THE EFFECTS OF MECHANICAL STIMULUS INDUCED OSTEOCYTE APOPTOSIS ON VASCULARIZATION AND MECHANICAL PROPERTIES OF BONE

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ABSTRACT

The Effects of Mechanical Stimulus Induced Osteocyte Apoptosis On Vascularization and Mechanical Properties of Bone

Osteoporosis and age-related bone loss are characterized by a chronic deterioration in bone density and bone strength over time, resulting in an increased risk for bone fracture. This decline is generally due to excess resorption of bone compared to new bone formation, hormonal changes, nutritional habits, and environmental mechanical stimuli. Currently fifty percent of women and twenty percent of men over the age of 50 will experience an osteoporotic fracture during their remaining life. These instances as well as morbidity, mortality, financial, and social consequences, as well as current substandard remedies drive the need for successful treatment options to eliminate these debilitating diseases. Better understanding of the cellular mechanisms involved in osteocyte apoptosis and vascularization in bone adaptation processes will help progress toward promising therapies.

The aims of this current project were to determine the effects of normal bone adaptation through mechanical loading on vascularization and osteocyte apoptosis in cortical bone tissue. Specifically this study attempted to understand these mechanistic relationships by introducing a loss of function study inhibiting osteocyte apoptosis through the Fas ligand signaling pathway. Ten week old FasL knockout (n=10) and B6 control mice were subjected to axial tibia loading of -1N to -3.2 N at 1200 cycles and 4 Hz 5 days a week for two weeks. Mice were sacrificed 5 days post surgery and processed for characterizing mechanical properties as well as regional changes in osteocyte apoptosis and vascularization through three-point bending mechanical testing, TUNEL assays, and biotinylated lectin endothelial staining. Results showed mechanical loading caused no significant differences in mechanical properties of the mouse tibiae between FasL KO and control mice as well as no significant influence on regional osteocyte apoptosis density or percentage. Baseline values indicated comparable results of osteocyte apoptosis in FasL KO and controls. Vessel density was significantly higher in the posterior region of FasL KO but loading tended to reduce vessel density in the posterior and overall tibia at midshaft.

Collectively these data may suggest inhibition of FasL has no effect on osteocyte apoptosis and that the current load, two-week time point, sacrifice post loading regimen, or a combination are not effective in activating osteocyte apoptosis at the tibia midshaft. Furthermore, these data imply FasL inhibition may cause a decrease in vessel density in the posterior region of the tibia midshaft, and that loading of FasL KO mice attenuates rather than enhances vascularization in a spatially-defined manner. Although further work and optimization of the project design as mentioned remains, this project was the first in this application to lay the foundation as a pilot study in characterizing the relationship between osteocyte apoptosis, vascularization, and bone loading.

Keywords: Osteoporosis, Induced Remodeling, Mechanical Loading, Apoptosis, Vascularization
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Refuse to be average. Let your heart soar as high as it will.

– A.W. Tozer
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CHAPTER 1: INTRODUCTION

Bone Function - Remodeling

Bone tissue has been well documented as a structure protecting organs and providing the framework to the body, collaborating as lever systems in assistance with muscle tissue to create locomotion and movement of the body. Bone has also been characterized as a calcium reservoir necessary for appropriate muscle and nerve function as well as a support to the hematopoietic system. Cells that make up this tissue include osteoclasts, osteoblasts, osteocytes, bone lining cells, and their precursors. Osteoclasts, thought to emerge from macrophages, are multinucleated cells that secrete acidic enzymes to break up and resorb old or damaged bone, releasing calcium in its stead. Osteoblasts, known as bone forming cells differentiated from mesenchymal stem cells, are mono-nucleated and move into the resorbed area where the osteoclast activity was, secreting an unmineralized substance known as osteoid in the resorption area that will eventually become new bone. In cortical bone, osteoclasts resorb bone radially outward from the endosteal surface of bone while osteoblasts form new bone radially inward from the resorbed cavity. Thus osteoclasts and osteoblasts work in a coupled fashion to optimize bone mass, strength, and quality (Nakamura et al., 2007). As new bone is being laid down, osteoblasts become trapped in the bony matrix in pockets called lacunae where they differentiate into another cells type known as osteocytes. These cells are the most abundant cell type in bone tissue and reside within intracortical bone. Although placed in isolated pockets, osteocytes form communication networks through gap junctions with each other, bone lining cells, and cellular components within the bone marrow (Aguirre et al., 2006). As osteoblasts finish forming bone, quiescence occurs leaving a small inner void at the center known as a Haversian canal. This canal is essential for nerves and vasculature to reside to provide nutrients for all the bone cells.
Osteoblasts left on this finished surface become bone lining cells, lining all surfaces of bone. Once bone lining cells are activated, it is thought they can turn back again into osteoblasts to undergo new formation of bone. The whole process of osteoclast resorption, osteoblast formation, and quiescence is known as the remodeling process, and the final outcome produces a completed structure known as an osteon. Osteons are unique to cortical bone alone and together make up its densely-packed phenotype.

In terms of structure, bone is characterized as a dual composite material, composed of a hard inorganic layer of hydroxyapatite and a softer inorganic layer of mostly collagen fibers intermixed with proteoglycans, non-collagenous proteins, and water. The arrangement of these components produces two types of bone organization: cancellous (trabecular) and compact (cortical) bone. Cortical bone is classified as having 5-30% porosity, forming the outer shell of almost all bones and diaphysis specifically of long bones. Trabecular bone on the other hand is classified as having 30-90% porosity forming a three-dimensional lattice continuous with the outer cortical bone located mainly at the epiphyses of long bones (Carter et al., 1976). This porosity of bone allows for the optimization of high strength to low weight, creating the ability to bear external and internally generated loads. Bone tissue, although seemingly structurally sound, is unique for being in a constant state of modeling, remodeling, and regeneration. The idea that bones will grow and be shaped throughout life to adapt to their mechanical environment is built off of Wolff’s Law (Pearson, Lieberson, 2004. This process has many stages, with cells “sensing” mechanical stimuli in a process called mechanotransduction. This macroscopic stimulus is thought to cause a shear stress in the oscillatory fluid housed within the bone tissue over the osteocytes. These cells then translate this signal into a response to form or resorb material appropriately as to withstand the mechanical load placed on the tissue (Pearson, Lieberson,
This mechanism involving bone remodeling occurs through coordinated signaling between bone-resorbing osteoclasts and bone-forming osteoblasts upon all environmental stimuli and trauma. The regenerative process relies on a network of spatial and temporal cues from biomechanical and biochemical regulators to enable cell proliferation, differentiation, and recruitment (Pearson, Lieberson, 2004). Very little is known of the specific mechanisms involved, however it is clear that disuse, aging, and the inability of the coordinated remodeling and regeneration to occur can result in devastating diseases such as osteoporosis and osteoarthritis.

**Bone Disease - Osteoporosis**

Osteoporosis is defined as a chronic metabolic bone disease which leads to an eventual deterioration in bone strength and provokes a higher risk for bone fracture (Schuiling, Nye, 2011). This disease has become a global concern as the World Health Organization estimated more than 75 million people in Europe, Japan, and the US combined have osteoporosis (Schuiling, Nye, 2011). Currently fifty percent of women and twenty percent of men over the age of 50 will experience an osteoporotic fracture at some point during their life (Holroyd, Cooper, 2011). Hormonal changes, especially of estrogen in females and testosterone in males, are thought to be one of the primary causes in the imbalance of resorption and deposition that lead to osteoporosis (Pearson, Lieberson, 2004).

It has been reported over 300,000 hospitalizations occur annually in the United States, with a one in four patient mortality rate within the first year (US Congress, 1994). Hip fractures for one are particularly devastating. One study found those patients with hip fracture that do survive past the one year mark will have spent 17% of their remaining life in a nursing facility (Braithwaite, Wong, 2003). Moreover hip fractures have economical consequences estimated to
cost of $81,300 per patient (Braithwaite, Wong, 2003). Osteoporosis is responsible for financial and social costs as well as increased overall morbidity and mortality rates. Ideally researchers desire to identify multiple processes involved in bone healing and regeneration to create novel therapies more valuable in enhancing the healing process, particularly in people identified with a higher risk for osteoporosis/low-bone mass. An understanding of the molecular regulation is critical for the development of treatments that can specifically target and direct these processes toward restoration, reduction in fracture risk, and enhancing proper bone function.

Due to the detrimental activity seen in osteoporotic patients, various therapies have been pursued to reverse or prevent the loss of bone mass. A practical option is by mechanical stimulation, a natural anabolic agent for regulating bone mass and enhancing bone formation. However, aging bones have been to be less responsive to mechanical stimuli (Lieberman, Crompton, 2003) and prescribing loads capable of osteogenesis is problematic to osteoporotic patients who are already at an increased risk of fracture (Sugiyama, Lanyon, 2008). For instance in pre-clinical studies, there is evidence that growing rats are able to repair faster in long bone fractures than aged rats (Meyer, Garges, 2003). Also specifically with age, reports have shown a delay and attenuation in the healing response, possibly caused by a reduction in angiogenesis, vascularization, cell differentiation, bone formation, periosteal reaction, and bone remodeling rates (Castillo). Another study examining aging effects in rats reported tibiae responding to loading grew less frequently and required an increased load microstrain in older rats to get the same osteogenic response as their adult counterparts (Turner, Owen, 1995). This decrease in responsiveness therefore leads to increased bone loss and potential fracture risk. Although the fracture itself may not be life threatening, when individuals experience a fracture they are more likely to obtain another fracture (Dretakis, Christodoulou, 1981 and Boston, 1982). Patients may
then experience greater rates of non-union and necessary reoperation of the injured site (Pugh, McKee, 2003).

Instead a hormonal attempt involves administration of intermittent parathyroid hormone (iPTH) to increase osteoblast differentiation and activity while decreasing osteoclast apoptosis (Sugiyama, Lanyon, 2008). This in turn increases trabecular thickness and number, trabecular connectivity, cortical thickness, and cortical strength (Goldring, 2002). However, at continuous high dose delivery it has been known to increase bone resorption causing severe bone loss (Sugiyama, Lanyon, 2008). Furthermore, iPTH is the only current licensed anti-resorptive agent and may be attenuated with low physical activity (Sugiyama, Lanyon, 2008). The pharmacological intervention for bone-related diseases using bisphosphonates has also been shown to have positive and negative effects. Long-term treatment is associated with fracture reduction but may increase the risk of osteonecrosis of the jaw and atypical fractures with guides for treatment duration still debatable (Compston and Bilezikian, 2012). Osteoinducive agents such as BMP-2 and BMP-7 have been shown to significantly reduce nonunion rates, but not all patients have been able to respond as efficiently to BMP treatment alone.

These current shortcomings have led to the production of research in developing better strategies to treat osteoporosis and bone fractures trying to avoid compromising the integrity and function of the diseased bone tissue. Abundant studies in the literature have well established protocols studying fracture modeling and massive trauma/damage in vivo (Devine, 2002). Although this is advantageous when studying the mechanisms for bone healing and injury, osteoporosis is more characterized as an imbalance in the bone remodeling process rather than a dysfunction in bone repair. Therefore this study wanted to approach an understanding of bone
disease by encouraging bone remodeling under normal physiologic conditions rather than bone healing mechanisms.

**Mechanical Loading and Osteocyte Apoptosis**

Previous evidence has shown either low or high levels of mechanical loading enhance osteocyte apoptosis. Under the mechanism of osteocyte apoptosis, osteocytes undergo a programmed cell death causing cell shrinkage, chromatin condensation, and nuclear fragmentation (Chan et al., 2010). Apoptosis is a process that takes place during embryonic development, normal cell turnover, or due to cytotoxicity of a cell (Steller, 1995). This process occurs before and is associated with recruiting osteoclasts to the apoptotic region initiating an increase in bone resorption (Aguirre et al., 2006). Recent studies have used a transgenic mouse model of inducible osteocyte ablation to show osteocyte apoptosis is able to trigger osteoclast recruitment and bone resorption (Tatsumi et al). This may suggest that by disrupting the osteocyte communication network through events such as osteocyte apoptosis, the self-repairing process of bone will be activated and potentially enable bone to adequately adapt to its environmental strains. The same is true for mechanical stimuli that are insufficient in their stimuli. In a tail suspension mouse model for example, osteocyte apoptosis was increased in both trabecular and cortical bone, with resorption most severe near endosteal surfaces. This was also followed by increased osteoclast activity and porosity in cortical bone as well as an reduced bone mineral density of the spine and vertebral strength (Aguirre et al., 2006). In vitro studies have also shown osteocytes subjected to oscillatory fluid flow had attenuated apoptosis compared to no flow-conditions, supporting again the idea that mechanical loading does influence osteocyte apoptosis. (Wing-Yee Cheung, Chao Liu, Rachel M.L. Tonelli-Zasarsky, Craig A. Simmons, and Lidan You). These outcomes suggests that too low of mechanical forces on
bone may also abolish signals that maintain osteocyte viability, and therefore leads to apoptosis (Raisz, T. John Martin, Principles of Bone Biology, p. 248).

The importance of establishing the connection between mechanical loading and initiated osteocyte apoptosis is to understand their effects on bone mechanical properties. Some *in vitro* work by Aguierre and coworkers found evidence to suggest osteocyte apoptosis is a critical determinant of bone strength (Aguierre *et al.*, 2006). Estrogen deficiency has also been shown to enhance osteocyte apoptosis in humans and mice, disrupting the osteocyte network and may contribute to bone fragility (John P. Bilezikian, Lawrence G. Raisz, T. John Martin, Principles of Bone Biology, p. 249). In Sprague Dawley rats, osteocyte apoptosis was shown to be necessary for intracortial remodeling after fatigue microdamage (Corodoso *et al.*, 2009). These studies aforementioned provide a clear interpretation that bone loading has a positive effect on initiating the remodeling process in bone, and remodeled bone creates stiff and stronger bone material.

**Mechanical Loading and Vascularization**

Along with remodeling, it has been suggested osteoclast precursors are recruited from the bone marrow through capillaries to the desired bone surface site. For instance, angiogenesis has been reported near bone resorption sites in human trabecular bone possibly used for the transfer of osteoclasts to the bone surface. Other studies have suggested osteocyte apoptosis is produced from paracrine signaling with VEGF, a known angiogenic cytokine, upon regional angiogenesis (Wing-Yee Cheung,1 Chao Liu,2 Rachel M.L. Tonelli-Zasarsky,3 Craig A. Simmons,1,2,4 and Lidan You, 2010). Also it was found four days after tibial bending in mice, there was an increase in angiogenic gene expression (Baylink *et al.*, 2005). Regarding the temporal expression, a rat model instigated with woven bone formation by a damage fatigue ulna
loading protocol found angiogenic genes *VEGF* and *hif1a* upregulated after one hour and still strongly expressed 1 to 3 days after loading along with an increase in vessel volume and number (Mckenzie and Silva, 2010). These data reveal the cohesiveness of many tissues, cells, and pathways important for optimizing and strengthening bone tissue function.

Although many studies have approached mechanisms for bone remodeling coupled to apoptosis and angiogenesis *in vitro*, work done *in vivo*, specifically with a mouse model regarding bone osteocytes, is not as well characterized. One small type II membrane protein known to play a role in apoptosis is Fas ligand (FasL) (French et al., 1996). This protein has been shown to be expressed in many organs of the body including the thymus, lung, spleen, small intestine, small vesicle, prostate, and uterus, as well as lymphoid tissues in the adult (French et al., 1996). Fas receptor (FasR) is a type I transmembrane receptor part of the tumor necrosis factor (TNF) family, and when FasL binds to FasR, apoptosis of cells is thought to occur quickly through a process called the “death domain” (Itoh et al., 1991; Trauth et al., 1989). FasL has been well documented in the lymphatic system as an important regulator of deleting autoimmune cells (Singer and Abbas, 1994) as well as a necessary component activating cytolytic T lymphocytes (Kagi et al., 1994; Lowin et al., 1994). In terms of bone tissue, one study did find the Fas ligand/Fas receptor signaling cascade, upon activation by estrogen, promoted osteoclast apoptosis in a mouse model (Nakamura et al., 2007). Fas Ligand has also been shown to stimulate chondrocyte apoptosis in vitro (Lotz et al., 1999, Lee et al., 2004). Although FasL has been well documented in these tissue areas, its role in bone tissue apoptosis, specifically toward osteocyte apoptosis, is not as well understood. Moreover the characterization of apoptotic effects when FasL is inhibited is still under investigation, especially in terms of osteocyte function and bone remodeling.
Thus the use of a Fas ligand (FasL) knockout model was chosen in this current study to investigate the effects of Fas inhibition in terms of osteocyte apoptosis, in order to create a loss of function study when osteocyte apoptosis is not able to occur. These aforementioned studies and more provided the foundation for attempting to resolve the unknown aspects of bone function, in order to develop better remedies when this function is impaired as in osteoporosis and other related bone diseases. The first aim of this study was to development of a loss-of-function study through inhibition of apoptosis and its effects of mechanical properties of bone. Through this aim stemmed to hypotheses, the first being a murine model inhibition of the Fas ligand is capable of effectively reducing apoptosis in osteocytes, and consequently bone remodeling is also reduced. The second hypothesis stated was animals with FasL knocked out will have reduced osteocyte apoptosis necessary for bone remodeling and hence have reduced mechanical strength. Conversely control animals with Fas ligand still present will have enhanced osteocyte apoptosis when mechanically loaded compared to the KO mice, and therefore rescued/restored bone remodeling and mechanical strength properties normal levels.

The second aim of the project was to determine the effects of mechanical loading on vascularization in cortical bone tissue. From this aim prompted two hypotheses: The first was that control animals with Fas ligand still present will have enhanced vascularization while FasL KO animals will have reduced regional vascularization after mechanical tibia loading. The second hypothesis was loading induces osteocyte apoptosis, creating a hypoxic environment, and therefore resulting in increased vascularization in and surrounding the local bone tissue. The idea is when osteocyte apoptosis occurs, bone remodeling and angiogenesis are initiated. When bone remodeling and angiogenesis take place in the cortical bone tissue, microdamage is taken out and replaced making the bone inherently stronger.
The vast limitations existing with treatments for osteoporosis and age-related bone loss as well as the continued uncertainties regarding mechanisms in proper bone function, indicate a critical importance in better characterizing these pathways and relating bone loading to apoptosis to neovascularization. A mechanism that could increase osteocyte apoptosis and instigate normal bone remodeling as well as reduce or reverse age-related decline of osteoprogenitors while enhancing new intracortical vessel growth would be an invaluable tool for improving patient fracture healing outcomes.
CHAPTER 2: METHODS

Animal Model

FasL and its transmembrane receptor Fas (FasR) are known to function in the body instigating programmed cell death, or apoptosis. The inhibition of FasL from binding to FasR would therefore potentially not allow apoptosis to occur. To investigate whether inhibition of FasL would influence osteocyte apoptosis, and to determine the interaction between apoptosis and neovascularization upon mechanical loading, a mutant mouse model was used in which the FasL protein was genetically knocked out. Six-week-old mutant mice (n=10) and six-week-old B6 control mice (n=10) were purchased from Jackson Laboratories for the experimentation. Five mice from each group were separated into two analysis categories: (1) mechanical testing by three-point bending to determine mechanical properties, and (2) histological analyses to quantify regional osteocyte apoptosis and vessel densities. All animal experiments were carried out following review and approval in accordance with IACUC guidelines.

Mechanical Tibia Loading

All mice were subjected to in vivo axial compression of the left tibia under anesthesia by isoflurane inhalation. Right tibiae of each animal were used as an internal control. Mechanical loading was performed using a Bose Enduratec compressive force system with a load range from -1.0 N to -3.2 N at 1200 cycles and a frequency of 4 Hz. Loading was performed 5 days per week for 2 weeks as performed in previous experiments, and all mice were sacrificed 5 days after the last loading cycle. FasL KO mice were separated from control mice following each loading cycle in micro-isolation cages, 5 mice per cage, with a twelve hour light/dark cycle and
access to feed and water ad libitum. Optimization of the loading cell and fixture, calibration, as well as all calculations and software integration was performed by Ian Mahaffey and is discussed in greater detail within his applications project report.

**Tissue Processing**

All mice were sacrificed 5 days following mechanical loading by isoflurane inhalation and cervical dislocation. Left and right tibiae were harvested from each specimen and those designated for mechanical testing were immediate frozen for later use. Left and right tibiae specified for histological analysis were isolated and instead immediately fixed in Histachoice (Electron Microscopy Sciences, Hatfield, PA) for 48 hours. These specimens were then placed in an ethylenediaminetetraacetic acid (EDTA) solution to decalcify the tibiae tissue for approximately 7 to 10 days. Complete decalcification was identified when no hard material could be felt within the bone tissue. Once fully decalcified, specimens were washed 10 minutes in 1x PBS and re-fixed in Histachoice for 12 hours. After this time, tibia were cut transversely at the midshaft and placed cut-side down into blocks for paraffin embedding. Each block contained either a single left or right tibiae. From there, specimens were infiltrated with paraffin wax by an automated processing and embedding system.

After tibiae were embedded in paraffin, each mold was placed at 4°C at least 30 minutes prior to sectioning in order to avoid tissue breakage upon sectioning. All paraffin sectioning was performed using a RM225 Leica microtome (Leica Microsystems, Buffalo Grove, IL) with low-profile disposable microtome blades (Leica Microsystems, Buffalo Grove, IL) and blocks wetted with deionized water after each section to avoid tissue shredding for high quality sectioning (**Figure 1**). Paraffin blocks cut in 6 micron sections from the tibiae midshaft. Each section was
then rehydrated immediately in a 37°C water bath for 2 to 4 minutes and then mounted onto Super Bond-Rite microscope slides (ThermoFisher Scientific, Houston, TX) for use in histological staining. All staining processes were done approximately 30 minutes after specimens were sectioned to ensure integrity and avoid oxidization of the tissue.

**Figure 1: Paraffin sectioning microtome.** RM225 Lecia Microtome used for all tissue paraffin sectioning, illustrating horizontal block placement and vertical microtome blade cutting positions.

**Osteocyte Apoptosis Detection with TUNEL staining**

Immunohistochemistry (IHC) by the DeadEnd Calorimetric TUNEL System (Promgea, Madison, WI) was used to quantify the amount of osteocyte apoptosis in situ at the single cell level in both loaded and nonleaded hindlimbs. The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick-End Labeling (TUNEL) assay is used to measure nuclear DNA fragmentation which is an indicator of apoptosis. The osteocyte apoptosis analysis was
performed in FasL KO and B6 control mouse tibiae from paraffin-embedded blocks as previously mentioned. Paraffin specimens were sectioned transversely at a thickness of 6 microns using a RM225 Leicca (Leica Microsystems, Buffalo Grove, IL) microtome. Sectioned specimens were immediately placed in a 37°C water bath to rehydrate and placed onto Bond-Rite slides (ThermoFisher Scientific, Houston, TX) as described previously. Slides were kept out to air dry approximately 20 minutes before being placed in an oven at 80°C for 10 minutes to initiate melting of the paraffin wax (Figure 2). Once all wax was observably transparent, sections were fully deparaffinized with fresh Xylene, dehydrated through a graded ethanol series, and set out to air dry 20 minutes at room temperature. After this time, slides were washed, fixed in HistoChoice, and washed again before exposed to a Proteinase K solution. This solution produces holes in the cell membrane, preparing the tissue for uptake of DNA labeling. After Proteinase K, slides were washed, fixed, and washed again, followed by a 10 minute, room temperature exposure to Equilibration Buffer as provided in the TUNEL kit. During this time the biotinylated nucleotide mixture rTDT was created. Equilibration Buffer was blotted off the tissue by KimWipe tissues, 100 ul rTDT was placed directly onto the tissue, and each specimen slide was immediately covered with a plastic coverslip to avoid drying out. All slides were placed horizontally into a slide rack, and placed inside a humidified chamber at 37°C for a 60 minute incubation period (Figure 2). This step causes the end-labeling of DNA fragmentation to occur. Following this incubation period, slides were uncovered and placed in 2x saline sodium citrate buffer (SSC) to terminate the end-labeling reaction. Before the staining reagent, diaminobenzidine (DAB), could be applied the slides were first exposed to 0.3% hydrogen peroxide to block endogenous peroxidases followed by a 100 ul 1:500 Streptavidin HRP-PBS for 30 minutes. Then DAB was mixed according to the manufacturer’s instructions and placed onto
each slide for approximately 10 minutes, until a light brown color was visible on the tissue. Once the desired color was reached, the slides were rinsed carefully in deionized water and mounted with aPerm mount mounting medium (Fischer Scientific, Houston, TX). Samples were observed using a bright field microscope (Olympus, Center Valley, PA) and a Retiga Exi camera with software (Q Imaging, BC, Canada). All evaluations were performed using ImageJ software.

Figure 2: Microscope slide configurations. (A) Indicates proper position of Bond-Rite slides after tissue sectioning in 80°C oven to allow melting of paraffin wax before de-paraffinizing steps. (B) and (C) show illustration of creating humidified chamber for incubation period in heat oven in which DNA fragmentation labeling occurs. Slides are placed horizontal in a slide rack, which is then slid into glass slide holder wrapped in paper towel soaked in distilled water.
**Osteocyte Viability with 2% Methyl Green Staining**

The methyl green protocol was performed in conjunction with the TUNEL osteocyte apoptosis protocol in order to differentiate apoptotic from viable osteocytes. Ordinarily the two stains would be administered together with the methyl green as a counterstain to the DAB substrate DNA fragmentation labeling. However, previous lab experience had shown results were optimized when the two protocols were separated, comparing each sectioned specimen approximately 1-2 sectins apart, or 6 to 12 microns. Methyl green is a potent stain used to label viable cell types in the tissue of interest. After sectioning, each specimen went through the same procedures as described in the TUNEL assay up to the dehydration and 20 minute air drying steps. Following this, the specimens were immersed in deionized water for 5 minutes followed by 2% methyl green (Fisher Scientific, Houston, TX) for no longer than 45 seconds. Slides were then rinsed briefly in distilled water, deionized water, and mounted with Permount mounting medium Fischer Scientific, Houston, TX). All imaging and analysis processes were performed using the same procedure as the TUNEL assay samples.

**Cortical bone Vascularization Detection**

Detection of angiogenesis was evaluated through the identification of endothelial cells lining blood vessels in the cortical layer of bone at the tibia midshaft for both FasL knockout and control mice. This was performed using an endothelial-specific biotinylated glycoprotein lectin I staining protocol. The biotinylated lectin conjugate is made from purified lectin labeled with biotin specific for endothelial cells. With the addition of avidin, its high affinity to bind to biotin makes this stain capable to identify endothelial cells, and hense vessels, readily in intracortical tissue. After tissue fixation and decalcification, specimens were paraffin-embedded and
sectioned onto Bond-Rite slides as described previously. Slide were then deparaffinized and hydrated through a series of Xylene and graded Ethanol washes followed by antigen retrieval with Citrate Buffer (RICCA Chemical Corporation) incubated 10 minutes at 95°C in a pressure cooker. This retrieval was necessary in breaking the protein cross-links formed during fixation, uncovering hidden antigen sites and improving overall antibody penetration. All slides were then rinsed in PBS 10 minutes. To validate the antibody performance, the bottom sections on each slide were then incubated in 10 ug/ml primary antibody biotinylated GS lectin 1 (Vector Laboratories, Burlingame, CA), and the top specimens of each slide remained in PBS only. All specimens were incubated in this step for one hour at room temperature in a humidified chamber. A secondary streptavidin antibody, Vectastain Elite® ABC R.T.U (Vector Laboratories, Burlingame, CA), was then added to each slide for 30 minutes at room temperature. After washing with PBS, DAB substrate was prepared according to the manufacture instructions and was placed onto all specimens for 15 minutes to produce staining of the endothelium within the cortical bone tissue. Slides were then rinsed with deionized water, dehydrated in a graded ethanol and xylene series, and mounted with Permount mounting medium (Fischer Scientific, Houston, TX). Vascularization from stained slides was evaluated using a bright field microscope (Olympus, Center Valley, PA) and a Retiga Exi camera with software (Q Imaging, BC, Canada). All evaluations were performed using ImageJ software. See Appendix B for full protocol description.
**Figure 3: Reagent Placement.** Depiction of reagent droplets placed onto each miscroscope slide during TUNEL and lectin staining procedures. The top tissue (closest to the green slide label) was designated as a negative control and contains no primary antibody during lectin staining and the negative control during the TUNEL assay.

![Image of reagent placement](image)

**Quantification of Immunohistochemistry**

Lectin endothelial detection was quantified at the tibia transverse midshaft by separating each tissue region into four quadrants: anterior, posterior, medial, and lateral (**Figure 4**). Lines and angles designated for each quadrant were imitated from previous work contributed to regional quantification techniques as shown in Figure 4. (Govea, 2011). Cross sectional areas and circumferential distances of the total bone as well as the four regions were calculated individually for left and right tibiae of both control and FasL KO mice. Then vessel density was quantified expressed as the number of vessels present per area for each region. Calculations were done to compare the vessel density at baseline values (non-loaded limbs) and percent difference comparisons for both control and FasL KO mice.

Osteocyte apoptosis was quantified using the same regional technique described separating the tibia midshaft into four quadrant regions for both left and right tibiae of FasL KO and control mice. Areas and circumferential distances were calculated as was done for vessel
quantification, and osteocyte densities were determined from the number of apoptotic osteocytes per regional area. Osteocyte apoptosis was confirmed by comparing TUNEL-stained sections to its methyl green stained counterpart, approximately one tissue slice apart, in order to not mistake a apoptotic osteocyte for a viable cell. All apoptotic osteocytes and all viable cells per region were counted and recorded as a percentage to the total number of cells per region as well as a density of number per area. Confirmation of apoptotic osteocytes was performed from previous work on confirmed apoptotic osteocyte from different cell types (Dufour et al., 2006; Chan, 2011). All imaging calculations were performed in Microsoft Excel and using ImageJ software (NIH, Bethesda, MD).

**Mechanical Testing**

Half of the loaded and non-loaded tibia samples for both FasL KO and B6 control mice that were not subjected to decalcification were fixed and immediately frozen upon harvest to be used for mechanical testing purposes. These sample groups were used to determine the effects of compressive tibial loading on mechanical properties, namely cortical bone stiffness and strength, at the tibia midshaft where highest compressive strains are generated. Before testing, all bones were thawed in 1x PBS until measured at room temperature. The testing was performed using a three-point bending apparatus in which each tibia bone was placed horizontally between three fixtures, one on top of the bone and two on the bottom. A compressive force was then applied vertically onto the bone until failure, characterized as a complete fracture and severance of the cortical bone. The load was measured temporally until failure, and each bone’s cross sectional area was measured in order to obtain the stress, strain, Young’s modulus, and moment of inertia of each specimen. The configuration and procedures used for the mechanical testing was all performed by Ian Mahaffey and is extensively elaborated on in his application project report.
Statistical Analysis

One-way ANOVA in Microsoft Excel was used to evaluate statistical differences in regional comparisons for both osteocyte apoptosis and vessel as a percentage and density. Comparisons were also made between the loaded and non-loaded limb, and between control and FasL KO mice using a Student’s t-Test. Results are expressed as a mean ± standard deviation. Differences were considered significant with a p-value < 0.05.
CHAPTER 3: RESULTS

Parameters quantified in this study included regional bone area and cross sectional circumference, vessel number, vessel density, osteocyte apoptotic number as a percentage of total cell number, osteocyte density as a function of anterior, posterior, medial, lateral, and whole bone regions. All parameters were determined for the left and right tibiae at midshaft for both B6 control and FasLKO mice. These parameters are beneficial in understanding the amount of osteocyte apoptosis at baseline when Fas ligand is present and when it is inhibited as well as an indicator in how mechanical loading effects osteocyte apoptosis and vascularization in the midshaft of mice tibia when the Fas ligand is functioning as normal as well as inhibited in B6 control and FasL KO mice, respectively. These data provide insight into the relationship between vasculature remodeling and osteocyte apoptosis in regards to bone structure.

![Figure 4: Regional Quadrants of the Tibia Midshaft](image)

Figure 4: Regional Quadrants of the Tibia Midshaft. Transverse cross section of the mouse tibia midshift depicting the four representative quadrants used for calculating regional vessel and osteocyte apoptosis densities: anterior, posterior, medial, and lateral. Image reproduced from M. Govea thesis work using Magnification = 4x. Images produced using bright field microscope and Retiga Exi camera with Q Imaging software.
**Intracortical Vasculariation from Lectin Endothelial Immunhistochemistry**

Vessel density was significantly higher at baseline levels at the posterior region of the tibia midshaft in the FasL KO model compared to controls (Figure 15). All other regions had comparable vessel density values between KO and controls. Analyzing vessels in the control mice only of the tibia cortical bone showed no significant difference between loaded and non-loaded limbs for any region of the tibia midshaft (Figure 17). Moreover evaluating vessel density in the FasL KO mice also showed no significant difference in the loaded limbs compared to the non-loaded limbs at the tibia midshaft (Figure 18). This indicates a lack of regional specificity of vessel density when tibiae are mechanically loaded at this load level and two-week time point.

**Osteocyte Apoptosis as a Function of Mechanical Loading**

Results from the TUNEL assay regarding baseline values for osteocyte apoptosis as a percentage of total cell number showed no significant difference between FasL KO and control mice in any region of the tibial midshaft (Figure 5). When comparing the FasL KO mice to control mice that were loaded after two weeks, it was again found there was no significant difference between the two groups at any region of the tibia midshaft (Figure 6). Baseline levels of control mice alone and FasL KO alone were also calculated to examine how mechanical loading effected osteocyte apoptosis directly. In the control non-loaded versus loaded tibiae, results showed there was no significant difference between the limbs in any region analyzed, suggesting osteocyte apoptosis is unaffected by mechanical loading at this current load and time point (Figure 7). When comparing FasL KO non-loaded versus loaded tibia, results found no significant difference in osteocyte apoptotic density in any region specified (Figure 8). This again may indicate loading had no effect on the apoptosis of osteocytes at the tibial midshaft in the FasL KO mouse at the present load and time point.
Figure 5: Baseline Osteocyte Apoptosis Percentage. Osteocyte apoptosis specified as a percentage in each region of the tibia midshaft during non-loading conditions of both B6 control and FasL KO mice.

Figure 6: Loading effects on regional osteocyte apoptosis percentage. Osteocyte apoptosis specified as a percentage in each region of the tibia midshaft two weeks after mechanical loading comparing both B6 control and FasL KO mice.
Figure 7: Loading effects on Control Mice. Comparing mechanical loading effects on osteocyte apoptosis density in each region of the tibia midshaft mechanical loading for B6 control mice.

Figure 8: Loading on FasL KO mice. Comparing mechanical loading effects on osteocyte apoptosis density in each region of the tibia midshaft mechanical loading for FasL KO mice.
**Figure 9: Images of Tibia Midshaft for Loaded and non-loaded Controls.** Representative images of TUNEL staining for intracortical osteocyte apoptosis in the midshaft region of the (A) right non-loaded and (B) left loaded tibia of the B6 control mice. Image magnification = 10x

**Figure 10: Images of Tibia Midshaft for Loaded and non-loaded FasL KO.** Representative images of TUNEL staining for intracortical osteocyte apoptosis in the midshaft region of the (A) right non-loaded and (B) left loaded tibia of the FasL KO mice. Image magnification = 10x
Figure 11: Osteocyte Apoptosis and Viable Detection Strategy. B6 Control mouse transverse tibia at medial-anterior midshaft stained using the TUNEL assay (Top) for osteocyte apoptotic cells and 2% methyl green staining (B) for viable osteocytes within the cortical bone. Black arrows indicate apoptotic osteocytes, red arrows indicate viable osteocytes. Image magnification = 10x
Mechanical Loading Effects on Bone Mechanical Properties

Mechanical testing results performed by Ian are shown below. Results from the three-point bending revealed no significant difference in stiffness of the loaded limb between FasL KO and controls. Although this value was not significant, there was a trend with control mice having reduced stiffness after loading comparing to FasL KO mice (Figure 12). This raises the possibility that an increase in sample size may support the idea that less osteocyte apoptosis leads to enhanced stiffness in bone. This idea, however, remains to be conclusively determined.

Further analysis of mechanical properties including elastic modulus and ultimate stress showed no difference in loaded FasL KO and control tibiae (Figure 13, 14). These results are consistent with the osteocyte apoptosis data, indicating that without a significant reduction or enhancement of apoptosis in the osteocytes, mechanical structure properties of bone also remain unchanged.

Figure 12: Loading Effects of Tibia Mechanical Stiffness. Mechanical Testing with three-point bending of control and FasL KO tibiae midshaft comparing loading and non-loading tibia stiffness properties. a representative of a p-value <0.06.
Figure 13: Loading Effects of Tibia Elastic Modulus. Mechanical Testing with three-point bending of control and FasL KO tibiae midshaft comparing loading and non-loading tibia elastic modulus.

Figure 14: Loading Effects of Tibia Ultimate Stress. Mechanical Testing with three-point bending of control and FasL KO tibiae midshaft comparing loading and non-loading tibia ultimate stress.
Figure 15: Vascularization Differences in FasL KO and Control Tibiae. Illustrating baseline values (non-loaded limbs) for vessel density in each region of the tibia midshaft mechanical loading for B6 control and FasL KO mice.

Figure 16: Illustrating percent differences comparing mechanical loading effects on vessel density in each region of the tibia midshaft mechanical loading for B6 control and FasL KO mice.
Figure 17: Effect of Loading on Vessel Density in Control Mice. Comparing mechanical loading effects on vessel density in each region of the tibia midshaft mechanical loading for B6 control mice.

Figure 18: Effect of Loading on Vessel Density in FasL KO Mice. Comparing mechanical loading effects on vessel density in each region of the tibia midshaft mechanical loading for FasL KO control mice.
**Figure 19:** Representative FasL KO mouse transverse tibia at midshaft stained using the Biotinylated lectin endothelial assay for vessel number and density. Black arrows indicate vessels within and surrounding the cortical bone. Tearing and warping seen of bone tissue possibly related to processing during paraffin embedding and sectioning. Magnification = 10x
CHAPTER 4: DISCUSSION

Osteoporosis is characterized by a dysfunction of bone’s natural ability to remodel and repair itself. This imbalance is defined as a chronic metabolic disease which leads to a deterioration in bone density and strength, further provoking a higher risk for bone fracture. One in three women and one in five men over the age of 50 are thought to experience an osteoporotic fracture within their remaining life. Recent studies in the past year have also brought to life the severity of the disease as osteoporotic fractures were found to occur more than twice as often as lung cancer, prostate cancer, and Alzheimer’s disease, and reach about 600,000 new incidences per year compared to the approximated 550,000 and 400,000 new cases per year of heart disease and stroke, respectively (Binkley, 2011). Due to the higher likelihood of obtaining a second osteoporotic fracture after a first and the high one year mortality rate after obtaining an osteoporotic fracture, it is obviously clear the critical need for treatments of this disease. Before treatments can be approved, however, there must be an accurate understanding of the underlying pathways involved in both function and dysfunction. The objective of this study therefore was to take knowledge and experience obtained from Stanford CIRM internship to translate to Cal Poly in order to better understand the mechanisms of bone adaptation on bone structure and vascularization.

Through the aims of this project, four major hypotheses were developed. First hypothesized was a murine model inhibition of the Fas ligand is capable of effectively reducing apoptosis in osteocytes, and consequently bone remodeling is also reduced. The second hypothesis stated was animals with FasL knocked out will have reduced osteocyte apoptosis necessary for bone remodeling and hence have reduced mechanical strength. Conversely control animals with Fas ligand still present will have enhanced osteocyte apoptosis when mechanically
loaded compared to the KO mice, and therefore rescued/restored bone remodeling and mechanical strength properties normal levels. Third, control animals with Fas ligand still present will have enhanced vascularization while FasL KO animals will have reduced regional vascularization after mechanical tibia loading. The fourth hypothesis tied in the other points together, stating loading induces osteocyte apoptosis, creating a hypoxic environment, and therefore resulting in increased vascularization in and surrounding the local bone tissue. The data from the project results did not conclusively support the aforementioned hypotheses, but noteworthy outcomes were made that will be advantageous toward future studies desiring to create an established protocol in studying osteocyte apoptosis and vascularization on bone structure and remodeling processes.

Since tibia vessel density was higher in the FasL KO model compared to controls in the posterior region, the FasL may be affecting vascularization in inhibiting apoptosis in the vasculature and resulting in an increased number of total vessels present at the tibia midshaft. After a two-week loading period, FasL KO mice sowed a further reduction in vessel density in the posterior and total bone regions. Interestingly, previous work had shown after a two-week load period, there was a significant increase in vessel density in a loaded limb in regards to the lateral region of the tibia midshaft. Although the regions are in different places and for two different mouse models, it is noteworthy that loading does seem to have a regional affect on vessel density. These studies results may indicate the effect of mechanical loading is most profound in the posterior region of the tibia midshaft when the apoptotic Fas signaling cascade is disrupted. Analyzing vessels in the control mice only of the tibia cortical bone showed no significant difference between loaded and non-loaded limbs for any region of the tibia midshaft. This is contrasted to previous work that had shown a significant increase in the loaded hindlimb
compared to non-loaded limbs after a two-week loading period (Govea, 2011). Moreover evaluating vessel density in the FasL KO mice also showed no significant difference in the loaded limbs compared to the non-loaded limbs at the tibia midshaft.

These results on vessel density may suggest the FasL KO model have more vessels in the tibia posterior region initially, and that loading causes a more profound attenuation of vascularization in the cortical tibia midshaft, most notably in the posterior region. During axial loading, the posterior region has been shown to be placed more in compression, and the anterior region to be more subjected to tension. This may mean during a compressive response, vascularization is decreased at least when the apoptotic FasL/FasR signaling cascade is impaired. The exact correlation between FasL and vascularization remains to be investigated.

The lack of significant difference between baseline apoptotic osteocytes suggests FasL inhibition does not affect osteocyte apoptosis at the tibia midshaft during normal functioning. When comparing the FasL KO mice to control mice that were loaded after two weeks, it was again found there was no significant difference between the two groups at any region of the tibia midshaft. This may indicate mechanical tibial loading had comparable effects whether the FasL was inhibited constitutively or not. This also brings into question the efficiency of the FasL KO to inhibit apoptosis in osteocytes. It may be possible the FasL is not specific enough for bone osteocytes. Another possibility to consider is that the current load was unable to generate a higher enough force to initiate osteocytes to undergo enhanced apoptosis. One way to resolve the issue would be to redo the experiment at increasing force loads and compare the amount of osteocyte apoptosis to see if apoptosis is actually instigated properly at higher loads. An alternative solution may be to keep the load value the same but used on a different mutant mouse, potentially one with a more specific inhibition of apoptosis in osteocytes. A third
alternative approach would be to use B6 mice, the same strain as the controls, and inject a potent reagent known to efficiently ablate apoptotic signaling in the osteocyte before mechanical loading the tibia.

Since no significance was determined between osteocyte apoptosis for any region or being controls and FasL KO mice during loading and non-loading conditions, it was critical to rule out other possible unforeseen variables that had accounted for these outcomes. Since values were based on a density and therefore indirectly related to area, each region of bone area (total bone, anterior, posterior, medial, and lateral) was compared between control and FasL KO bones to see if the lack of significance was due a difference in area. Analysis revealed, however, there was no significant difference between total bone area, as well as anterior, posterior, medial, and lateral areas for each control and FasL KO mouse. Furthermore, total cell number were compared in each region between FasL KO and control mice to see if the amount of cells present were the cause for the lack of significance. ANOVA analysis showed however, there was comparable total cell numbers between FasL KO and control mice in the tibia midshaft in all regions. These data support that results from this study are due to comparable apoptotic osteocyte densities rather than due to differences in bone area or cell number.

A limitation remains for both vessel number and osteocyte apoptosis count, however, in that both are subjective to the person counting. Although using guidelines from previous work, it must be considered the possibility in the miscount of an osteoblast undergoing apoptosis rather than an osteocyte for example. Another subjective user error could be due the proximity of defining the circumference of each quadrant region. Since each region is outline with the Polygon Selections tool in ImageJ, the less precise the tracing is to the actual tissue, the farther the approximation of the circumference and subsequent calculated area will be. Also, since the
tracing was performed manually, it is difficult to repeat the process in tracing for each section. Future work could eliminate this flaw in analysis by a automated or semi-automated tracing system.

Tissue quality was another limitation in the analysis of both vessel density, osteocyte apoptosis density and, osteocyte apoptosis percentage. The tissue sectioned developed tears in the bone tissue itself, or the marrow cavity leaked out onto the cortical bone region, impairing the view of all osteocytes within the cortical bone. This made it difficult to accurately count all osteocytes, or perhaps was the cause of false positive vessels or apoptotic osteocytes since the marrow was also stained. Speculation for this tearing may be due to the dullness of the microtome blade during sectioning, or may be due to the PBS rinse done before the samples were submerged in Histachoice for paraffin embedding processing. Future work in sectioning tibia samples would be to eliminate the PBS rinse as well as using a new microtome blade for each section to ensure clean cutting of the bone tissue.

Further future work studies should also look at other areas of the tibia besides midshaft. Although this location in the tibia is known to generate the highest strain and produce the greatest response in terms of vascularization and remodeling, it would be advantageous to a holistic view of the effects of loading on the tibia bone. This would provide a better understanding in characterizing the role of osteocyte apoptosis and vascularization in areas of minimal and maximal strain.

Due to the noted significance of vascularization to bone function, other avenues to investigate would be application of angiogenic factors such as VEGF or loss of function studies using known hypoxic regulators such as Hif-1a to determine how involved vascularization truly is during bone adaptation.
CHAPTER 5: CONCLUSION

This study was performed to investigate the effects of mechanical adaptation through tibial loading on bone structure and bone function related to osteocyte apoptosis and intracortical vascularization. The study involved in a murine model in which the Fas ligand was genetically knocked out to inhibit apoptosis in vivo, with B6 mice retaining normal apoptotic function serving as experimental controls. Results from the present study revealed mechanical loading had no effect on FasL KO mice or control mice at the midshaft of the tibia. Data further indicated comparable osteocyte percentage and osteocyte density in all regions of the tibia midshaft, and no significant difference in these parameters between FasL KO and control mice. Regarding vascularization, results showed FasL KO mice regionally significant vessel density compared to controls and a significant reduction in vessel density upon loading as compared to controls. Collectively these data may suggest inhibition of FasL has no effect on osteocyte apoptosis, and that the current load, two-week time point, sacrifice post loading regimen, or a combination are not effective in activating osteocyte apoptosis at the tibia midshaft. Furthermore, these data imply FasL inhibition may cause a decrease in vessel density in the posterior region of the tibia midshaft, and that loading of FasL KO mice attenuates rather than enhances vascularization in a spatially-defined manner. Although further work and optimization of the project design as mentioned remains, this project was the first in this application to lay the foundation as a pilot study in characterizing the relationship between osteocyte apoptosis, vascularization, and bone loading. These findings may be beneficial in increasing the understanding of bone function and vascularization for further research and clinical application of diseases such as osteoporosis and age-related bone loss.
REFERENCES


http://books.google.com/books?id=LEDkfnAljdkC&pg=PA249&lpg=PA249&dq=Fas+ligand+in+osteocyte+apoptosis&source=bl&ots=Q8YBUlLpMk&sig=yesAyhlA8MAg1gQGT_Nw3Ssf5wY&hl=en&sa=X&ei=AUKcT7b1KLT8iQL87ZidAQ&ved=0CDkQ6AEwAQ#v=onepage&q=Fas%20ligand%20in%20osteocyte%20apoptosis&f=false


4. Chan, J., A Study of Osteocyte Apoptosis By Region and Quadrant in Murine Cortical Bone, 2011, Digital Commons Cal Poly Library Services, 1-78


APPENDIX A: 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 ~3 ml·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 tibae) with Histachoice
____109. Store vials at room temperature and in an appropriate location
APPENDIX B: Immunohistochemistry

Endothelial Cell Marker Lectin Protocol: Paraffin Embedded Sections

Incubation Time: 
Reagents:

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 1° Lectin (GSL 1 in PBS; 0.1 ml Lectin and 4.9 ml PBS = 10ug/ml)
30 min 2° Streptavidin (Vector AB Kit: 2.5ml PBS, 1 Drop A Mix, 1 Drop B Mix)
3 min D-PBS pH 7.4
10 min DAB Substrate – when light brown color is observed put deionized water (MiliQ)
3 min Distilled Water
10 seconds 95% EtOH
10 seconds 100% EtOH
30 seconds Xylene
Xylene + permount

Note: All steps were performed at room temperature in a humidified chamber unless otherwise stated.

APPENDIX C: Immunohistochemistry
1. **Deparaffinize the tissue sections**
   Immerse the slides in xylene for 5 minutes

2. **Immerse** in 100% ethanol for 8 minutes (wash)

3. **Rehydrate through graded ethanol washes**
   - 95% ethanol for 3 minutes
   - 85% ethanol for 3 minutes
   - 70% ethanol for 3 minutes
   - 50% ethanol for 3 minutes

4. Air dry for 20 minutes

5. **Immerse** in PBS for 5 minutes (wash)

6. **Immerse** in HistoChoice for 15 minutes (fix)

7. **Immerse** in PBS for 10 minutes (wash)

8. **Remove liquid from tissue and place slides on a flat surface**
   Prepare a 20 µg/ml proteinase k solution: 1 part 10 mg/ml proteinase k stock solution (**see Appendix D for procedure**) to 499 parts PBS
   Add 100 µL of the proteinase k solution to each slide to cover the tissue section
   Incubate slides for 10 minutes set out to dry
   *Use shorter incubation times because of the thinner sections.*

9. **Immerse** in PBS for 5 minutes (wash)

10. **Immerse** in HistaChoice for 5 minutes (refix)

11. **Immerse** in PBS for 5 minutes (wash)

*For positive control: treat sample with DNase 1 → causes DNA fragmentation*

*For negative control: Prepare rTDT reaction mixture without rTDT (However be sure to keep all other concentrations the same!)*

12. **Tap the slides to remove excess liquid** (use Kimwipes)

   Cover the cells with 100 µL of equilibration buffer and equilibrate for 5 minutes

13. **Thaw biotinylated nucleotide mixture on ice**

    Prepare rTdT reaction mixture (keep on ice)

    Place 100 µL of the reaction mixture per slide

    *For positive control:*

    Combine: 98 µL of equilibration buffer + 1 µL of biotinylated nucleotide mixture + 1 µL of rTdT

    *(Omit rTdT for negative control)*

14. **Blot around equilibrated areas with tissue paper**

    Add 100 µL of the rTdT reaction mixture to the sections on each slide
Do not allow the sections to dry out

15. Cover the sections with plastic coverslips to evenly distribute the reagent

Incubate at 37 degrees C for 60 minutes inside of a humidified chamber (wet paper towel over dish)

This step allows the end-labeling reaction to occur

16. Dilute: 1 part 20x SSC solution to 10 parts deionized water

Remove coverslips

Immerse in the 2x SSC solution for 15 minutes

This step terminates the end-labeling reaction

17. Immerse in fresh PBS for 15 minutes (wash)

This step removes unincorporated biotinylated nucleotides

18. Dilute: 1 part 3% hydrogen peroxide to 10 parts PBS

Immerse in the 0.3% hydrogen peroxide solution for 3 to 5 minutes

Do not use the 20x hydrogen peroxide

This step blocks endogenous peroxidases

19. Immerse in PBS for 15 minutes (wash)

20. Dilute streptavidin HRP solution 1:500 in PBS

Add 100 µL to each slide

Incubate for 30 minutes (set out to air dry)

21. Immerse in PBS for 15 minutes (wash)

22. Combine DAB components right before use: Add 50 µL of DAB substrate 20x buffer to 950 µL of deionized water. Next add 50 µL of DAB chromagen and 50 µL of 20x hydrogen peroxide

Add 100 µL of this DAB mixture to each slide and develop until there is a light brown background, approximately 10 minutes

Keep DAB components and mixture away from light and use mixture within 30 minutes

23. Rinse several times in deionized water

24. Mount slides with permount
**APPENDIX D: Proteinase K Reconstitution Procedure**

Proteinase K comes in a powdered form and needs to be reconstituted with a proteinase K buffer solution.

Proteinase k Buffer:

- 100mM Tris-HCl (pH 8.0)
- 50mM EDTA

1. Micropipette 0.100 mL of 1M Tris-HCl (pH 8.0) into a 1 mL aliquot.
2. Micropipette 0.100 mL of 0.5M EDTA (pH 8.0) into the aliquot.
3. Bring the volume to 1 mL with deionized water (MiliQ)
4. Mix 10 mg of the proteinase k powder with 1 mL of the buffer.

*Note: This reconstituted proteinase k will be a 10mg/mL solution.*
APPENDIX E: Methyl Green Staining Procedure

1. Do steps 1 through 4 of TUNEL procedure

To do simultaneously with the TUNEL procedure, offset the Methyl Green slides by 8 minutes behind.

2. Immerse in deionized water for 5 minutes

3. Immerse in 2% Methyl Green for 45 seconds (no longer than this)

4. Rinse in distilled water

5. Rinse in deionized water

6. Mount slides with permount