentage of positively stained vs. unstained vessels, the protocol could be improved with work pertaining to section integrity, heated antigen retrieval, and peroxidase incubation times in order to increase image clarity and stain contrast. An example of potentially unstained vessels is shown below, **Figure 25**.

One improvement would be optimization of peroxidase incubation times, reduction of the temperature for the antigen retrieval, and more tightly controlled heating apparatus. Also, the addition of a future counterstain may help improve image quality and vessel identification.

Figure 25: Example Unstained Vessels.

40x view (A, B, C) of 2 Week loaded bone. Positively stained vessels are marked with black arrows and possible unstained vessels are marked with white arrows (A, C). The serial control (no primary antibody) vessels are marked with dashed arrows, B. White scale bar is 50 μm .

Aside from the specific Lectin application, the bone-tissue preparation and basic procedure enables the protocol and tissues to be utilized for different IHC staining and apoptosis staining, with some optimization. The basic IHC protocol can accept a different primary antibody other than the Lectin, for labeling other structures.

Aim 2: Ischemic Effects on Vascularization and Mechanical Properties.

Overview

Due to the correlation between osteoporosis and PAD, the vascular system may play a critical role in the development of these diseases. One way to understand and isolate the involvement of the vasculature in bone processes is through induction of ischemia. Any resultant remodeling response in bone would most likely be driven primarily by the vascular response, as minimal mechanical changes are directly induced. Understanding the vascular contribution (and if it is necessary) to bone processes and properties is vital to better understand the bone remodeling process and osteoporosis implications.

Inducing Ischemia and Physiologic Response

From the pulse oximetry data presented in Figure 7, we can conclude that ischemia was induced as a result of the surgical procedure. Angiogenesis does occur in muscle as a result of the ischemia and hypoxia [15, 29], with vessel density in muscle increased at day 7 through day 35 [69]. Additionally, proliferative activity of endothelial cells is a maximum at day 7, still increased at day 14, but subsequently falls back to control levels by day 21 [69]. In line with these findings, we would have expected to see a decreased pulse oximetry in the operated leg up until day 14 but the data presented in **Figure 8** did not reflect this, most likely due to low instrument consistency and accuracy. Ultimately, if the vascular and bone remodeling processes are linked, hypoxia and resulting angiogenesis would be the initial processes involved in explaining the relationship. Angiogenesis is necessary for bone modeling, so we

are testing the hypothesis that independent angiogenesis will be sufficient for bone modeling and/or remodeling. We are relying on hypoxia to drive an angiogenic response that is independent of mechanical stimulation.

Vascularization in Bone

The vascular system is, at the minimum, implicated in the bone modeling and remodeling processes, as evidenced by previous work associated with aim 3. In response to the induced ischemia there is a marked increase in vascular density in the bone by day 14 and that increase from the day 0 and day 7 baseline is sustained until day 28, **Figure 16**. This result points to increased angiogenesis stemming primarily from the ischemic condition. In order to support the robust angiogenic response, there has to be extensive endothelial proliferation. Some pruning of vessels, or rarefaction, may also occur after the initial angiogenic response, evidenced by lack of difference between the day 28 operated and unoperated limbs, while the operated limb is still greater than day 0 and 7.

Since the vasculature is changing, the next step is to determine how this change may affect the bone processes. Most often, tissue necrosis occurs to some degree in an ischemic environment, necessitating removal and repair of damaged tissue. And since the osteoclasts are the cells responsible for tissue removal in bone, the vasculature may interact with the osteoclasts in the following way. As previously discussed, osteoclast recruitment is tied to the RANK / RANKL complex interaction; RANK is an osteoclast membrane receptor and RANKL is the associated ligand expressed on endothelial cells and osteoblasts [9]. While poorly understood, it is

thought that endothelial cell adhesion, through the RANK complex, relocates the osteoclasts from preexisting vasculature into the tissue of interest where they execute their function [9]. Thus, increased endothelial cell proliferation could lead to increased osteoclast activation and extravasation. Applied to this study, if proliferation is occurring between day 7 and 14, osteoclast-enabled tissue removal could be occurring during a similar time frame, with removal being the first step in the remodeling process after initial hypoxia induced angiogenesis.

Confounding these findings is the notion of bone disuse. Due to the surgical procedure, the animals tended to favor the un-operated leg, possibly leading to a disuse case, leading to bone removal and diminished bone growth [61]. If this is occurring, then the necrosis and remodeling bone removal would no longer be the sole contributing factor. No study has specifically addressed the vascular implications of unloading, but trabecular bone formation and PECAM expression decreased with unloading by day 7 and was back to baseline levels after 7 days of normal activity [70]. In this case, the vascular implications weren't directly assessed, as only PCR data was used, but as previously described, the PECAM protein could play a role in the vascular response including angiogenesis. This finding may support my hypothesis as disuse appears to have an inhibitory effect of vascular pathways. Conversely, but not directly translatable, increased osteogenesis and angiogenesis occur in response to HIF upregulation in defect repair [71], which points to the ischemia related response influencing the processes independently.

The vascular factor, VEGF, involved in the ischemic response may also have substantial effects on osteoclasts, as the osteoclasts express VEGF receptors tied to

the RANK complex [35]. VEGF expression is upregulated in bone with exercise, and VEGF inhibition prevented bone gain and vascular adaptation in exercising rats [35]. Additionally, it is thought that VEGF mediates bone angiogenesis (mirroring its role in muscle) and is coupled with increased osteoblast activity during early response to exercise [35]. Furthermore, only cells adjacent to capillaries give rise to bone tissue and eventual mineralization (because of the RANK / RANKL interactions between the osteoblasts, osteoclasts, and endothelial cells), therefore implicating any surrounding vasculature in the bone processes [9, 34]. From these findings we can deduce that the invading vasculature is a primary factor implicated in the bone remodeling processes.

A higher level discussion of the effect on the processes themselves will come in the following section, but as it pertains to this research and sequence of vascular and bone processing events, the most significant increase in vasculature occurs at day 14, prior to any observed mechanical changes. Because of this and for the reasons just outlined, it appears that the vascular response is driving the bone remodeling response, such that an increase in vasculature, resulting from the ischemia, is directly affecting bone cell function. Although similar interpretations have been made for bone modeling in fracture repair, this is the first examination of direct ischemia effects.

Mechanical Property Implications

The data presented in Figure 10 lends itself to a variety of conclusions that tie nicely into the observed vascular response. Starting with the sham hindlimb, the data suggests that there may be bone modeling in the sham tibia by day 7, evidenced by the significant increase in stiffness. Furthermore, the data suggest bone removal and/or remodeling is occurring in the sham hindlimb by day 28, as the stiffness returns to a pre-surgical level. This change can be attributed to increased loading of the sham tibia for a week post-operatively, as the animal tends to favor the non-operated limb, primarily based on every other day observation until the animal was no longer 'limping'. Since bone modeling results from increased loading, the bone properties will change, and when the increased stimulus is removed when the animal regains normal function, the bone will likely undergo some tissue removal and remodeling.

For the ischemic hindlimb, it seems that remodeling is occurring in response to ischemia, evidenced by the increase in stiffness at day 28 and the trending of the ultimate load data. Coupled with the vascular data presented and the possible molecular interactions discussed prior, it appears that the ischemia alone is triggering the remodeling response, most likely through angiogenesis related processes (endothelial cell proliferation, VEGF expression, or both). The emergence of a difference in mechanical properties at day 28 fits with the idea of vascular changes driving remodeling changes.

Remodeling results in an increased mineralization of the bone tissue changing the mechanical properties [55]. Most notably, an increase in mineralization, and resulting

increase in stiffness, enhances the brittle nature of the bone leading to an increased susceptibility to microcracks or whole bone fractures [55]. Because there is an increase in stiffness at day 28, we can conclude that remodeling may be transpiring. We hypothesize that the following is resulting from the ischemia and influencing bone remodeling:

1. Ischemia is induced with resulting hypoxia and tissue necrosis
2. Osteoclast recruitment closely follows the resulting angiogenic response through RANK/ VEGF interactions
3. Tissue removal occurs between day 7 and day 14 (commencing the remodeling process) as vasculature advances
4. Remodeling process proceeds and systemic changes are observed by day 28

From this hypothesis it appears that the bone remodeling process can be stimulated solely by a vascular response to ischemia. Previous work seems to support facets of this finding and help explain how these interpretations were made. In patients with PAD, where tissue necrosis is increased, there is a reduction in nutrient supply which impairs the bone repair process, which points to the necessity of a vascular response [3]. There is also a decrease in bone formation attributed to ischemia at day 10 and 14, again indirectly implicating the vascular system with bone processes [72]. In regards to time scale, in humans it takes 20 days for initiation and tissue removal by osteoclasts prior to any bone formation occurring in a specified 2 dimensional plane [61], and although not specifically defined, due to the similarity between BMUs in mice and humans, we may be able to assume the removal

timescale in mice is similar. This removal time scale fits well with the hypothesis, as remodeling processes may be occurring by day 28. The hypothesis outlining the possible interaction between the vascular and bone processes is highly relatable to osteoporosis treatment, and will be discussed later, especially in understanding disease onset in patients with osteoporosis and PAD.

With osteoporosis, the patient has lower BMD and stiffer bones (tied to the brittleness of the tissue). If tissue necrosis occurs, the vasculature and osteoclasts will respond. This will lead to even more bone resorption and greater risk to the patient. In a patient with PAD, however, the result may be more critical. With PAD, a greater amount of necrosis may occur and the vascular response will most likely be diminished due to the disease pathogenesis. This may be problematic as the response to tissue hypoxia will still result in VEGF expression, effectively recruiting more osteoclasts. And without the vasculature in place to allow for the full remodeling and bone deposition process, the bone tissue will necrose and be resorbed, amplifying the osteoporotic condition. This hypothesis may explain the connection between PAD and osteoporosis.

It is known that the vasculature is required for bone remodeling, but the seemingly direct connection of the ischemic environment to stimulated remodeling is a novel observation. Furthermore, it appears that angiogenesis is sufficient for bone remodeling and this ischemic influence on bone remodeling could further our understanding of the system interactions. Thus, the stimulation of angiogenesis alone could have an effect on bone, in the long term. There would likely be a period of damaged (microcrack or necrosis) tissue removal, but over time, the angiogenic

response would provide the framework for bone growth and repair. In this capacity, stimulating angiogenesis could provide new treatment strategies for patients with both PAD and osteoporosis.

Future Work

Continuation of this research should involve quantification of any occurring remodeling, so that the proposed hypothesis would be better supported. Additional experiments involving isolated, non-exercising post-operational recovery could also be performed to further isolate the ischemic response. To determine the role of disuse, an array of treatments could be applied. One treatment could look at disuse and ischemia vs. disuse control and other would be observing disuse in the short term for both hindlimbs, followed by normal use. Disuse could be accomplished by nerve crushing or tail suspension. These varying treatments would reveal contributions of the disuse mechanism, as well and furthering the ischemic coupled bone remodeling hypothesis.

Validation of the time course and possible causal relationship of the vasculature to remodeling could also be performed. Angiogenesis could be stimulated at particular intervals and for varying durations in order to reveal the time course for any resultant bone remodeling. One angiogenesis stimulant could be a HIF gene therapy. Upregulation of HIF, and use of bone marrow-derived mesenchymal stem cells (BMCs), and increased osteogenesis and angiogenesis in defect repair have been detailed in previous studies [71]. By directly inducing an angiogenic response, the bone remodeling response could be defined as a function of both time and

stimulation amplitude. Some of these experimental treatments could translate into the clinical realm.

Aim 3: Vascularization in Response to Induced Remodeling

Overview

Bone remodeling and the BMU are highly implicated in the development of osteoporosis [34, 39]. Additionally, the microvasculature is critical in the bone remodeling process [9], but the degree to which vasculature is involved in osteoporotic onset is not well defined. Establishing the vascular contribution to the remodeling process may reveal treatment opportunities for remodeling-dependent pathologies such as osteoporosis.

Previous Research Comparison

Although not specifically quantified, prior studies indicated that a majority of the increases in vascularization occurred in the periosteal region [52], however this study shows no such findings. Additionally, at day 14, and at a similar strain value, previously quantified increases in vessel number were around 200% [52], compared to 70% increase in vessel density seen in this study. Several factors, including methodology and analysis, may explain this discrepancy. As previously mentioned, the IHC most likely stained less vessels than were actually present in the tissue, compared to other published vascular data [52], and despite a significant result presented in this paper, it was not as robust as previous findings. Due to the IHC resolution issues, data was presented as vessel density, opposed to total vessel number or percent change from day 0, which may shield additional similarities between the two studies.

Vascularization in Response to Induced Remodeling

Based on the objective, the results support the observation of an increase in vasculature from induced mechanical loading. This occurrence was only seen in the day 14 loaded animals and not in the day 35 animals. A similar result has been shown previously, vessel size and number increases with mechanical loading at day 14 [52], and can be explained by osteocyte responses. Fluid shear and tissue strain increases due to the load, triggering the Wnt signaling pathway leading to osteogenesis [68]. As previously established, vasculature is required for bone formation, thus an increase in vasculature is seen by day 14 in order for bone formation to occur. However, the initial stimulus, rather than the long-term stimulus, has a greater impact on bone formation [61], which can explain the vessel density disparity at the day 35 time point compared to day 14. A reasonable explanation for the dependence of bone formation on initial loading stimulus, and why changes are observed at two weeks and not at five weeks, are 'desensitization' of bone cells occurring with increased loading durations, evidenced by a bone formation plateau [61]. Through cytoskeletal reorganization within the osteocyte due to routine loading, the mechanosensitivity and activation threshold is altered, resulting in diminished bone formation and remodeling over time [61]. If there is a correlation between vascularization and bone formation/ remodeling, Figure 20 would support the desensitization hypothesis. It is also possible that by day 35, some pre-study existing bone is no longer utilized (due to the change in applied load), leading to its removal, along with any associated vasculature [61]. This hypothesis is supported by the decrease of vessel density in the day 35 time point compared to day 14. However, without directly quantifying the

amount of remodeling that is occurring over time, it is hard to speculate on what the changes in vascular density are supporting; whether it's modeling and maintenance or the remodeling process.

Examining vascular density changes from a more specific view, regional analysis revealed an increased vessel density in the lateral region and may be described by increased load and decreased sclerostin in the region of formation. Sclerostin is a molecule normally secreted by osteocytes in a homeostatic environment and acts to suppress the osteogenic response. However, strain suppresses production of sclerostin, leading to increased bone formation through the diminishment of osteogenic regulation [68]. In the tibial loading model, some of the highest compressive strain is seen in lateral region [68, 73], stimulating bone growth in that area, with previous research showing increased matrix mineralization in the lateral region in response to mechanical loading [68]. And consistent with prior conclusions, these changes are only observed at the 2 week loading time point. In both of these scenarios, vascularization is necessary in order for the osteogenic process to occur, thus increased levels of vessel density are seen in the lateral region of the loaded tibiae.

Understanding of induced bone modeling and remodeling has accompanied observations regarding bone vasculature. Bone modeling appears to occur in the subperiosteal region with mechanical loading [53], so one can hypothesize that if remodeling is occurring, it would arise in the cortical bone. Because the main mechanical stimulus for remodeling is changing strain and microdamage, this can only occur in fully mature bone, which in this experimental case, only exists in the

cortical region. With this assumption, and the fact that vasculature is required for bone remodeling, the increase in cortical vessel density at 2 weeks appears to be indicative of remodeling events. If that is indeed the case, it seems that the apparent remodeling profile is time and location dependent. Extrapolating to osteoporosis, if the vasculature is intimately involved with the normal remodeling response, this interaction and time dependence could be targeted as a possible treatment target or diagnostic; for example, using contrast CT to quantify vascularization in the bone tissue to draw conclusions about the bone tissue health (necrosis, density).

As the most basic conclusion from this study, we can interpret the data as vascularization being concurrently implicated in the bone response to mechanical loading. And since we know that modeling (in response to the applied loads and resultant strain changes) and remodeling (in response to induced microdamage and subsequent repair) occurs in response to loading, we can conclude that the vascularization is concurrently implicated in the remodeling and modeling of bone. Moving forward from this study, we know that vascularization is necessary for bone remodeling [9], and based on the presented data, vascularization seems to be stimulated by mechanical loading in areas of preexisting bone. Since vascular growth precedes remodeling and bone formation, these findings can be applied as a treatment for osteoporotic patients; as it appears that loading can provide stimulation for increased vascularization in preexisting bone, hopefully resulting in bone growth and/or remodeling. One way to accomplish mechanical stimulation is using vibration, which has shown increased bone mass [74]. Although the effect of loading has been

proven helpful [74], our data suggest that the positive remodeling effect can be increased and modulated by loading duration and frequency.

Future work

It needs to be seen whether the observed increase in vasculature is correlated to increased bone remodeling. Quantification of the integrity of osteocytes, with an apoptosis stain, both regionally and between cortical and periosteal tissue, will lead to more robust interpretations and understanding of the interaction between the vasculature and bone remodeling. Similarly, a model of short-term loading cycle and lengthened recovery along with remodeling analysis might elucidate the sequence between bone formation and loading stimulus. If a sequence is fully defined, we can then interpret the findings as a causal relationship between the vascular and bone systems. Furthermore, quantification of skeletally mature (6 month) C57 day 0 animals could lead to more definitive conclusions regarding time dependence.

C57 mice are more sensitive than most animals to mechanical loads, including more bone gain per unit of strain [75], so future experiments involving other animal models may more closely simulate the human physiologic response. Additionally, experiments examining only vascular gain of function treatments would be beneficial to observe if a mechanical stimulus is required for bone remodeling, or if only a purely vascular stimulus will result in bone formation and remodeling. For this type of study, all involved cells (osteoclasts, osteoblasts and osteocytes) as well as osteocyte apoptosis would need to be quantified in order to determine the amount of remodeling. Associated findings would be directly relatable to the treatment of

osteoporosis as modulation of the bone remodeling process is the key component of the disease.

Final Thoughts

We have shown the concurrence and causal relationship of the vasculature to the bone remodeling process and have furthered our understanding of the seemingly intimate interaction of the two physiologic systems. These findings could be especially helpful in new treatment strategies that mainly target the vascular system or that incorporate the interaction of the two systems. There are some emerging treatments that can benefit from increased understanding.

Vitamin D administration has seen positive results for patients with both PAD and osteoporosis, as vitamin D increases BMD in osteoporotic patients with PAD and alleviates symptoms associated with lack of vitamin D, including bone aches, pain, and weakness [51]. Prolyl hydroxylase (PHD) inhibitors increase angiogenesis and could increase capacity for bone regeneration if used in osteoporotic patients [57]. Exercise and low level vibration can increase bone formation, decrease bone resorption, raise peak bone mass, and enhance bone strength, and may be able to be used concurrently with a vascular treatment to improve efficacy [74]. Also, with bisphosphonates (BP) treatment, osteoprotegerin (OPG) is positively correlated with BMD [76], which is to be expected because OPG regulates the osteoclasts and may have an effect on osteoblasts.

By taking into account the vascular and bone systems, the application of this study could be instrumental in further research and clinical application.

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APPENDIX A: PROTOCOLS

Hindlimb Ischemia Surgery - Resection

Mouse Information

DOB: _____
 Sex: _____
 Tag: _____
 Genotype/strain: _____
 Cage: _____

Materials

Autoclave in Surgical Instrument Container

- ____1. Standard pattern forceps (1)
- ____2. S&T forceps (2)
- ____3. 5-45 forceps (1)
- ____4. Iris scissors (1)
- ____5. Microdissection scissors (1)
- ____6. Castroviejo (1)
- ____7. Retractor (1)

Autoclave in Surgical Pack

- ____8. 4 x 4 gauze sponge (3)
- ____9. cotton swabs (12)
- ____10. 6.0 silk suture (2 x 1-inch)
- ____11. Surgical drape (1)

Obtained in surgery suite

- ____12. sterile Petri dish w/ sterile saline
- ____13. sterile gloves
- ____14. sterile 7.0 prolene suture
- ____15. heat-cautery
- ____16. FST heat pad w/ rectal probe
- ____17. Recovery heat pad
- ____18. recovery bin & weigh boat
- ____19. depilatory cream
- ____20. non-sterile cotton swabs
- ____21. non-sterile 2 x2 gauze sponge
- ____22. isolation mask & cap
- ____23. analgesic (Buprenorphine)

Surgery preparation

- ____24. Spray surgery area with Nolvasan
- ____25. Weigh animal in weight boat
- ____26. Place animal in anesthesia box
- ____27. Open the oxygen cylinder and set anesthesia-machine flow meter to ~3 l·min⁻¹
- ____28. Anesthetize animal w/ 5% isoflurane
- ____29. Reduce flow rate to 0.5-1.0 l·min⁻¹ and the isoflurane to 1-3%
- ____30. Apply ear tag high on left ear
- ____31. Lay animal supine with nose in nose-cone
- ____32. Shave hair on the right and left hindlimb & lower abdomen with clippers
- ____33. Remove excess hair with depilatory cream
- ____34. Spray right and left hindlimb with Nolvasan
- ____35. Return animal to anesthesia box

- ____36. Lay animal supine on circulating heat pad w/ nose in nose-cone
- ____37. Insert rectal probe and set thermo-controller to 35°C
- ____38. Apply veterinary ointment to eyes to avoid drying during procedure

Surgery

- ____39. Make a small incision on the middle, medial aspect of the left thigh
- ____40. Extend the incision up to the abdominal wall
- ____41. Blunt dissect the subcutaneous connective tissue to maximize surgical exposure
- ____42. Use cautery to remove fat pad overlying femoral a-v pair & cauterize epigastric a-v pair
- ____43. Blunt dissect the femoral artery-vein pair from the nerve starting just upstream to the muscular branch & extending to half-way between the knee & ankle
- ____44. Tie off the saphenous a-v pair with 6.0 silk suture, halfway between the knee & ankle
- ____45. Tie off the femoral a-v pair with 6.0 silk suture, halfway just upstream to the muscular branch
- ____46. Grasping the distal ligature, use the cautery & microscissors to remove the a-v pair up to the upstream ligature
- ____47. Gently rest cotton swabs on hemorrhage sites
- ____48. Use 6.0 polypropylene suture to close the skin
- ____49. Make a small incision on the middle, medial aspect of the right thigh
- ____50. Extend the incision up to the abdominal wall
- ____51. Blunt dissect the subcutaneous connective tissue to maximize surgical exposure
- ____52. Use 6.0 polypropylene suture to close the skin

Post-Surgical

- ____53. Give the animal an subcutaneous injection of buprenorphine (0.075mg/kg)
- ____54. Place the animal in the recovery bin, on a blue bench cover, above a heat pad and allow to recover
- ____55. Turn flow meter down to 0, turn off isoflurane, and close the oxygen cylinder
- ____56. Indicate surgery on cage card

Notes

Figure 26: Surgical station with instruments.
Key components are labeled and indicated with arrows.

Tibial Dissection – Mechanical Testing

Mouse Information

DOB: _____

Sex: _____

Tag: _____

Genotype/strain: _____

Cage: _____

Materials

- ___57. Standard pattern forceps
- ___58. S&T forceps
- ___59. Iris scissors
- ___60. Microdissection scissors
- ___61. Bone scissors

- ___62. Kimwipes (2)
- ___63. Centrifuge vials (2)
- ___64. Tape

Dissection preparation

- ___65. Place animal in anesthesia box
- ___66. Open the oxygen cylinder and set anesthesia-machine flow meter to $\sim 3 \text{ ml} \cdot \text{min}^{-1}$
- ___67. Anesthetize animal w/ 3% isoflurane
- ___68. Perform cervical dislocation on animal to euthanize
- ___69. Lay animal supine and tape forepaws down

Dissection

- ___70. Make a small incision on the middle, medial aspect of the left thigh
- ___71. Extend the incision up to the proximal head of the femur and down to the ankle
- ___72. Blunt dissect the subcutaneous connective tissue and remove the skin around the hindlimb
- ___73. Using the microdissection scissors, cut the tendons and ligaments at the ankle joint including the Achilles
- ___74. Sever the patellar tendon with the microdissection scissors
- ___75. Peel off all surrounding musculature from tibia and fibula
- ___76. Using the bone scissors, cut at the knee and ankle joints to separate the tibia/fibula
- ___77. Remove the fibula using the bone scissors
- ___78. Wipe off any remaining tissue from the tibia using the Kimwipe
- ___79. Place the excised tibia in a properly labeled vial
- ___80. Repeat process for right hindlimb

Post-Dissection

- ___81. Record animal usage in the log
- ___82. Place the animal in the in a sealed plastic bag and then into the biohazard bag
- ___83. Put the vials (containing the tibiae) into the freezer

Tibial Dissection – Histology

Mouse Information

DOB: _____

Sex: _____

Tag: _____

Genotype/strain: _____

Cage: _____

Materials

- ___ 84. Standard pattern forceps
- ___ 85. S&T forceps
- ___ 86. Iris scissors
- ___ 87. Microdissection scissors
- ___ 88. Bone scissors

- ___ 89. Kimwipes (2)
- ___ 90. Centrifuge vials (2)
- ___ 91. Tape

Dissection preparation

- ___ 92. Place animal in anesthesia box
- ___ 93. Open the oxygen cylinder and set anesthesia-machine flow meter to $\sim 3 \text{ ml} \cdot \text{min}^{-1}$
- ___ 94. Anesthetize animal w/ 3% isoflurane
- ___ 95. Perform cervical dislocation on animal to euthanize
- ___ 96. Lay animal supine and tape forepaws down

Dissection

- ___ 97. Make a small incision on the middle, medial aspect of the left thigh
- ___ 98. Extend the incision up to the proximal head of the femur and down to the ankle
- ___ 99. Blunt dissect the subcutaneous connective tissue and remove the skin around the hindlimb
- ___ 100. Using the microdissection scissors, cut the Achilles tendon
- ___ 101. Sever the patellar tendon with the microdissection scissors
- ___ 102. Peel off the gastrocnemius muscle
- ___ 103. Using the bone scissors, cut at the knee and ankle joints to separate the tibia/fibula
- ___ 104. Place the excised tibia in a properly labeled vial
- ___ 105. Repeat process for right hindlimb

Post-Dissection

- ___ 106. Record animal usage in the log
- ___ 107. Place the animal in the in a sealed plastic bag and then into the biohazard bag
- ___ 108. Fill vials (containing the tibiae) with Histachoice
- ___ 109. Store vials at room temperature and in an appropriate location

Immunohistochemistry

Endothelial Cell Marker Lectin Checklist: Paraffin Embedded Sections

<u>Incubation Time</u>	<u>Reagent</u>
10 min	Oven at 80 C (setting 7) after sections mounted on slides
10 min	Xylene
3 min	100% EtOH
3 min	100% EtOH
3 min	95% EtOH
3 min	85% EtOH
3 min	70% EtOH
3 min	50% EtOH
5 min	Distilled Water
Antigen Retrieval	Rice cooker at 95 C for 10 min (samples in Citrate Buffer)
10 min	D-PBS pH 7.4
60 min RT°	1° Lectin GS1 in D-PBS Use .1 ml Lectin and 4.9 ml PBS (10 ug/ml)
30 min, RT	2° Streptavidin Use Vector AB Kit :2.5ml PBS, 1 Drop A and Mix, 1 Drop B and Mix
3 min	D-PBS pH 7.4
15 min	DAB Substrate – when light brown color is observed put in ddH ₂ O
3 min	Distilled Water
10 seconds	95% EtOH
10 seconds	100% EtOH
30 seconds	Xylene
	Xylene + permount

Study _____.

Staining performed by _____

Date ____/____/____

Associated protocol description and notes

After sectioning, place the slides in the tray and place the tray in the oven in the orientation shown in **Figure 31** to ensure tissue attachment on the edges of the

slides. Additionally, 10 minutes is an estimate, leave in the oven until the slides are translucent, at which time place immediately into the xylene.

Figure 27: Slide tray placement in the oven.
Tray is indicated by white arrow.

The xylene and ethanol hydration steps should be performed in the hood and in the solution dishes. Place and remove the slide tray from dish to dish slowly and gently to avoid tissue detachment. Illustration of a dish with the slide tray and the array of dishes are shown below in **Figure 32**.

Figure 28: Dish array and dish with slide tray.

During hydration steps, fill the rice cooker pot 1/3 with water, turn on the rice cooker to the ‘Cook’ position, and place a dish with citrate buffer in the cooker. Cover the pot and periodically check the temperature with the thermometer until 95° C is reached. For the antigen retrieval step, place the slide tray and thermometer in the hot citrate buffer dish and replace the lid. The proper arrangement is illustrated in **Figure 33**. Monitor the temperature and remove the lid for several seconds at a time if the temperature is too high.

Figure 29: Antigen retrieval set up.
Dish and thermometer is visible, with the 'Cook' light illuminated.

After the 10 minutes remove the slide dish and unplug the rice cooker. With the hydrophobic pen, outline the tissues from each section to isolate them. **Figure 34** below illustrates this technique.

Figure 30: Hydrophobic tissue outline
Blue circles represent the tissue, and the dashed lines represent the hydrophobic pen outline.

After tissue outlining, place the slides into the paper towel lined incubation Tupperware. Pipette the reagents, using the 1 ml pipette, onto the tissue, within the hydrophobic outline. An example of incubating slides is shown in **Figures 35** below.

Figure 31: Slides with reagent applied.

Top image, Lectin incubation. Bottom image, DAB incubation, with pipette visible.

To remove the reagents, use the vacuum shown in **Figure 36**. Following all reagent incubations, remove the any residual fluid before putting the slides back into the tray. After all slides have been subject to 30 of xylene, take the slides out one at a time and immediately apply mounting medium and coverslip. To prevent tissue detachment, apply the mounting medium directly on top of the tissue. Let slides dry overnight. The final step is shown in **Figure 37** below

Figure 32: Reagent removal with vacuum.

Figure 33: Completed slides.
Key components are labeled and indicated with arrows.

APPENDIX B: APPARATUS AND EQUIPMENT FIGURES

Figure 34: Overview of the 3 Point Bending apparatus.
Key components are labeled and indicated by arrows.

Figure 35: Zoom view of bending apparatus.
Bone tray, load cell and point of contact are visible.
Arrow indicates location of tibia.

Figure 36: Overview of the cortical thickness measurement apparatus
Key components are labeled and indicated with arrow.

Figure 37: Pulse oximeter device.
Hindpaw measurement cuff indicated with arrow

APPENDIX C: ADDITIONAL DATA

Day	Ultimate Stress (MPa)		Elastic Modulus (Gpa)	
	<i>Sham</i>	<i>Resection</i>	<i>Sham</i>	<i>Resection</i>
28	282.4 ± 30.6	371.8 ± 77.5	4.4 ± .5	6.1 ± 1.5
14	276.0 ± 22.6	251.8 ± 28.9	4.2 ± .4	3.7 ± .5
7	265.1 ± 5.7	247.2 ± 51.4	3.9 ± .1	3.7 ± .9
0	218.6 ± 12.0	265.1 ± 24.6	3.2 ± .2	4.0 ± .5