THE EFFECT OF AGE ON BONE AND ITS RESPONSE TO MECHANICAL STIMULATION

by

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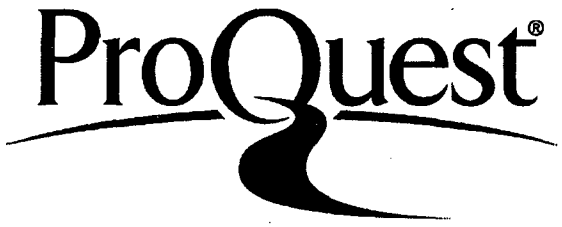

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DEDICATION

To my wonderful family who always supported and believed in me.

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Behind every successful woman is a substantial amount of coffee

~Stephanie Piro

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PREFACE

Dear Reader,

It is important to include in this thesis a brief interview with bones cells. They were previously interviewed in 2004 by Dr. C. E. Hoffler and it is time for an update.

Danese: Thanks for agreeing to an update interview. It has been a few years since the last one. First why don't you all introduce yourselves?

Osteoblast: My name is OB for short and my job is quite important. I am the one primarily responsible for bone formation.

Osteoclast: My job is to mess up all the work the osteoblast has done. I go by OC and I spend most of my time resorbing or removing bone when appropriate.

Osteocyte: However, I have the coolest job of all. I get to sense mechanical stimulation and cause all sorts of stuff to happen. By the way for the rest of the interview you can call me Big Poppa.

Danese: Ok Big Poppa. Why don't you give us an update?

Osteocyte: Well I've aged a bit since the last interview. I was beginning to wonder if it were time for me to undergo an examination.

Danese: What do you mean?

Osteocyte: Well I am just not sure things in the old body are working the way they used to. I think my extensions eh processes may be thinning and I'm just not sure I'm as adept at my job as I used to be.

Danese: What about you OB and OC? Has age had an effect in your performances?

OB: That is an interesting question and I think researchers are working on it; however an exact answer remains unknown. I know that I'm not able to reproduce quite like I used to and there could be changes in my ability to respond to mechanical stimulation.

Danese: I didn't know that you can also respond to mechanical stimulation.

OB: Mos' def'. The whole process in bone is not completely understood, however investigators have shown that all sorts of things can happen when I'm mechanically stimulated.

Danese: What about you OC? You have been pretty quiet.

OC: I feel better than ever. I feel even more productive than I did five years ago.

Danese: This is interesting, but how might these changes with age effect the influence of mechanical forces on bone?

Osteocyte: I don't know. Sounds like you just found yourself a thesis project.

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ABSTRACT

Bone is a specialized connective tissue system which is able to regulate its own bone mass and architecture to meet the daily demands of its external environment. Mechanical loading directly or indirectly influences the activity of cell populations to deposit, maintain, or remove bone tissue as appropriate, which is integral to skeletal adaptation to load. With advancing age there are alterations in bone structure and mineralization which are often associated with an increase in osteoporotic fracture risk. The transduction of mechanical cues affects bone structure and mineralization and could be altered with advancing age. Current *in vivo* and *in vitro* data suggest age may affect the capacity of bone cells to respond to mechanical stimulation; however the effect of age on this response to mechanical stimulation in the regenerative skeleton and on osteocytes, which are thought to be the primary mechanical sensors, is unknown.

It was hypothesized that the influence of mechanical factors on the maintenance or repair of bone is influenced by age. In this study regenerative specimens primarily composed of osteocytes were produced in young and old animals. Their response to mechanical loading via nitric oxide (NO), prostaglandin E_2 (PGE_2), connexin 43 (Cx43), MAP Kinase, and c-fos signaling was assessed via ELISA and western blot and compared to the response of age matched mature bone. Regenerative specimens from young animals had a higher net increase in NO, PGE_2, Cx43 and c-fos after mechanical stimulation than regenerative specimens from old animals. The mechanical stimulation

of regenerative tissue resulted in a higher net increase of mechanical response molecules than mature bone in both age groups. This was observed at an earlier time point of regeneration in specimens produced in young animals which could initiate earlier remodeling and thus maintain a mean tissue age that is fairly constant and less susceptible to brittle fracture.

Progenitor cells from old animals exhibited delayed mineralization and a decrease response to mechanical stimulation throughout differentiation. The data from this study suggests primary cells from old donors with appropriate differentiation time and mechanical stimulus may promote bone formation, which could make them useful for tissue engineering applications. In addition, key differences in mechanical response were highlighted which have the potential for further investigation to develop therapeutics for bone loss in aging populations.

CHAPTER I

INTRODUCTION

I'm just trying to find a decent melody a song that I can sing in my own company

-Stuck in a Moment

Bone is a specialized connective tissue consisting of various cells, a calcified extracellular matrix, and extracellular fluid. It is able to support the body, is a site for muscle attachment, protects vital organs, and serves as a calcium, magnesium, and phosphate reservoir which allows the homeostasis of these ions in the blood (Bernardo, P. et al., 2002). The skeletal system is able to regulate its own bone mass and architecture to meet the daily demands of its external environment. These observations support the well accepted theory that mechanical loading directly or indirectly influences the activity of cell populations to deposit, maintain, or remove bone tissue as appropriate which is integral to skeletal adaptation to load (Buchholz et al., 2007). Although the exact mechanism is not completely understood, cells can respond to a mechanical stimulus through chemical activities which ultimately result in an adaptive response during a process typically termed mechanotransduction.

With advancing age there are alterations in bone structure and mineralization which are often associated with an increase in osteoporotic fracture risk. Osteoporosis is a disease characterized by low bone mineral density (BMD) and micro architectural deterioration of bone and has great implications on health care cost and quality of

living (Schuit, S. et al., 2003). Every year 1.5 million fractures occur in the United States due to osteoporosis and an estimated $60 billion will be spent per year by 2025 on this health care problem. The number of Americans that will have osteoporosis by 2015 could exceed 41 million. Osteoporosis is an important clinical and public health problem among men and women. Estimates suggest 1-2 million men age 50 and over in the United States have osteoporosis and 8-13 million have osteopenia. There were 0.5 million hip fractures in men in 1990 and this number will increase to 1.2 million by 2025. Furthermore, men account for about 20% of the direct cost of osteoporosis in the United States (Cauley, J. et al., 2001). Bone mineral density and distribution, which are heavily influenced by the mechanical environment, are major risk factors for osteoporotic fracture.

The goal of this dissertation is to study the effect of age on bone under various conditions of maturation and differentiation and its response to mechanical stimulation. Of specific interest is the effect age has on the ability of osteocytes and osteoblasts to respond appropriately to mechanical cues as they mature. These cells are thought to be involved in the transduction of mechanical stimulation, however little is known about the ways in which age affects their response, especially osteocytes which are deeply embedded in the matrix. If alterations in the adaptive mechanisms to load are observed between young and old animals it could highlight key targets for therapeutic agents and reveal the role mechanotransduction plays in maturing bone in elderly populations. Understanding the ways in which bone adapts to mechanical load with advancing age could help identify new approaches to treat musculoskeletal diseases and injuries (Rubin, C. et al., 1999; McCreadie, B. et al., 2000).

1.1 Bone Structure

Long bones consist of a shaft termed the diaphysis and two ends known as the epiphysis. The flared component of the epiphysis directly adjacent to the diaphysis is called the metaphysis and extends from the diaphysis to the epiphyseal line. The marrow or medullary cavity is a large cavity filled with bone marrow which forms the inner portion of bone and is surrounded by bone tissue. The tissue lining both the bone facing the medullary cavity and the trabeculae is termed endosteum which contains progenitor cells.

Blood is supplied to the shaft of long bones primarily through arteries that enter the medullary cavity through nutrient foramina in the diaphysis and epiphysis. The bone is drained by veins that leave through the nutrient foramina or through the bone tissue of the diaphysis and out via the periosteum. Volkmann's cannals provide the main route within compact bone enabling nourishment to bone tissue. The diaphysis of the adult human bone contains units of bone structure known as a Haversian system or secondary osteons. Interstitial lamellae are lamellar territories between the Haversian system and are the remnants of older osteons or of circumferential lamellae. The lamellar interface may play an important role in torsional yielding by keeping cracks physically isolated from one another and delaying microcrack coalescence (Jepsen, K. et al., 1999). Smaller blood vessels enter the Haversian canals, and periosteal tissue allows lymphatic drainage.

In the inorganic matrix calcium in the form of hydroxyapatite crystals provides strength and stiffness to bone tissue and enhances load bearing capacity; however at increased level of mineralization the tissue can become brittle reducing the energy required for fracture (Burr, D. et al., 2002; Aarden, A. et al., 1994). Phosphate,

carbonate, and other impurities (sodium, magnesium, potassium, citrate, fluoride, and HPO^{3-}) also comprise the mineral phase (Nyman, J. et al, 2005). The bone matrix consists primarily of type I collagen fibers which likely play a role in post yield properties and overall toughness of bone due to its ductile nature (Burr, D. et al., 2002).

The organic part of bone matrix is primarily composed of type I collagen. Osteocalcin is the most abundant noncollagenous protein and is produced by the osteoblast and released during degradation of the osteoclast. Matrix Gla Protein is similar to osteocalcin and a member of the vitamin K-dependent gamma-carboxyglutamic acid proteins, is synthesized by osteoblasts, and may be a regulator of extracellular matrix calcification. Osteopontin is an abundant non collagenous sailoprotein in the bone matrix produced by osteoblasts and plays a role in osteoclast attachment and resorption. It stimulates bone formation *in vitro* and may mediate cell-cell interactions via integrin binding. Osteonectin is a noncollagenous bone matrix protein that is involved in cell attachment and supports bone remodeling and the maintenance of bone mass (van Leeuwen, J. et al., 2004)

Marrow stroma makes up the hematopoietic microenvironment (Allen, T. et al., 1981) which is involved in the maintenance and structural support of marrow hemopoiesis. It contains cells of several derivations and is made up of a network of fibroblastic cells, reticular cells, adipocytes, macrophages, and endothelial and smooth muscle cells. Stem cell origins of osteoblast and other connective tissue cells from undifferentiated pluripotent cells of the mesenchyme (Maximow, A. et al., 1924; Levander, G. et al., 1940) are referred to as mesenchymal stem cells (MSC) (Caplan, A. et al., 1991).

Osteoblasts are bone forming cells which arise from mesenchymal cells and synthesize and regulate the deposition and mineralization of the ECM. Their development begins with local proliferation of MSCs in the marrow and periosteum and Runx2, Dlx5, and Msx2 push the precursors towards the osteoblast lineage. Additional differentiation requires Runx2, osterix, and components of the Wnt signaling pathway. The mature osteoblast expresses matrix proteins type I collagen and osteocalcin and alkaline phosphatase (Robling, A. et al., 2006).

The osteocyte is the most abundant cell found in bone and is a terminally differentiated relic of a once prolific osteoblast. As a row of active osteoblasts secrete un-mineralized matrix (osteoid) and moves away from the bone's surface a small number of cells fall behind and are incorporated into the matrix. They generate long cytoplasmic gap junction coupled processes passing through the matrix via small channels (canaliculi) to remain in communication with surrounding cells. It is thought that osteocytes play a role in the arrest of fatigue cracks, mineral exchange, osteocytic osteolysis, the guidance of osteoclastic cutting cones involved in mineral exchange and the repair of micro-damage, renewed remodeling activity after release by resorption, and strain detection (Robling, A. et al., 2006).

Osteoclasts are derived from hematopoetic stem cells in the marrow and spleen. They are stimulated to generate mononuclear cells and are introduced into the blood where they fuse with one another to form a multinucleated immature osteoclast. Once an osteoclast becomes mature its bone resorbing capacity and survival is regulated by RANK-L. Osteoclasts participate in bone resorption through peripheral attachment to the matrix which creates micro-compartments. H+ ions are pumped into the compartment to

solubilized bone mineral followed by protease degradation of the organic matrix (Robling, A. et al., 2006).

1.2 Adaptive Response to Mechanical Load

The strength of bone and its resistance to fracture is dependent on its mass, geometry, and intrinsic properties (Burr, D. et al., 2002). Bone tissue adjusts its architecture in relation to its functional load bearing and reflects a balance between the form and mass required for adequate strength and the metabolic benefits (Rubin, C. et al., 1984). It is hypothesized that blood flow and cell matrix deformation can create a pressure gradient which leads to interstitial fluid flow in the lacunar-canalicular network. This provides nutrients and creates shear stress. The latter influences the transmission of signals to bone lining cells to release paracrine factors which stimulate osteoprogenitor cells to divide and differentiate into pre-osteoblasts (Duncan, R. et al., 1995). Skeletal unloading can result in reduced matrix production, mineral content, bone formation, and increases in bone resorption which is often observed in cases of prolonged bed rest and during space flight. Skeletal loading depending on the duration, magnitude, and frequency can result in an increase in bone mass and decreased resorption (Rubin, C. et al., 1994). Mechanical stress improves bone strength by influencing collagen alignment as new bone is formed and daily loading can result in increased bone formation rate, bone mineral apposition rate, and labeled bone surface area (Robling, A. et al., 2006; Kim, C. et al., 2003).

1.3 Global Mechanotransduction

Mechanotransduciton can be categorized into four stages; mechanocoupling, biochemical coupling, transmission of a signal, and effector cell response. During

mechanocoupling mechanical loads result in *in vivo* deformations in bone that stretch bone cells within and lining the bone matrix. It has been hypothesized that this creates fluid movement within the canaliculi and the creation of streaming potentials. *In vivo* changes in surface strain charge has been recorded in the radius of sheep during locomotion (Lanyon, L. E. et al., 1977). During biochemical coupling a force transduction occurs through the integrin-cytoskeleton-nuclear matrix structure, stretch activated cation channels within the cell membrane, G protein dependant pathways, and a linkage between the cytoskeleton and phospholipase C or phospholipase A pathways (van Leeuwen, J. et al., 2004).

1.3.1 Signal Transduction

There are several signaling pathways and intracellular molecules likely involved in the mediation of bone cell response to strain and mechanical forces. Studies suggest that strain induces activation of extracellular signal-related kinase (ERK)-1/2 which is involved in the mechanical strain inhibition of RANKL, c-jun N terminal kinase (JNK), phospholipase C and protein kinase C, and intracellular calcium mobilization. *In vitro* stretch, strain, compressive forces, pulsating fluid flow, and intermittent hydrostatic compression can induce prostaglandin E_2 (PGE_2) release in bone cells which is important for the induction of gap junctions between osteocyte like cells in response to mechanical strain and plays a major role as a local mediator of the anabolic effects of mechanical forces on bone cells. The increase in PGE_2 is often accompanied by an increase cAMP and cGMP levels. (van Leeuwen, J. et al., 2004).

Mechanical stimulation can induce a rapid and transient increase in nitric oxide (NO) which is produced by osteoblasts in response to mechanical stimulation and is a

mediator of mechanical effects in bone cells which leads to increased PGE_2 release in osteocytes. Mechanical forces can also induce the expression of inducible cycloxygenase COX-2 in osteoblasts and osteocytes and this effect depends on cytoskeletal-integrin interactions and occurs through the ERK-signaling pathway in osteoblasts.

Induced COX-2 expression is mediated by C/EBP beta, cAMP-response element binding proteins, and activator protein-1 (AP-1) in osteoblast cells. Mechanical strain can also increase intracellular levels of inositol triphosohate which is partially dependent on prostaglandin synthesis. The inositol phosphate pathway may be involved in the mechanical-strain induced proliferation of bone cells. Thus the transduction of a mechanical stimulus into a biochemical response in bone cells likely involves an increase in calcium levels, which occurs before the activation of protein kianse A and protein kinase C. Increased inositol triphosphate activates *c-fos*, COX-2 transcription and results in the production of PGE_2, intracellular cAMP levels and downstream target molecules such as IGF-I and osteocalcin in osteoblasts. The activation of *c-fos* anf *c-jun* may also modulate osteoblast and osteoclast replication or differentiation through activation of target genes whose promoters have functional AP-1 sites (van Leeuwen, J. et al., 2004).

Mechanical stretching upregulates the expression of DNA binding of the osteoblast transcription factor Runx2/Cbfa1. Microgravity affects TGF β expression in the hind limb and mechanical stimulation of osteoblasts *in vitro* increases the expression of TGF β transcripts. Microgravity also increases the expression by osteoblasts of interlukin-6 which activates osteoclast formation. Mechanical loading through cyclic stretch and fluid shear have been shown to enhance phosphorylation of connexin 43, the protein that forms gap junction channels, and gap junction communication in osteoblasts

and osteocyte like cells (Cheng, B. et al., 2001). *In vitro* studies have shown that osteocyte like cells (MLO-Y4) respond to shear stress through the establishment of more gap junctions and increased phosphorylation of connexin 43 when compared to un-loaded controls (Alford, A. et al, 2003). Once activated connexon hemichannels mediate the release of paracrine factors that activate intracellular signaling pathways (Riddle, R. et al, 2008)

1.3.2 Mechanical Receptors

The cell surface proteoglycan layer (glycocalix) is a likely sensor of mechanical signals and can transmit force to the plasma membrane or submembrane cortex-actin cortical skeleton. Lipid rafts and caveolae may serve as cell surface mechanotransduction sites within the plasma membrane and transduction might also occur at intracellular junctions (adherens junctions) and cell matrix contacts (focal adhesions) (Liedert, A. et al., 2006).

Integrins are transmembrane proteins that link extracellular matrix proteins to the cytoskeleton and control cell deformation, focal adhesion, and cell adherence to the matrix. The integrin-cytoskeletal system may also play a role in the transmission of signals in lining cells, osteoblasts, or osteoctyes. Integrin-mediated binding is necessary for resistance to strain by osteoblast cells and mechanical stimulation and cell adhesion stimulate the expression of integrins in osteoblasts. The transduction of mechanical signals in bone cells requires cytoskeleton integrity, and mechanical stimulation in osteoblasts revises focal contacts and cytoskeleton and induces tyrosine phosphorylation of several proteins linked to the cytoskeleton including focal adhesion kinase (FAK)

It is thought that the mobilization of FAK, MAP Kinase and the small G protein signaling molecules Ras, Rac, and Rho mediate the early bone cell cytoskeletal response to load. Studies have shown that the application of fluid shear to osteoblasts results in reorganization of actin filaments into contractile stress fibers that involve the recruitment of β1-integrins and α-actinin to focal adhesions. An increasing amount of evidence suggests that the development of internal tension by actin and myosin is important in signal transduction from the ECM to the nucleus to regulate gene transcription (Malone, A. et al., 2007).

In addition to the integrin-cytoskeletal system, membrane proteins may also respond to strain and mechanical forces. Long-lasting (L-type) voltage-sensitive channels are involved in the influx of calcium into bone cells and might initiate molecular signals involved in mechanotransduction. Stretch sensitive channels that respond to mechanical stimulation are present in several cell types and are upregulated by strain in osteoblasts. Glutamate receptors present in osteoblasts, osteocytes, and osteoclasts may also be involved in the effects of strain in bone cells and estrogen receptors likely interact with mechanical forces to modify the response to mechanical loading which is supported by several studies (van Leeuwen, J. et al., 2004).

1.4 The Effect of Age on Bone

Changes in the integrity of bone structural architecture and bone mineral density are a well established function of age. *In vivo* studies have shown decreases in cortical thickness, cancellous bone volume fraction, bone mineral density, trabecular number, structure model index and connectivity, and increases in trabecular spacing and porosity with advancing age in the spine, femur, and tibia (Halloran, B. et al., 2002; Majumdar, S.

et al., 1997). Studies utilizing infrared spectrometry suggest larger crystals are present in the bone of older osteoporotic women which could damage the mechanical properties of the tissue (Burr, D. et al., 2002). The organization of calcium phosphate crystals of various sizes embedded in and around fibrils of the type I collagen lattice are influenced by hormones, cytokines, and functional stimuli all of which may also be influenced by aging (Schuit, S. et al., 2003; Rubin, C. et al., 1999). Collagen deteriorates with advancing age specifically through a decrease in collagen content; however it remains unclear whether its functional properties are altered (Schuit, S. et al., 2003).

With advancing age there is a decrease in the number of bone forming osteoblasts and an increase in the number of marrow adipocytes. Stromal cells from aged animals have a much greater ability than stromal cells from young or adult animals to induce osteoclastogenesis. Osteoprotegrin which blocks the osteoclast-stimulatory effects of RANK ligand and osteoclastogenesis, decreases with age in humans and RANKL and macrophage colony stimulating factor (M-CSF) increase (Cao, J. et al., 2005; Cao, J. et al., 2003). Studies suggest lacunar number and number of actively remodeling osteons per bone area decreases with advancing age and lacunar area is significantly reduced in osteoporotic specimens (Mullender, M. et al., 2005). An increase in micro-damage accumulation with advancing age was also observed in loaded human cortical bone tensile specimens when compared to unloaded controls (Nyman, J. et al., 2005).

Experimental studies suggest cells and tissue may lose the ability to respond to the daily mechanical demands of its environment with advancing age through alterations in structure and/or functional capability (Bonewald et al, 2002). For example, aging neurons undergo morphological changes including a reduction in the complexity of

dendrite arborization and length, and a decrease in spine number, a major site of excitatory synapses (Dickstein, D. et al., 2007). Turner and colleagues showed that the relative bone formation rate in old rats was over 16-fold less than that reported for younger rats at an applied four point bending load of 64N and the relative bone forming surface in old rats was 5-fold less than in younger rats under similar loading conditions. In addition, the mechanical threshold in young rats for lamellar bone formation was 1050$\mu\varepsilon$ for the endochondral bone surface and old rats required over 1700$\mu\varepsilon$ on the endochondral surface before bone formation was increased (Martin, D. et al., 1993 and Turner, C. et al., 1995). In an aged mouse population exercise led to increases in structural and tissue level mechanical properties compared with weight-matched control mice without changes in bone size or shape (Kohn, D. et al., 2009). Interestingly exercise is limited in its ability to maintain or restore bone mass in postmenopausal women (Wallace, B. et al., 2000).

In vitro data suggests that age may affect the percent of cells responding to mechanical load via calcium signaling, however; not the calcium signal magnitude (Donahue, S. et al., 2001). *In vitro* studies have also shown that osteocyte stiffness across the nucleus increases with advancing age which could alter the local strain environment of the osteocyte and its ability to sense and respond to load. However, whether changes in material properties affects signaling pathways thought to be involved in mechanotransduction has yet to be elucidated (Wenger, K. et al., 2007). *In vitro* studies suggest that the diffusion coefficient of molecules through gap junctions in the lacunar-canalicular network is decreased with aging in mice, however changes in load induced transport of molecules through this network as a function of age is currently unknown

(Zhang, X. et al., 2007). These studies demonstrate conflicting and complicated influences of age on cellular response to mechanical loading.

To date research on aging osteocytes has primarily been conducted *in vitro.* While *in vitro* studies offer the advantage of a high cell yield and a highly controlled testing environment, they neglect the analysis of cells in their native three dimensional environments, which is particularly important in the study of osteocytes due to their stellate morphology. The importance of a 3D ECM has been recognized for epithelial cells, where 3D environments facilitate a normal epithelial polarity and differentiation. The culture of fibroblast cells on flat 2D substrates introduces an artificial polarity between lower and upper surfaces of these normally non polar cells and their morphology and migration differ once suspended in collagen gels (Hoffler, C. E. et al., 2006; Raab, D. et al., 1990).

Furthermore, *in vitro* studies lack the bone matrix which is an important medium through which osteocytes perceive physical phenomenon. Bone matrix micro cracks are more prevalent in aging adults in the femoral neck and osteocyte apoptosis has been linked to fatigue induced micro crack development and subsequent resorption. Thus, changes in bone ECM mechanical properties might be a factor in the etiology of these fractures.

In *in vitro* studies it is difficult to isolate a pure population of osteocytes since many of their biochemical characteristics are similar to that of the osteoblast. *In vivo* osteocytes express mRNA for β-actin, osteocalcin, connexin-43, insulin like growth factor I, c-fos, phosphate-regulating gene with homology to endopeptidases on the X chromosome (PHEX), dentin matrix protein 1 (DMP1), and c-jun (Mason, D. et al., 1996

and Gu, G. et al., 2006). *In vivo* they also exhibit receptors for estrogen ERα and 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] and contain hyaluronan lacunae. *In vitro* they produce smaller amounts of collagen and fibronectin, greater amounts of osteocalcin, osteonectin, and osteopontin, and decreased alkaline phosphatase activity when compared to osteoblasts.

In vitro analysis of the osteocyte suggests the cell body and processes are surrounded by a thin layer of un-mineralized matrix of a different composition than the mineralized interlacunar matrix (Raisz, L. et al., 2001). This matrix is PAS+, contains randomly oriented collagen fibers, and proteoglycans which consist of dermatan sulfate, keratin sulfate, and chondroitin 4 sulfate. Prominent actin bundles have been observed in osteocyte cell culture as well as an abundant presence of the actin protein fimbrin. OF45 and RGD containing matrix protein are particularly expressed by bone embedded osteocytes, as well as CD44, which is involved in cell attachment and movement (Murshid, S. et al., 2007). Evidence for receptors for PTH has been observed both *in vivo* and *in vitro*, however currently the only mAb specific to the osteocyte exists for avian species.

Lanyon and Skerry proposed that osteoporosis is the result of a mal-adaptation to loading (Raisz, L. et al., 2001, Turner, C. et al., 1995 and Turner, C. et al., 1994). Studies support this concept including work by Tatsumi et al. in which osteocyte-ablated mice exhibited fragile bone with intracortical porosity and micro-fractures, osteoblastic dysfunction, and trabecular bone loss with micro-structural deterioration and adipose tissue proliferation in the marrow space, and resistance to unloading induced bone loss (Tatsumi, S. et al., 2007). In a recent study, *in vivo* analysis of the amount of ERα

protein per osteocyte in stained sections from control and loaded ulna and the *in vitro* effect of exogenous estrogen and mechanical strain on the expression of ERα mRNA levels was assessed. Overiectomy was associated with a decrease in ERα protein expression per osteocyte suggesting bone cell's responses to both strain and estrogen involve ERα but only estrogen regulates its cellular concentration. These finding are consistent with the hypothesis that bone loss is associated with estrogen deficiency and is a consequence of reduction in ERα number and activity associated with lower estrogen concentration, reducing the effectiveness of bone cells' anabolic response to strain (Zaman, G. et al., 2006).

While this hypothesis may shed light on the alterations in bone structure and integrity observed in post menopausal women, however it fails to address the structural and functional changes in the osteocyte that may occur as a function of age despite menopause and decreased estrogen levels. Osteoporosis is also an important clinical and public health problem among older men. As noted earlier, men account for 20% of the direct cost of osteoporosis in the United States and mortality after osteoporotic fracture may be greater in men that women (Van der Plas, A. et al., 1994). Therefore it is important to assess osteocyte function and response to mechanical loading with advancing age independent of decreases estrogen levels.

Current experimental techniques to explore osteocytes and bone matrix strains include animal studies with implants or external loading devices, bone biopsy culture and bone organ culture. The main mechanical limitation of these models is the inability to directly calculate strains in the bone matrix and around the osteocyte. Biopsies pose a limitation in that they require severing the canalicular network which may be vital to

osteocyte mechanotransduction. Bone organ cultures surpass this later limitation, however irregular shapes impede uniform distribution of applied mechanical loads.

1.5 Global Hypothesis

The effector cell response during mechanotransduction is the focus of this dissertation. The global hypothesis of this dissertation is that the influence of mechanical factors on the maintenance or the repair of bone is influenced by age. In addition it is hypothesized that the influence of mechanical factors during bone repair and regeneration is influenced by age. Finally it is hypothesized that the influence of mechanical factors during the maintenance of mature bone is influenced by age.

1.6 Chapter Overviews

Studies have shown contradicting results of the effect of mechanical stimulation on bone weight, density, and strength in old and young populations (Turner, C. E. et al., 1995, Donahue, S. et al., 2001). These variations could result from differences in animal species, length and/or intensity of exercise training or methods to assess mechanical parameters. The majority of these assessments have been at the macroscopic level, thus it remains unknown if there are differences with aging in the cellular and molecular responses to mechanical load.

Furthermore, it is important to understand the effects of mechanical stimulation on cells within a repair system to predict the response and success of implants *in* vivo. To thoroughly address the effect of age on material properties of bone and response to a comparable mechanical stimulation at various stages of cell maturation, three specific aims were developed. The overall goal of this thesis is to examine responses in the aging skeleton during conditions of repair and mechanical stimulation. Specific aim one is to

create a model of regenerative bone in a mature and aged skeleton and to determine if and how aging affects mineralization and the mechanical response of the regenerative bone tissue. Specific aim two is to examine mature bone in a mature and aged skeleton and to determine if and how aging affects mineralization and the mechanical response of mature bone tissue. Specific aim three is to determine if and how aging and maturation time affects marrow stromal cell mineralization capacity and their response to mechanical stimulation.

These three aims offer the advantage of investigating changes in mechanical response as a function of age using a dimensionally controlled experimental model. The specimen geometry allows the application of standard engineering principles to characterize stress fields under load. It also allows observation of biological activity, deformation, and communication of osteocytes in response to controlled mechanical loading applied to the matrix. Furthermore, investigation of the effect of age on mechanical response of regenerative bone tissue and marrow stromal cells over a time course of differentiation may be a good representation of the role age plays in mechanotransduction during remodeling conditions. Additionally, analysis of the effect of age on mechanotransduction both *in vitro* and *in vivo* will allow the comparison of these two experimental paradigms in answering the question; does age affect the response of bone to mechanical loading

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CHAPTER II

THE EFFECT OF AGE ON REGENERATIVE BONE TISSUE AND ITS RESPONSE TO MECHANICAL STIMULATION

And if the night runs over and if the day won't last and if our way should falter along the stony pass it's just a moment this time will pass

-Stuck in a Moment

2.1. Introduction

It is well established that bone responds to the demands of its daily mechanical environment during development, repair, and adaptation. Mechanical stimulation enhances the expression of phenotypic markers for osteoblastic cells and induces the release of paracrine factors necessary for the anabolic response of bone to mechanical load. Unloading, which can occur during bed rest, can decrease cell proliferation potential and impair differentiation capacity.

With advancing age bone exhibits decreases in mass and area which may adversely affect its ability to support daily mechanical loads. In both men and women the mechanical properties of cortical and trabecular bone decline with age. In human cortical bone after age 20 the elastic modulus decreases 2% per decade, the ultimate strength declines 2-5% per decade, and incurred deformation and energy absorbed before fracture decreases 5-12% per decade. In human trabecular bone there is a reduction in the amount of bone which may compromise the integrity of the trabecular network. Trabecular bone

from the iliac crest matched for density exhibited a 40% decrease in compressive strength in older donors when compared to young. Trabecular number and thickness decline with aging and the separation distance between trabeculae increases with advancing age. Studies indicate that men and women undergo endosteal resorption and periosteal expansion, and may have a decrease in cortical area and moment of inertia Rosen, C. et al., 1999).

The adult skeleton is continuously remodeled by osteoclast removal of old matrix and osteoblast deposition of new bone. Systemic hormones and local factors affect the replication of undifferentiated cells, the recruitment of cells, and the differentiated function of cells. Polypeptides, steroid and thyroid hormones and local factors such as growth factors, cytokines, and prostaglandins can directly or indirectly act on skeletal cells to resorb or deposit bone as appropriate. With aging there is evidence that the rate of bone repair is progressively reduced. Studies have shown that the repair process is delayed and that there is also an increase in the number of non unions with advancing age (Naik, A. et al., 2009).

Studies *in vivo* suggest the ability to adapt to mechanical loads is compromised with aging, however the exact mechanism is not well understood. *In vitro* data highlights contradicting results on the effect age has on bone cell's ability to respond to mechanical load via Calcium signaling. Currently, there is no data on the effect of age on regenerative bone and its capacity to respond to mechanical load. To investigate the effect of age on regenerative bone tissue and its response to mechanical loading, an animal model was adapted from the bilateral-explant system originally developed by Hoffler (Hoffler, C. E. et al., 2006).

2.2 Materials and Methods

The animal model used in these experiments enabled the production of osteocytes in their native three dimensional matrix, which could facilitate real time measurements of osteocyte biological activity during mechanical loading **(Figure 2.2-2.4)** (Hoffler, C. E. et al., 2006).

2.2.1 Surgical Procedures

Five 6 month old and five 21 month old male Sprague Dawley rats were bilaterally implanted with a custom implant-explant system. The ages were selected based on previous data collected for an aging male rat experimental model (n=32). Micro-CT, four point bending, and compression analysis indicated statistically significant changes in bone morphology and geometry as a function of age **(Figure 2.1A-2.1F)**. The data suggested maximum properties at 8 months followed by dramatic changes which tended to plateau at 23 and 33 months. In an effort to observe the earliest alterations in bone morphology, geometry, and material properties with age 6 and 21 month old animals were selected for respective young and old groups.

Animals received 5ml of warmed lactated ringers mixed with butorphenol (1mg/kg) followed by atropine (0.1mg/kg) subcutaneously. After induction of anesthesia and sterile preparation, the anterolateral femoral diaphysis was approached through a 2cm incision and the lateral intermuscular septum subsequently separated **(Figure 2.2A)**. The sub-periosteal cortex was exposed with a ¼" periosteal elevator. A custom clamp held the diaphysis while the defect was drilled with a #62 drill **(Figure 2.2B)**. A cross cut carbide dental burr (100-4895) was used to create a full thickness cortical defect and the dimensions were verified with a custom probe. The implant was fitted to the diaphysis

with the channel plate suspended in the defect and the back plate resting on the periosteal surface. The #62 drill was used to pre-drill both cortices for a #001-1/4" self tapping screw. The screw was placed in the diaphysis with the implant clamped to the diaphysis. The implant was allowed to align with the natural bony architecture prior to tightening the screw **(Figure 2.2C)**. Next, the wound was flushed with 10mL saline and closed in layers with 3-0 polyglycolic acid and stapled. Radiographs were taken post-operatively **(Figure 2.2D)**. Animals were housed individually and monitored several times a week for pain and distress.

The implantation period was selected based on previous data from a study using the implant-explant model on fifty-one six month old male Sprague Dawley rats with pre-operative weights varying from 390-500g with a mean of 455g. This data suggested that maximum bone volume fraction and bone mineral density was reached on average after a twelve week implantation period. The specific aim in this study investigates aging effects; thus the implantation period was extended beyond this twelve week period to fourteen weeks and sixteen weeks in two distinct studies to enable the opportunity for the older animals to form adequate bone for analysis in the chambers.

2.2.2 Harvest Tissue Culture Procedure

Following the implantation period animals were prepared for surgery, cortical screws, back plate screw, and back plate were removed to expose the specimen **(Figure 2.3A,B)**. The chamber and in grown tissue was cooled with a sterile ice pack and the chamber and explant were extracted en block with a custom elevator placed in tissue culture media cooled with sterile ice. Distal and proximal connections with the host tissue were severed with a scalpel and only two explant surfaces required cutting to

separate tissue from the implant and host femur. These surfaces were as far as possible from the central bone tissue of interest. The procedure was repeated in the contra-lateral limb.

Animals were euthanized intra-operatively with intra-cardiac sodium pentobarbital (100mg/kg). Explants were removed from the chamber within the media and placed in wells with cold media, and transferred to an incubator at 37°C with 5% CO_2. Harvest media was replaced with fresh media within three hours. Tissue was cultured in BGJb with 10% heat inactivated fetal bovine serum, penicillin (100U/ml), streptomycin (100μg/ml), Amphotericin B (5μg/ml), and daily ascorbic acid (75μg/ml). Media was replenished every three days (Hoffler, C. E. 2004). Fourteen specimens were produced in young animals and fifteen specimens were produced in old animals after a 12 week implantation period. Seventeen specimens were produced in young animals and fifteen specimens were produced in old animals after a 16 week implantation period.

2.2.3 Micro Computed Tomography (μCT)

The degree of mineralization is an important component of bone strength and load bearing capacity. To determine tissue mineral density regenerative tissue was dissected, cleaned of soft tissue, and scanned on a μCT system at a resolution of 18μm/voxel. Data was calibrated to air, water, and hydroxyapatite. The reconstructed three dimensional images were thresholded on a value based on the average minimum Hounsfield Unit (HU) value between two peaks on a graph of frequency (voxels) versus HU value for the specimens to separate bone voxels from non bone voxels. The region of interest was created with the cortical tool which selected an ROI corresponding to the cortical shell of the bone and used a series of morphological operators to semi-automatically select

cortical bone components. A grey level threshold value and 2 scaling size parameters were used to improve the accuracy of the ROI tool. The threshold value selected separated out cortical bone voxels and essentially provides a cut off point. This value is typically determined from an average histogram from all the specimens of all the gray scale values in the scanned specimen and their frequency. Typical histograms have two peaks. The peak at the lower HU value has a lower degree of mineralization than the peak at the higher HU value.

2.2.4 Histology

Osteocytes are the most abundant cell in bone and thought to be the primary mechanosensors. They are deeply embedded in bone, surrounded by extracellular matrix and connected with other osteocytes and osteoblasts via processes. It is thought that deformation of the ECM can cause fluid flow and produce shear forces within the lacunar-canalicular network. In order to study the effect of age on the lacunar-canalicular network, regenerative bone tissue microspecimens and control femora were fixed for 48 hours in 10% neutral buffered formalin (NBF) and then fixed in 70% ethanol until further processing. Femora were decalcified in formic acid/sodium citrate for one week. Next, femora were rinsed in water and stored in 70% ethanol until 24 hour processing through graded alcohols, xylene, and then embedded in Paraplast Plus wax. Specimens were processed for 7.5 hours and then embedded in paraffin. Specimens were sectioned at 7 microns and stained with alcian blue hematoxylin and acid fuchsin eosin.

First, specimens were dewaxed and hydrated and then immersed in acid alcohol for 30 seconds and drained. Next, specimens were stained in alcian blue hematoxylin (1.5g hematoxylin in 250mL dH_2O, sol. of 1% iodine in 95% ETOH (0.5 g in 50mL 95%

ETOH), sol. of 10% aluminum ammonium sulfate in dH_2O (70g in 700mL dH_2O), and 10g Alcian blue) for 30 minutes at 20°C. Slides were then washed briefly in acid alcohol (1% Hydrochloric acid in 70% ETOH) for 2-3 seconds. Next slides were washed in tap water for 5 minutes and then stained in acid fuchsin-eosin (one part acid fuchsin (acid violet 19) and four parts eosin yellowish (acid red 87) mixed in powder form then prepared as a 1% solution in distilled water) for 3 minutes. Specimens were then washed in tap water for 5 minutes, dehydrated in alcohol, cleared, and mounted.

Cartilage stained blue, woven bone mauve-blue, mature bone mauve, muscle and necrotic bone red, and nuclei mauve-black. For quantitative histology 222μm x 166μm specimens spanning the (region) of each specimen were imaged at 40X with a Carl Zeiss (MicroImaging GmbH, Jena, Germany) light microscope. The number of lacunae and nuclei were counted for an average of 72 slices per 2 animals which spanned the entire microspecimen and an average of 47 slices per 5 animals which spanned the femora region used in micro CT analysis.

2.2.5 Three Point Bending

Three point bending is useful for measuring mechanical properties of long bones and was selected based on the small size of the specimens (794μm x 2490μm x 254μm) and it was assumed that shear stress was minimal. The simplicity of three point bending offers an advantage, however, an important disadvantage is the creation of high shear stresses near the midsection of the bone. Four point bending produces pure bending between the two upper loading points which ensures that transverse shear stress is zero, however four point bending requires that the force at each loading point be equal which is

easy to produce in regularly shaped specimens, but difficult in whole bone tests (Turner, C. et al., 2001).

A custom three point bending apparatus inserted into a microscope stage and allowed the tissue to be maintained in culture medium and in the incubator (37°C and 5% CO_2) while being loaded (**Figure 2.4A-2.4C**). The loading device was driven by a linear motor controlled by APT software. The loading system was mounted to an aluminum frame which could rest on the frame of a microscope. The loading platen connected to the load cell which was connected to the linear slide. The load cell measured up to 1000g and the fixed platen was continuous with the wall of the media well.

Specimens extracted from chambers were cyclically loaded at a 0.5μm/s rate for a total of sixty minutes to a maximum displacement of 14μm which produced ±17.63$\mu\varepsilon$. (1.7%). The loading rate and magnitude were selected based on previous data using this device on similar microspecimens. Under these loading conditions it was possible to observe a mechanical response and to observe and calculate deformations around the osteocyte pericellular and nuclear matrix (Hoffler, C. E. et al., 2004). Furthermore, other studies have elicited a mechanical response *in vitro* with the application of 1, 2.4, 5.3, and 8.8% surface strain and strains between 1% and 10% are necessary to activate bone cells (Riddle, R. et al., 2009 and Neidlinger-Wilke, C. et al., 1994). The time duration was selected based on previous data which demonstrated an increase in nitric oxide and prostaglandin E_2 production up to 3 hours of mechanical stimulation (Smalt, R. et al., 1997, Mcallister, T. et al., 199). During 3 point bending the load ranged from 19.5 to 30g. Media was harvested from the media well for each sample after fifteen, thirty, forty-

five, and sixty minutes of loading. Microspecimens were snap frozen in 1X PBS and stored at -80°C until further analysis.

2.2.6 Quantification of Prostaglandin E_2

Prostaglandin E_2 is released from bone cells after long term exposure to mechanical load and its pharmacological inhibition *in vivo* inhibits bone formation after the application of endogenous load (Riddle, R. et al. 2008). It has been identified as an important mediator in the regulation of bone turnover and regulates bone metabolism by both stimulation and inhibition of bone formation. In the osteosarcoma cell line UMR 106-01 PGE_2 enhances c-fos and c-jun mRNA (Glantschnig, H. et al 1996).

50 μL of media was assayed in triplicate for all samples with an EIA kit from Cayman Chemical (Ann Arbor, MI). The PGE_2 standard was reconstituted with 1.0ml of EIA buffer. Eight clean test tubes were numbered #1 through #8. 900μL EIA buffer was added to tube #1 and 500μL EIA buffer was added to tubes #2 - #8. 100μL of bulk standard was transferred to tube #1, mixed thoroughly, and the remaining standards serially diluted by removing 500μL from tube #2 and placing it in tube #3, mixing thoroughly, removing 500μL from tube #3 and placing it in tube #4 and the process was repeated for tubes #4 - #8. 50μL EIA buffer and 50μL serum free culture media was added to non-specific binding (NSB) wells and 50μL EIA buffer to maximum binding wells (B_o). 50μL of each standard and sample was added to the remaining wells.

50μL of prostaglandin E_2 AChE tracer was added to each well except total activity (TA), 50μL or prostaglandin monoclonal antibody was added to each well except TA, NSB, and blank (Blk) wells, and the plate was incubated for 18 hours at 4°C. After incubation wells were emptied and washed five times with wash buffer. 200μL of

reconstituted Ellman's Reagent was added to each well and 5μL of tracer to the TA well prior to a 90 minute incubation in the dark. The plate was read at 420 nm on a micro plate reader (Spectra Max v5 Molecular Devices, Sunnyvale, CA). Background optical density was subtracted from all specimens and a calibration curve constructed from which unknown samples PGE_2 concentration could be determined. These values were normalized by protein concentration prior to further analysis.

2.2.7 Quantification of Nitric Oxide

After long term exposure to mechanical loading bone cells increase endothelial nitric oxide synthase and ultimately increase nitric oxide production. Nitric oxide is a short-lived highly reactive free radical which is anti-apoptotic and contributes to osteoblast proliferation and differentiation via MAP Kinase signaling (Riddle, R. et al, 2008). NO reacts readily with oxygen to yield the stable metabolites nitrate (NO_3) and nitrite (NO_2) and these can be more easily assessed (van't Hof, R. J. et al., 2001). 30 μL of media was assayed in triplicate for all samples with a kit from Cayman Chemical (Ann Arbor, MI).

The nitrate standard was reconstituted with 1.0ml assay buffer. 0.9ml serum free culture media (SFM) and 0.1 ml reconstituted standard was then mixed and aliquated as follows to produce a standard curve: S1 (0μL standard and 80μL SFM), S2 (5μL standard and 75μL SFM), S3 (10μL standard and 70μL SFM), S4 (15μL standard and 65μL SFM), S5 (20 μL standard and 60μL SFM), S6 (25μL standard and 55μL SFM), S7 (30μL standard and 50μL SFM), and S8 (35μL standard and 45μL SFM). 50μL of SFM was added to each well of sample to bring the total volume to 80μL. 10μL of enzyme cofactor mixture was added to each well followed by 10μL nitrate reductase mixture.

The plate then incubated for 2.5 hours at room temperature. After incubation 50μL of Griess Reagent 1 was added to each well followed by 50μL Griess Reagent 2. After 10 minutes the plate was read at 550nm on a micro plate reader (Spectra Max v5 Molecular Devices, Sunnyvale, CA). Background optical density was subtracted from all specimens and a calibration curve constructed from which unknown samples NO concentration could be determined. These values were normalized by protein concentration prior to further analysis.

2.2.8 Quantification of Osteopontin

Osteopontin interacts with molecules in the bone matrix and is expressed in cells of the osteoblastic lineage. It is proposed to play a role in cell attachment and is a useful marker of osteoblastic differentiation. Immunohistochemical data has identified the presence of osteopontin in osteoblasts, osteocytes, and osteoprogenitor cells; and the kidney is another potential organ that may synthesize this protein (Yoon, K. et al., 1987). Its expression can be regulated by mechanical stress and it is deposited along the cement line and lamina limitans after the end of bone resorption by osteoclasts which may provide a signal to the osteoblasts that migrate to the bone resorption site and deposit bone matrix. Studies show that expression levels of mRNA for osteopontin were shown to be modulated in response to mechanical stimulation of bone cells *in* vitro (You, et al., 2001).

Standards were placed into the appropriate wells and 49.50μL of media assayed in duplicate for all samples with an osteopontin (rodent) EIA kit from Assay Designs (Ann Arbor, MI) 49.50μL of sample was added to the appropriate wells in addition to 0.5μL phenylmethylsulphonyl fluoride (PMSF) and 0.02μL protease inhibitor cocktail. The

plate was sealed and incubated for one hour at room temperature on a shaker. The contents of the well were emptied and rinsed with wash buffer four times. 50μL of antibody was added into each well except the blank, the plate sealed, and incubated for one hour at room temperature on a shaker. The contents of the well were emptied and the plate washed 4 times with wash buffer. 50μL of conjugate was added to each well except the blank, the plate sealed, and incubated for thirty minutes at room temperature on a shaker. The contents of the well were emptied and the plate washed four times with wash buffer. 50μL of substrate solution was added to each well and incubated for thirty minutes on a shaker at room temperature. 50μL of stop solution was added to each well and the plate read at 450nm on a micro plate reader (Spectra Max v5 Molecular Devices, Sunnyvale, CA). Background optical density was subtracted from all specimens and a calibration curve constructed from which unknown samples osteopontin concentration could be determined. These values were normalized by protein concentration prior to further analysis.

2.2.9 Raman Spectroscopy

Mineral to matrix ratios were measured with a custom built Raman microscope featuring a 785 nm diode laser and Holospec 1.8 spectrograph both from Kaiser Optical Systems. The phosphate ν_1 (958 cm^{-1}) peak area and the amide I (1600-1700 cm^{-1}) band area were used for a measure of the mineral content and collagen matrix abundance respectfully. The reference spectra were taken proximal to the implant.

2.2.10 Western Blot

After treatment specimens were ground with a polytron in a total of 1mL RIPA lysis buffer, sodium orthovanadate, PMSF, and protease inhibitor cocktail (1000:1:1:2;

Santa Cruz Biotechnology, Santa Cruz, CA), snap frozen in liquid nitrogen and stored at -80°C until further processing. Specimen protein concentration was determined with a BCA assay (Thermo Scientific, Rockford Il). Ten micro grams of protein were run on the lanes of a 10% Tris-HCL gel for ninety minutes at 150V and transferred to a PVDF membrane for forty minutes at 80V. Membranes were then blocked in 5% Blotto (5g dry milk:100mL 0.1% PBS-Tween) overnight and probed for connexin 43(1:8000 AbCam Cambridge, AM) and c-fos (1:1000, Cell Signaling Inc., Boston, MA).

2.2.11 Statistical Analysis

A two sided unpaired t test was used to determine statistical significance within the data set. P values less than 0.05 are expressed with * and p values less than 0.01 are expressed with **.

2.3 Results

Previously it was demonstrated that regenerative specimens never become a solid block of bone and most of the non-osseous tissue is a hypercellular marrow. Adipocytes were not present and occasionally there were fibrous tissue elements within the bony structure and at the implant interface. The bone tissue was uniformly intramembraneous and neither true cartilage nor endochondral remnants were observed. The tissue was a mixture of woven and lamellar bone and the lamellar components were observed less often and were often apposed to implant surfaces or pre-existing woven bone scaffolds. Lamellar tissue area did not increase significantly with longer implantation time periods. All bone cell types were present and the majority of cells were osteocytes. Bone lining cells were often present at the implant surface and osteoblasts were apparent but were not on the majority of surfaces. Osteoclasts were generally not visible and rare osteonal

remodeling was observed in specimens retrieved after a three month implantation period (Hoffler, C.E. et al., 2004, Hoffler, C.E. et al., 2006).

Osteocytes remained viable in the tissue culture through day eleven as verified by L-lactate dehydrogenase (LDH) activity. Uniform diffusion via the lacuna-canalicular channels and matrix microporosity was confirmed previously with diffusion experiments in which explants were imaged at 543 nm with a BioRad 600 laser scanning confocal microscope and exposed to 2% procion red. At the interface between regions of non-osseous tissue and dark mineralized tissue there was a bright region where the dye begins to diffuse into bone and within the bone were areas of matrix with diffuse yellow label generally located close to the marrow border (Hoffler, C. E. et al., 2004).

Regenerative bone was successfully produced in young and old animals during both a three month and four month implantation period. Representative isosurfaces of the microspecimens are shown in **Figure 2.5**. During the 3 month implantation period 15% of the chambers partially filled and 40% of the chambers completely filled with bone per young animal and 30% of the chambers partially filled and 20% the chambers completely filled with bone per old animal (**Figure 2.6A,B**). During a four month implantation period 30% of the chambers partially filled and 40% of the chambers completely filled with bone per young animal and 30% of the chambers partially filled and 40% of the chambers completely filled with bone per old animal (**Figure 2.6A,B**). During a 3 month implantation time period a higher percentage of bone specimens were produced in chambers placed in young animals, however a similar percentage, although greater than that for the 3 month implantation time period, of bone specimens were produced in

chambers placed in both age groups after a 4 month implantation time period (**Figure 2.6C**).

The average degree of mineralization was higher in regenerative specimens from old animals compared to young after both implantation periods (**Figure 2.7**). Significant differences were seen between young and old animals after a four month implantation period (**Figure 2.7**). There was a greater proportion of higher mineralized bone voxels in regenerative specimens produced in old animals after both implantation periods when compared to those specimens produced in young animals (**Figure 2.8A**). In **Figure 2.8B** the distribution of bone voxels (HU values > 500) appears uniform in regenerative specimens produced in 3 months in old animals, however the distribution is non uniform in regenerative specimens produced in 4 months in old animals **Figure 2.8C**. The distribution of bone voxels appears uniform in regenerative specimens produced during both implantation time periods in young animals (**Figure 2.8D,E**). The distribution of these highly mineralized voxels appears to be located along the center of regenerative specimens produced in 3 months in old animals (**Figure 2.9A**) and along the periphery of regenerative specimens produced in old animals during a 4 moth implantation time period (**Figure 2.9B**).

The TMD data is supported by Raman spectroscopy data in **Figure 2.10A** which shows a higher mineral to matrix ratio in regenerative specimens produced in old animals when compared to specimens produced in young animals for a four month implantation period. The regenerative mineral to matrix ratio was significantly lower than the mineral to matrix ratio of control femora bone for both age groups. The crystallinity of mature specimens was significantly higher than the crystallinity of regenerative bone tissue for

both age groups **(Figure 2.10B)**. There was no significant difference with age in the crystallinity of mature or regenerative tissue, however interestingly the average crystallinity of regenerative tissue was higher in young animals while the reverse was observed in mature tissue, although these findings were not statistically significant **(Figure 2.10B)**.

The mineral to matrix ratio of regenerative specimens produced during a 3 month implantation time period was less than mature control femora bone for both age groups which was statistically significant for young animals **(Figure 2.10C)**. A statistically significant increase in young mature control femora mineral to matrix ratio was observed compared to old animals **(Figure 2.10C)**. The ratio of regenerative mineral to matrix ratio to mature control femora mineral to matrix ratio was higher in old animals compared to young for both implantation time periods **(Figure 2.10D)**. The ratio was significantly higher in specimens produced during a 3 month implantation period in young animals compared to those produced during a 4 month implantation time period.

The regenerative specimens produced in young and old animals were primarily composed of woven bone **(Figure 2.11)**. Osteocytes within lacunae were clearly visible in regenerative specimens produced in young animals, however not in specimens from old animals **(Figure 2.11)**. The regenerative bone tissue from old animals was most likely damaged during sectioning and fixation. The tissue in specimens from young animals appears more organized than specimens produced in old animals which appear fragmented and fibrous **(Figure 2.11)**. Quantitative data was determined for regenerative specimens produced in young animals **(Figure 2.12)**. Approximately 95% of lacunae contained cells in the regenerative specimens produced in young animals **(Figure 2.12)**.

Pilot data suggested there may be a significantly lower proportion of nuclei within the lacunae of regenerative specimens produced in old animals when compared to those produced in the young, however quantitative histological data for regenerative specimens produced in old animals in this study is inconclusive.

Representative isosurfaces of control femoral bone are shown in **Figure 2.13**. Lacunae and osteocytes are present in mature femoral bone from both young and old animals **(Figure 2.14)**. The average number of lacunae and nuclei significantly decreased with increased implantation time, however the percent of lacunae filled with nuclei remained constant **(Figure 2.15A-2.15C)**. There was no significant difference between age groups in either implantation period study in the percentage of mature femoral bone lacunae filled with nuclei **(Figure 2.15C)**. The number of lacunae, osteocytes, and percent nuclei in lacunae was similar for mature femoral specimens from both young and old animals after both implantation periods **(Figure 2.15A-2.15C)**.

The load versus displacement was plotted for regenerative specimens from young animals and regenerative specimens produced in old animals **(Figure 2.16A,B)**. The calculated stiffness modulus was higher in regenerative specimens from old animals compared to those produced in young animals.

There was an increase in NO after all time points of mechanical stimulation in regenerative specimens produced in young animals over a three month time period **(Figure 2.17A)**. This increase was statistically significant after forty-five minutes of loading **(Figure 2.17A)**. There was an increase in NO concentration after all time points of mechanical loading in regenerative specimens produced in old animals over a three month time period **(Figure 2.17B)**. When the average three month implantation time

change in NO concentration per animal was compared it was higher in regenerative specimens from young animals after fifteen, forty-five, and sixty minutes of loading **(Figure 2.17C)**. However this finding was not statistically significant.

There was an increase in NO after all time points of mechanical stimulation in regenerative specimens produced in young animals over a four month time period **(Figure 2.17D)**. There was an increase in NO concentration after sixty minutes of mechanical loading in regenerative specimens produced in old animals over a four month time period **(Figure 2.17E)**. When the average four month implantation time change in NO concentration per animal was compared it was higher in regenerative specimens from young animals after all time points except forty-five minutes of loading **(Figure 2.17F)**.

Prostaglandin E_2 expression after mechanical stimulation is shown in **Figure 2.18A-F**. After a three month implantation period, specimens produced in young animals displayed an increase in PGE_2 expression after thirty and forty-five minutes of mechanical stimulation **(Figure 2.18A)**. Interestingly, basal levels of PGE_2 concentration were lower in regenerative specimens from old animals which is likely the result of extreme outliers **(Figure 2.18B)**. There was no increase in PGE_2 expression after mechanical loading of regenerative specimens produced over a three month time course in old animals **(Figure 2.18B)**. When the average change in PGE_2 expression in specimens produced during a three month implantation time period per animal was compared it was higher for specimens from young animals after fifteen, thirty, and forty-five minutes of loading **(Figure 2.18C)**. However none of these changes in PGE_2 were determined to be statistically significant.

An increase in PGE_2 was observed in specimens produced in four months in young animals after fifteen, thirty, and sixty minutes of mechanical stimulation **(Figure 2.18D)**. This increase was statistically significant after thirty minutes of mechanical loading **(Figure 2.18D)**. There was no measured increase in PGE_2 expression in regenerative specimens from old animals produced during a four month implantation period **(Figure 2.18E)**. When the change in PGE_2 concentration per animal was compared it was significantly higher in specimens from young animals produced after four months for all time points of loading except 45 minutes **(Figure 2.18F)**.

Connexin 43 protein was detected in regenerative specimens produced in young and old animals after both implantation periods **(Figure 2.19A,B)**. Although not statistically significant, there was an increase in connexin 43 protein expression in regenerative specimens produced during a three month implantation time period in young animals after mechanical stimulation **(Figure 2.20A)**. There was an increase in connexin 43 protein expression in regenerative specimens produced during a four month implantation time period in both age groups, however not statistically significant **(Figure 2.20B)**. C-fos protein was detected in specimens produced in young and old animals after a four month implantation period **(Figure 2.20C)**. There was a slight increase in c-fos protein expression in regenerative specimens from young animals after mechanical stimulation although not statistically significant **(Table 2.20C)**.

Osteopontin concentration was calculated to observe the anabolic response to mechanical stimulation. There was an increase in osteopontin concentration after all time points of mechanical stimulation in regenerative specimens produced in young animals after a three month implantation period **(Figure 2.21A)**. There was an increase in

osteopontin concentration after all time points except forty-five minutes of mechanical loading in regenerative specimens produced in old animals after a three month implantation period **(Figure 2.21B)**. When the average change in osteopontin concentration with mechanical load after a three month implantation period per animal was compared it was highest in specimens produced in old animals **(Figure 2.21C)**.

Specimens produced in young animals over four months had increased osteopontin expression after all time points of loading except sixty minutes **(Figure 2.21D)**. In specimens from old animals an increase was also observed with the exception of forty-five minutes of mechanical loading **(Figure 2.21E)**. When the average change in osteopontin concentration with mechanical load after a four month implantation period per animal was compared it was higher in specimens produced in old animals after thirty and sixty minutes of loading, while the reverse was observed after fifteen and forty-five minutes of loading **(Figure 2.21F)**.

2.4 Discussion

In this study the proportion of chambers that filled with bone tissue were similar between age groups for both implantation time periods. Previous work suggests age does not significantly affect the quantity of new bone formed (Sumner, D. et al., 2003). In this study the degree of mineralization was higher in regenerative specimens produced in old animals when compared to young for both implantation periods. This was supported by the higher mineral to matrix ratio observed in regenerative bone tissue from old animals compared to young. Mineralization provides strength and stiffness to the bone, which was higher in regenerative specimens produced in old animals; however excessive mineralization can have a negative effect on tissue ductility (Bonar, L.C. et al., 1983;

Brear, K.M. et al., 1990). Furthermore, bone from old animals may remodel slower than bone from young animals which could increase fracture susceptibility through an increase in proportion of highly mineralized tissue as opposed to maintenance of a mean tissue age that is fairly constant through secondary osteonal remodeling (Akkus, O. et al., 2003)

Studies suggest that tissue-level strength and stiffness can increase with increasing crystalline, an overall indicator of crystal size and stoichiometric perfection, while ductility is reduced (Yerramshetty, J. et al., 2008). In this study crystallinity was slightly higher in regenerative specimens produced in young animals; despite the observed reduction in stiffness, which could enhance its resistance to mechanical failure despite exhibiting lower values of TMD. Paschalis and colleagues found that newly deposited bone mineral is less crystallinity than older bone which was observed in this study (Paschalis, E. et al., 1997). However, osteoporotic bone is more crystalline than normal bone in anatomical regions of osteons and periosteal bone (Paschalis, E. et al., 1997).

Meyer and colleagues found that fractures in aged rats had reduced expression of *Indian hedgehog* (Ihh) and BMP-2. Other studies suggest fractures in aged mice have a delayed expression of the bone and cartilage matrix genes *col2*, *aggrecan*, and *osteocalcin*. Lu and colleagues found a decrease in fracture callus and the expression of *col2* and *colX* in aged animals, while a recent study also found that fractures in aged mice had reduced vascularization, callous formation, bone formation, and remodeling and early and delayed chondrogenesis (Lu, C. et al., 2005; Naik, A. A. et al., 2009). Lu and colleagues also found that mineralization was higher in regenerative tissue in young

animals after 10 and 14 days of fracture, however similar in young and old animals after 18 days of fracture healing (Lu, C. et al., 2005).

Regenerative specimens from old animals had a higher degree of mineralization than specimens from young animals and representative alpha blots and histograms suggest a difference in the distribution of mineral. Most of the highly mineralized bone tissue is located on the periphery of regenerative bone from old animals produced over a 4 month implantation time period. This could be the result of the reduced vascularization others have observed in fracture healing studies with advancing age. There appears to be a more uniform and consistent distribution of the mineral in regenerative specimens produced in young animals which could affect mechanical properties. The scattered locations of highly mineralized bone tissue in regenerative specimens produced in old animals might prevent an even distribution of strain throughout the tissue and perhaps affect its resistance to failure.

In this study nitric oxide was measured to assess response to mechanical stimulation. Nitric oxide functions as a sensitive mediator of intercellular communication in a wide variety of tissues. The binding of nitric oxide to guanylyl cyclase causes the activation of this enzyme and production of guanosine monophosphate (cGMP) which initiates a cascade of phosphorylation events and alters several cellular processes. Osteoclasts may possess constitutive and inducible nitric oxide synthase (NOS) and nitric oxide is a potent inhibitor of bone resorption. Human osteoblasts express the inducible NOS isoform, however high levels of NO can cause a decrease in proliferation or viability of UMR-106 and human osteoblasts.

Osteoblasts can also produce nitric oxide in response to pro-inflammatory cytokine stimulation. For example in cultured human osteoblasts low basal levels of NO production were increased along with cGMP levels after exposure of the cells to IL-1β in combination with TNF-α and IFN-γ. Low levels of NO may activate but high NO concentrations can inhibit the induction of cyclo-oxygenase (COX-2) (Collin-Osdoby, P. et al., 1995). It has been shown that osteoclasts migrate away from NO (Webster, S. et al., 2001). Data from this study suggests that regenerative specimens from young animals may increase their expression of nitric oxide more than regenerative specimens from old animals. There is a trend of a higher change in nitric oxide concentration over time for specimens from young animals for both implantation periods, with the exception of the four month implantation period after 45 minutes of loading, which could be the result of an outlier in the data set. This increased NO could reduce osteoclastogenesis in certain regions resulting in mineralization and distribution of the mineral that is highly able to resist mechanical failure.

Elevated mRNA levels of osteopontin precede observations of loading induced bone formation *in vivo*. Thus osteopontin is likely produced during the osteogenic response of bone to mechanical loading. The osteopontin data in this study is limited by the lack of a measurement of total microspecimen osteopontin content. Values of mRNA or cellular protein expression of the analyzed soluble mechanical response markers would be an important addition to understanding what proportion of there response markers were released from the cell due to fluid forces or the mechanotransduction process.

The media osteopontin concentration measured in this study could be linked to fluid forces which push the osteopontin out of the microspecimen. Donahue and

colleagues found that MC3T3 E1 cells responded to oscillating fluid flow with both an increase in intracellular calcium concentration and increased PGE_2 production, and these fluid flow induced responses were modulated by chemotransport (Donahue, T. L. et al., 2003). However, Owan and colleagues found that faster displacement rates which cause larger fluid forces induce osteopontin expression. In their *in vitro* experiment the rate of displacement was varied while the peak strain magnitude and maximal displacement were kept constant. Displacement rates of 0.2 and 1mm/s had no effect on osteopontin expression; however osteopontin expression was increased 3 to 4 fold when the displacement rate was increased to 4mm/s. In a separate study the strain magnitude and displacement were varied independently and neither had an effect on osteopontin expression (Owan, I. et al., 1997).

In this study, regenerative specimens from old animals secreted more osteopontin when loaded compared to specimens produced in young animals in the majority of cases. However, sham levels of osteopontin were higher in specimens from old animals than sham and loaded concentration levels of osteopontin in young animals. Perhaps specimens from old animals have a higher degree of mineralization and thus more osteopontin present at baseline before loading. Perhaps the high release of osteopontin from regenerative specimens produced in old animals affects the release of other markers of mechanical response analyzed such as nitric oxide. Nevertheless, whether or not osteopontin release is the direct or indirect result of chemotransport as opposed to fluid shear stress, the release of this protein into the media following mechanical stimulation could be an important step in the bone formation process.

Bone resorption is stimulated by PGE_2 in cultured fetal rat long bones via a cAMP pathway; however studies have also shown that PGE_2 causes a transitory inhibition of resorption by isolated osteoclasts. Furthermore, studies suggest prostaglandins may stimulate osteoclast development and at low doses may stimulate bone formation. Prostaglandins may inhibit NO production (Collin-Osdoby, P. et al., 1995). PGE_2 increases mRNA levels of osteoprotegerin ligand (OPG-L)/osteoclast differentiation factor (ODF) from osteoblastic lineage cells. OPG-L/ODF stimulates osteoclast differentiation and activity and inhibits osteoclast apoptosis (Webster, S. et al., 2001). Regenerative specimens from young animals may have a higher increase in PGE_2 after mechanical load than those produced in old and earlier initiation of resorption which would then be followed by bone formation.

Regenerative specimens from old animals have a higher stiffness, TMD, and MMR than specimens from young animals. The deformation of specimens from young animals is likely greater than it is for specimens from old animals. With a difference in deformation there is also a likely difference in strain. The strain on cells in regenerative specimens from young animals could be greater than the strain placed on cells within regenerative specimens produced in old animals. As a result of greater strain in specimens from young animals there could subsequently be a higher change in NO and PGE_2 after mechanical load. This higher increase in NO and PGE_2 observed in regenerative specimens produced in young animals could be mediated by increased intracellular communication through the establishment of more gap junctions, which the increase in connexin 43 suggests. The higher increase in NO in regenerative specimens from young animals could then initiate a higher increase in c-fos protein expression.

Other studies have found a substantial decrease in the concentration of the divalent reducible collagen cross links in osteoporotic patients when compared to sex and age matched controls, however no alterations in the concentrations of the pyridinolines. Thus there could be a reduction in bone strength even though the collagen density did not differ between age and sex matched groups (Oxlund, H. et al., 1996). Further work could investigate the collagen cross links in regenerative bone specimens, which could have an impact on deformation which can affect responses to mechanical stimulation. Studies have used microarray analysis to identify differences in gene expression in osteoporotic fracture and control individuals with no known bone pathology (Hopwood, B. et al., 2009). Future work could use microarray analysis with this regenerative bone tissue model to determine if there are any differences with age in gene expression during regeneration.

In this study regenerative specimens are produced in a confined geometrically controlled chamber, which is likely a very different mechanical environment from its natural one. There is a possibility that the implanted hardware alters the stresses that are placed on the specimens during their growth which could affect their ability to perceive mechanical stimulation and their subsequent response. This study is also limited by the small sample size. Repair studies in bone can produce data sets with high variability. High standard deviations could be curtailed by increasing the sample size and or using quantification methods less subject to human quantification variability such as RT-PCR instead of western blot densitometry.

In this study microspecimens were subjected to one loading regimen based on previous data (Hoffler, C. E. et al., 2004). An age difference was observed in regenerative

tissue degree of mineralization, thus there is likely a difference in the local strain placed on the cells embedded in the matrix. In this study only the global strain was controlled thus deformation of cells throughout the tissue could vary based on the degree of mineralization throughout the region. In addition studies suggest the perilacunar strain environment is distinctly different from its tissue level counterpart. Hoffler found that local strains were nearly an order of magnitude greater than global strains (Hoffler, C. E. et al., 2004).

In this study it was not possible to determine cellularity in regenerative specimens produced in old animals from the obtained tissue sections. Previous work suggests that there is no difference in cellularity with advancing age in regenerative specimens, however pilot data shown in **Appendix A** suggests that there may be a decrease in the number of cells present in regenerative specimens with advancing age. Fan and colleagues found evidence of a decrease in thickness and cell number in diaphyseal periosteum and fewer Stro1+ cells and more F4/80" TRAP+ cells and blood vessels in the cambial layer of metaphyseal periosteum (Fan, W. et al., 2008) which supports a decrease in cell number within regenerative specimens with increased age. Future work will focus on obtaining regenerative histological specimens from old animals in which cellularity can accurately be observed and quantified. A difference in the number of cells present which are able to respond to mechanical stimulation could result in the differences in NO, PGE_2, connexin 43, and c-fos increase observed in this study.

Furthermore assessing markers of mechanical response at the time points chosen also is a limitation. Data from this study suggests a mechanical response occurs in mechanically loaded regenerative specimens as early as fifteen minutes. C-fos is a

known early response marker of mechanotransduction (Chow, J. W. et al., 1998). Analyzing this protein after 60 minutes of loading may prevent observations of changes in its expression. Studies also suggest that short term recovery periods in loading can elicit a similar response in PGE_2 and osteopontin release from stimulated osteoblasts (Batra, N. et al., 2004).

The selected mechanical response variables also pose a limitation. Nitric oxide is a short lived highly reactive free radical which can present difficulty in its measurement and osteopontin mRNA levels can increase dramatically at 24 hours post loading (Jacobs, C. et al., 2000). While the data from this study suggests certain proteins and signaling molecules were up-regulated post loading there are many other load sensitive signaling molecules and proteins that were not measured in this study such as calcium which has an immediate transient response and components of the Wnt-β-Catenin pathway (Yellowley, C. E. et al., 2000). Studies have shown that MAPKs can be activated over a 2 hour time frame (You, J. et al., 2001).

In conclusion, regenerative specimens produced in old animals had a higher tissue mineral density and stiffness than regenerative specimens produced in young animals. Although not directly measured in this study mechanical loading of these specimens could result in more deformation in regenerative specimens from young animals compared to regenerative specimens from old animals. Perhaps due to less local deformation the regenerative specimens from old animals have less of a mechanical response than regenerative specimens from young animals as measured by NO, PGE_2, and connexin 43. There could then be delayed remodeling in regenerative specimens from old animals and remodeling which takes place in regenerative specimens produced

in young animals which may be reflected in the decreased tissue mineral density observed in regenerative specimens from young animals produced over a 4 month period compared to 3 month.

After a four month implantation time period regenerative tissue mineral density was again higher in specimens from old animals when compared to those produced in young animals. This could lead to smaller local deformations post mechanical stimulation within regenerative specimens produced in old animals when compared to those produced in young and may be reflected in the reduced mechanical response observed in specimens from old animals compared to those from young animals via NO, PGE_2, and connexin 43 analysis. Interestingly the tissue mineral density of specimens produced in young animals was lower after a 4 month implantation time period compared to a 3 month implantation time period. There could be more local deformation in the regenerative specimens from young animals produced after 4 months and may be reflected in the increased mechanical response measured by NO, PGE_2, and connexin 43 in regenerative specimens from young animals after a 4 month implantation period when compared to those produced during a 3 month implantation period. The increased tissue mineral density of regenerative specimens from old animals could render them susceptible to brittle failure, while remodeling and increased mechanoresponsiveness of regenerative specimens from young animals could result in maintenance of a mean tissue mineralization.

Figure 2.1A: Bone Volume Fraction (mm^3/mm^3)

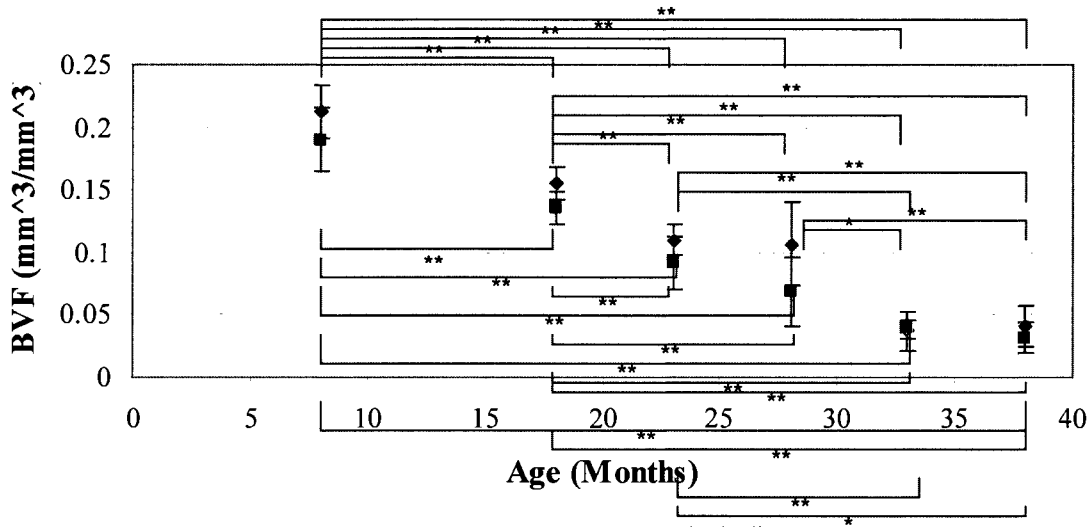
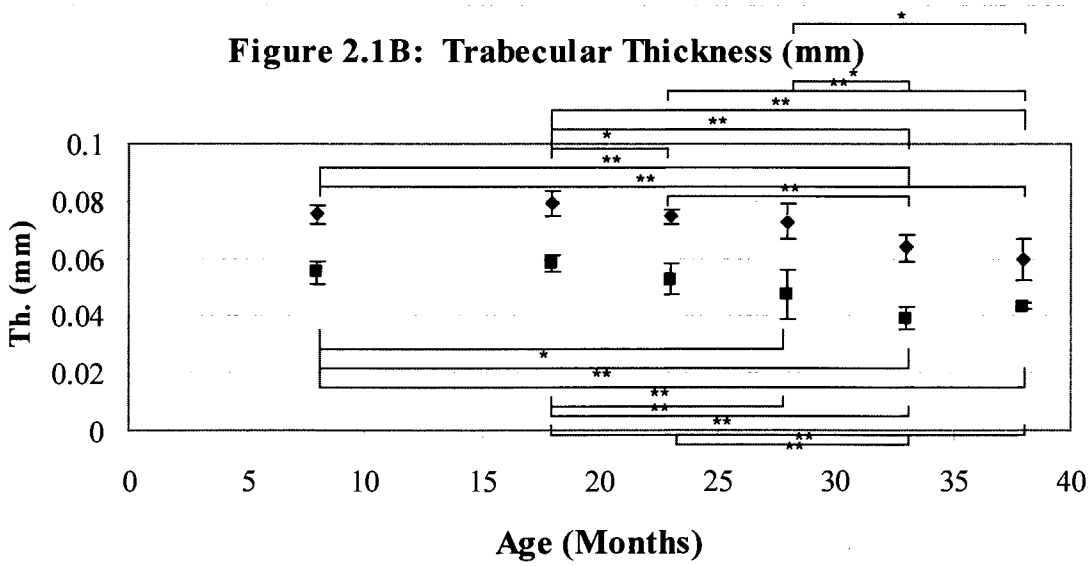


Figure 2.1B: Trabecular Thickness (mm)



(Adapted from Aging Rat Study Kreider, J. et al, 2006.) **(A)** The bone volume fraction of the tibia and femur decreases as a function of age. **(B)** The thickness of trabeculae decreases with advancing age in the femur and tibia.

Figure 2.1C: Trabecular Number

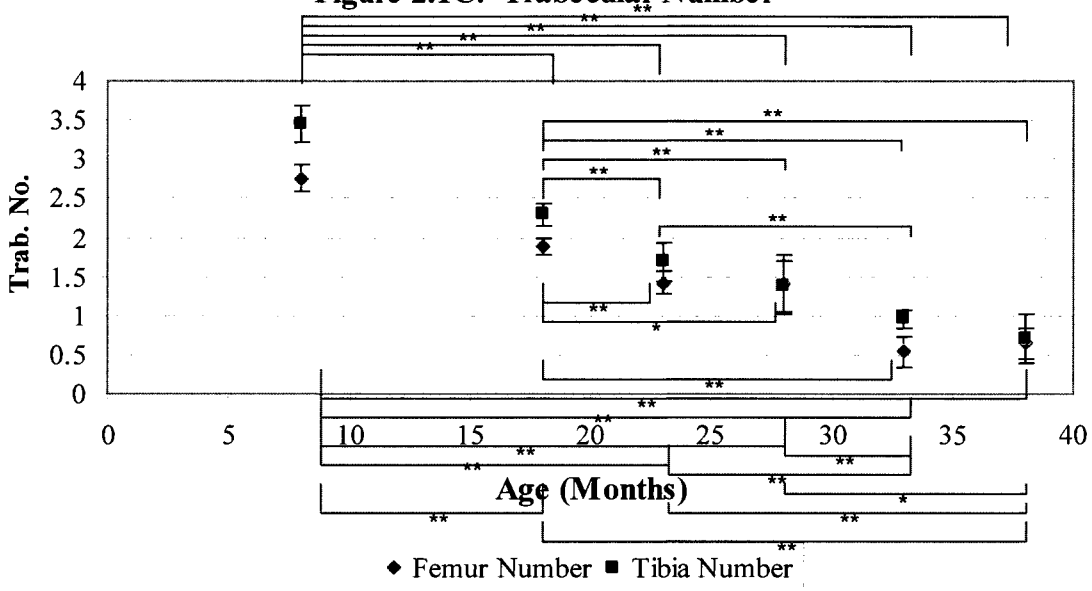
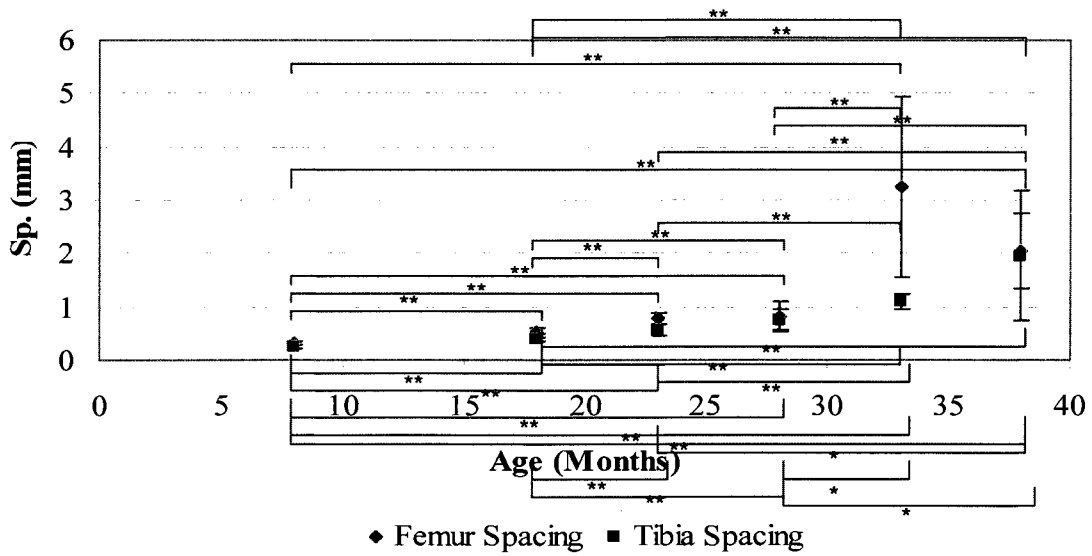


Figure 2.1D: Trabecular Spacing (mm)



(Adapted from Aging Rat Study Kreider, J. et al, 2006.) **(C)** The number of trabeculae in trabecular bone decreases with advancing age. **(D)** The spacing between trabeculae in the femur and tibia increases with advancing age.

Figure 2.1E: Cortical Bone Parameters

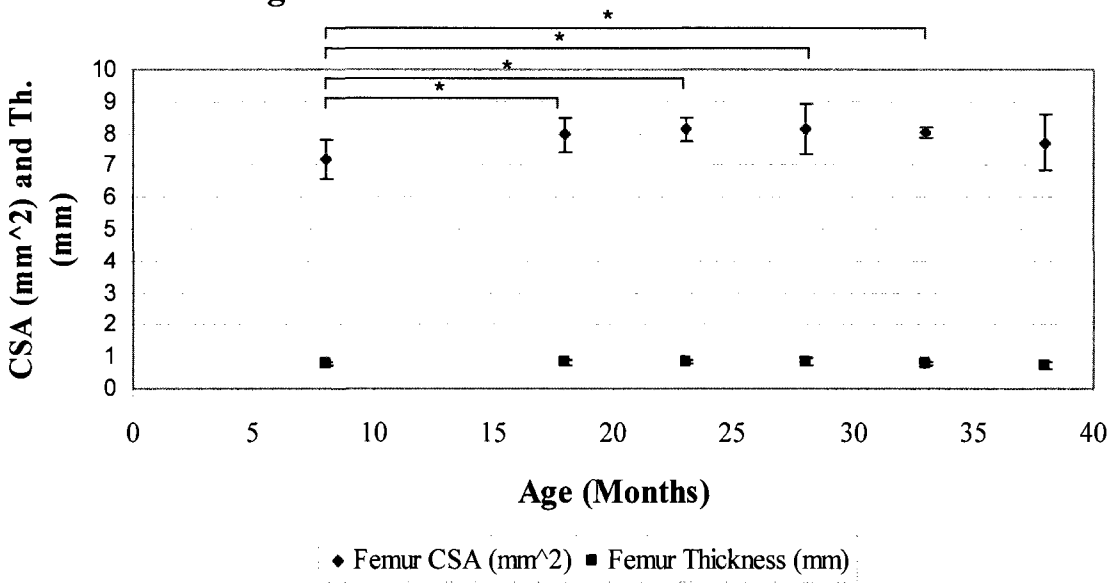
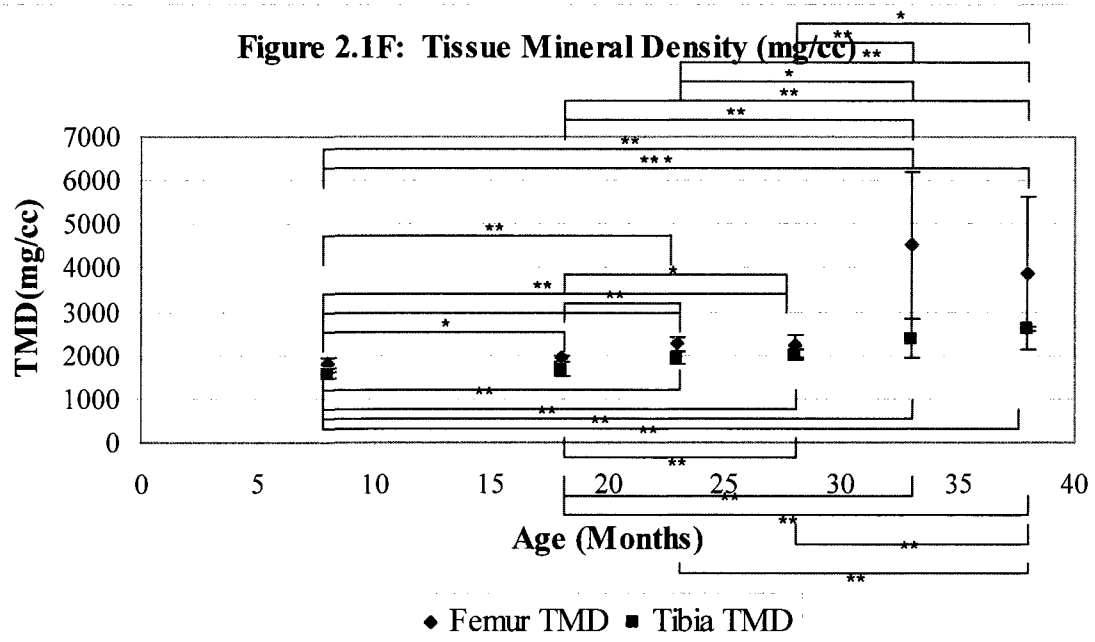


Figure 2.1F: Tissue Mineral Density (mg/cc)



(Adapted from Aging Rat Study Kreider, J. et al, 2006.) **(E)** Cortical thickness and cross sectional area increase and then decrease with advancing age. **(F)** The femur and tibia degree of mineralization increases with increasing age.

Figure 2.2A: Exposed Femora

Figure 2.2B: Cortical Defect

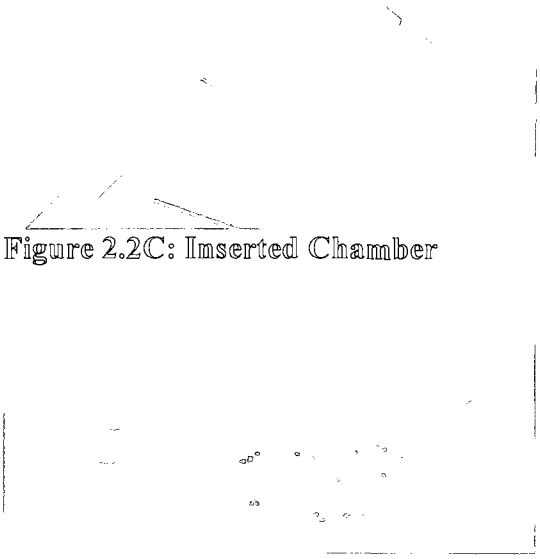


Figure 2.2C: Inserted Chamber

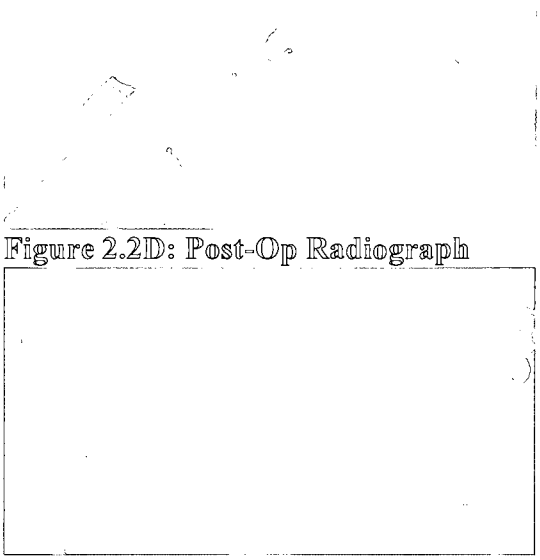
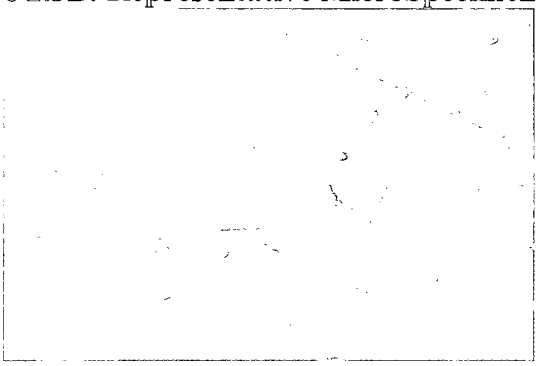


Figure 2.2D: Post-Op Radiograph

(A) Exposure of the femur (B) Cortical defect (C) Inserted chambers and internal plate (D) Representative radiograph *(A, B, and C Adapted from Hoffler, C.E. et al., 2004)*

Figure 2.3A: Regenerative Bone

Figure 2.3B: Representative MicroSpecimen



(A) Regenerative specimens in the chambers (B) Representative regenerative specimen *(Adapted from Hoffler, C.E. et al., 2004)*

Figure 2.4A: Mechanical Loading Apparatus

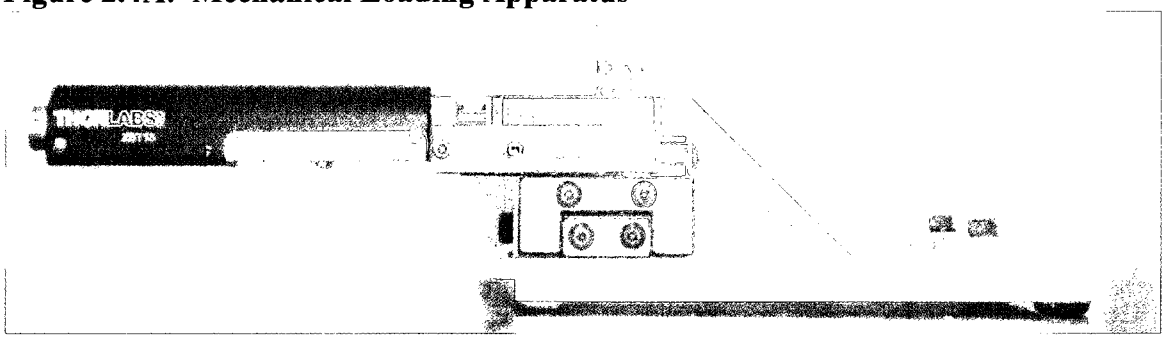


Figure 2.4 B: Three Point Bending

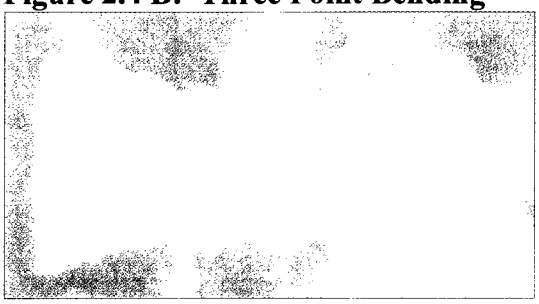
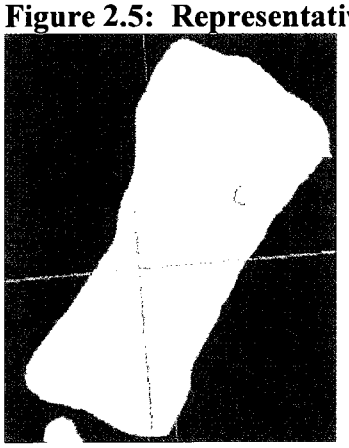


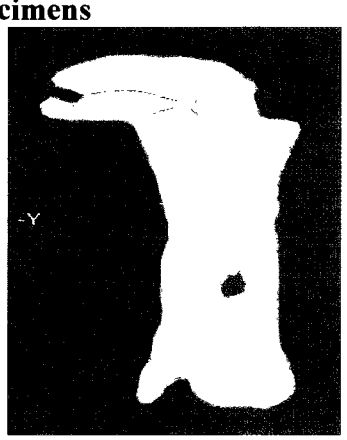
Figure 2.4C: Top View of Loading

(A) Side view of mechanical loading system attached to motor **(B)** Specimen under three point bending **(C)** Top view of mechanical loading system *(B and C Adapted from Hoffler, C.E.et al., 2004)*

Figure 2.5: Representative Isosurfaces of Regenerative Specimens



Old (n=6,6)



Young (n=3,7)

Regenerative bone tissue was successfully produced in chambers implanted into young and old animals.

Figure 2.6A: Percentage of Partially Filled Chambers per Animal

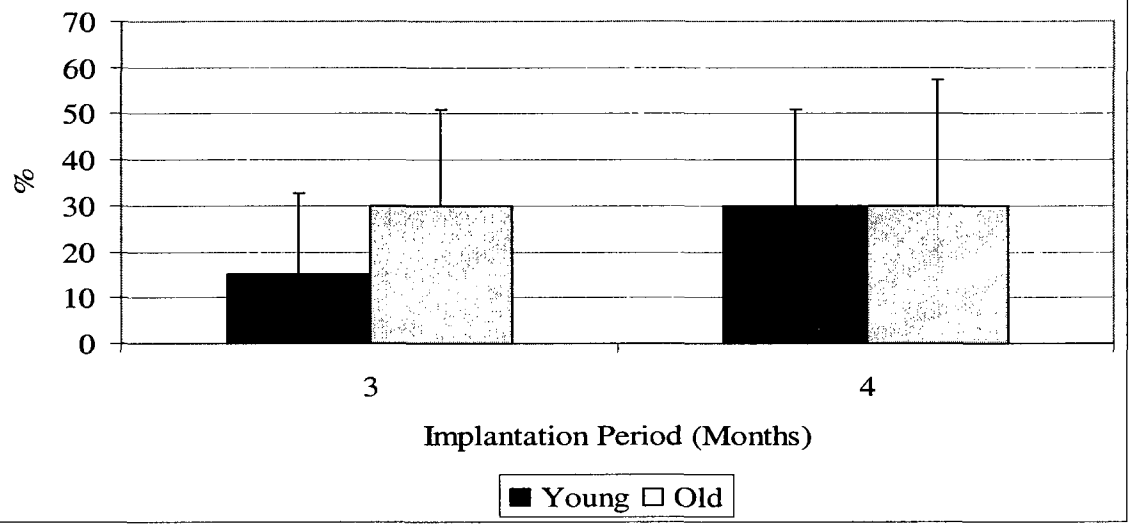
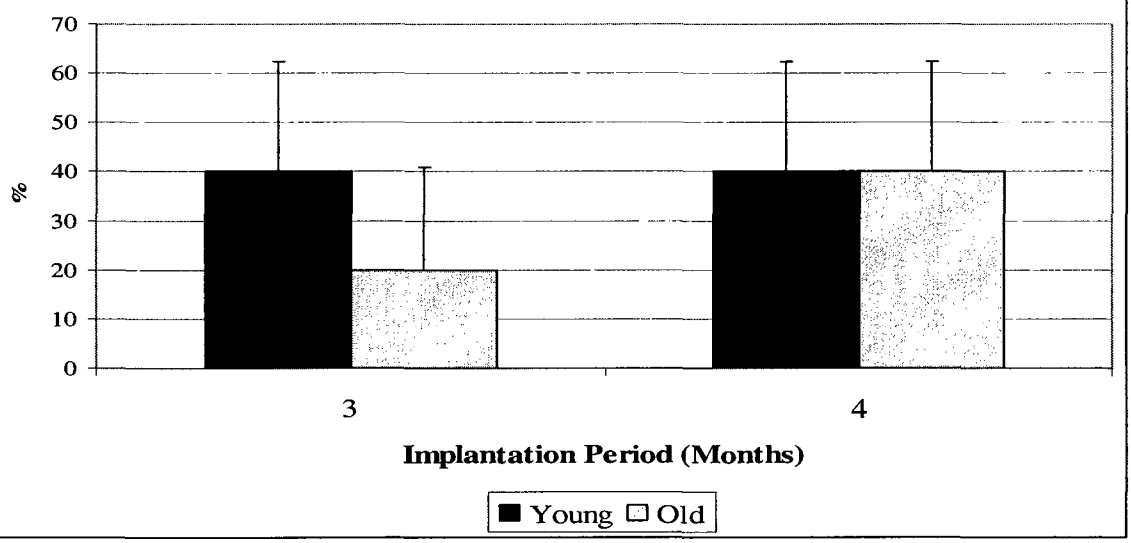
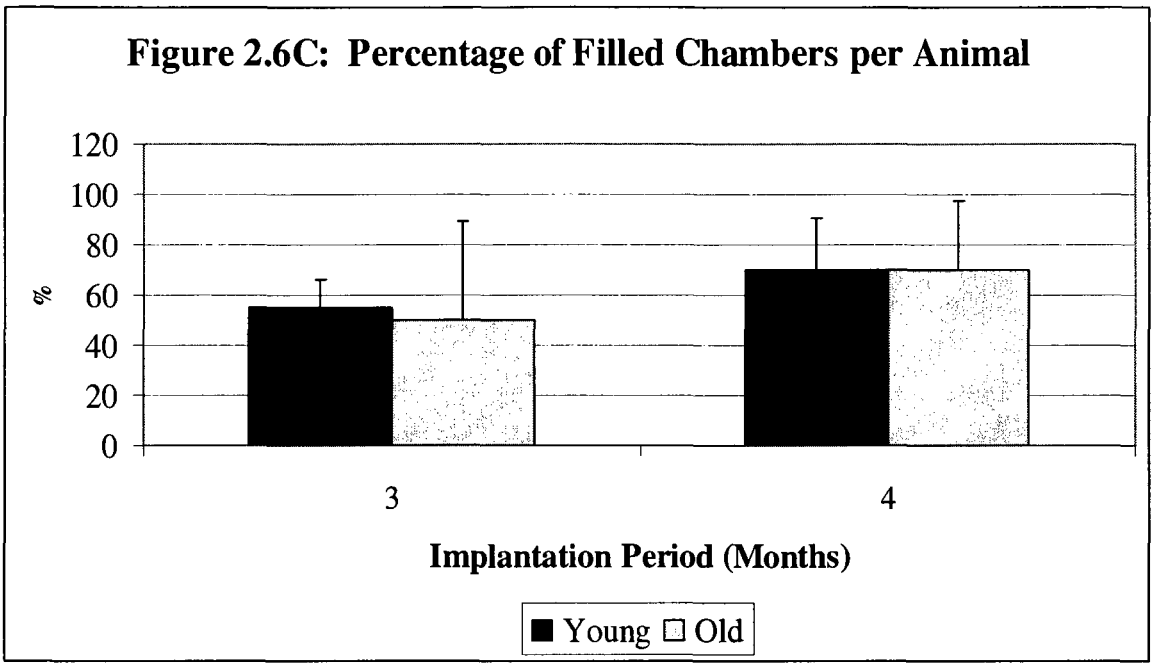


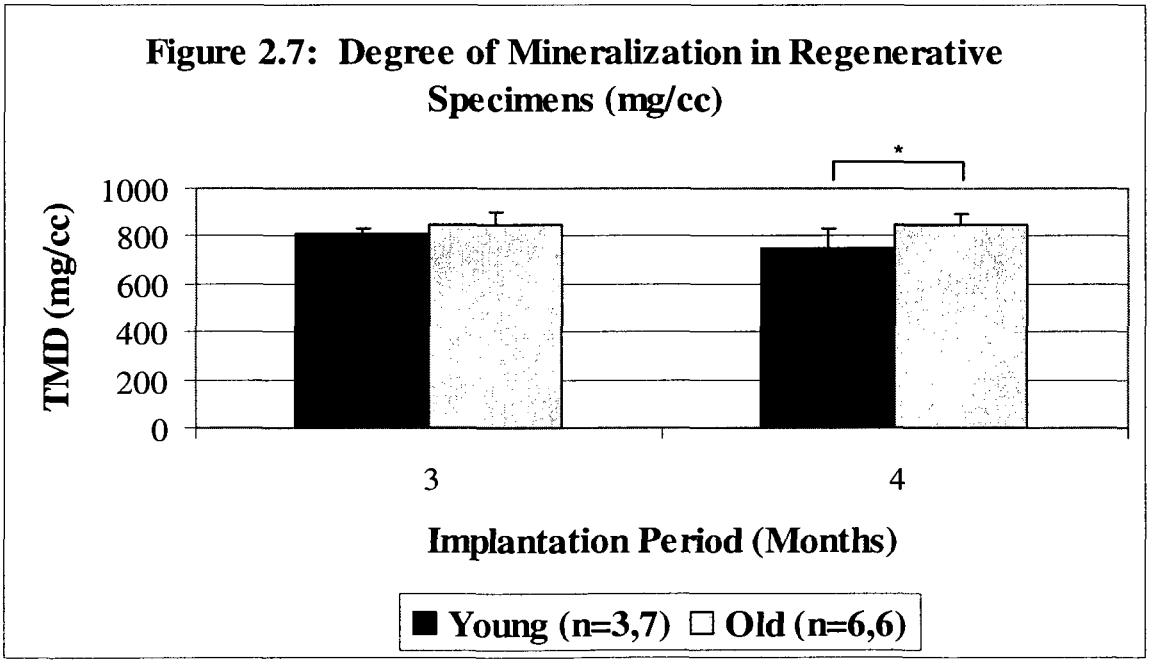
Figure 2.6B: Percentage of Completely Filled Chambers per Animal



(A) More partial regenerative specimens were produced in old than young animals after a 3 month implantation time period, however a similar number of partial regenerative specimens were produced in young and old animals after a 4 month implantation time period. **(B)** More complete regenerative specimens were produced in young than old animals after a 3 month implantation time period, however a similar number of complete regenerative specimens were produced in young and old animals after a 4 month implantation time period.



(C) The total number of regenerative specimens produced in young and old animals after a 4 month implantation time period was higher than the number produced after a 3 month implantation time period. Old animals produced less regenerative specimens than young after a 3 month implantation time period however a similar amount after a 4 month implantation time period.



Regenerative tissue mineral density was higher in specimens from old animals for both implantation periods. This difference was significant after a four month implantation.

Figure 2.8A: Histograms of Regenerative Specimens

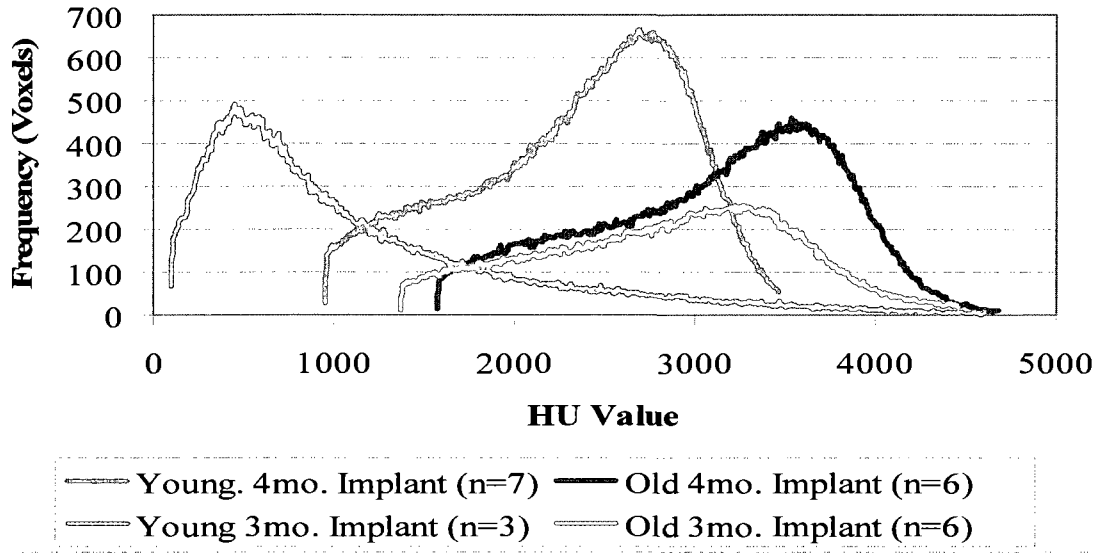
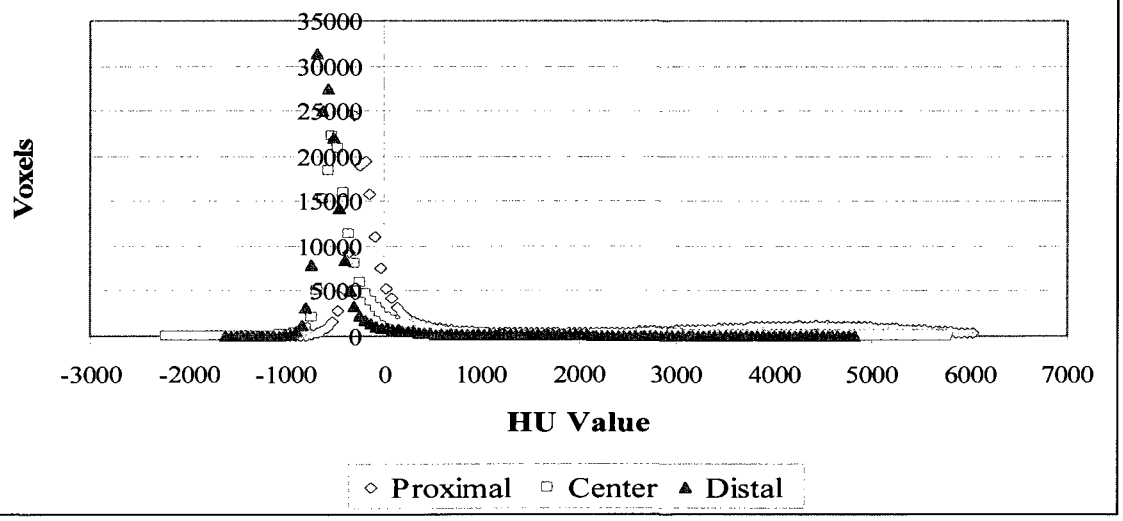


Figure 2.8B: Old Animals Regenerative Tissue Mineral Distribution (3 Month Implantation Period)



(A) There appears to be a shift increase in the proportion of highly mineralized bone voxels in regenerative specimens produced in old animals compared to those produced in young animals. **(B)** The mineral distribution of bone voxels appears uniform in regenerative specimens produced during a 3 month implantation time period in old animals.

Figure 2.8C: Old Animals Regenerative Tissue Mineral Distribution (4 Month Implantation Period)

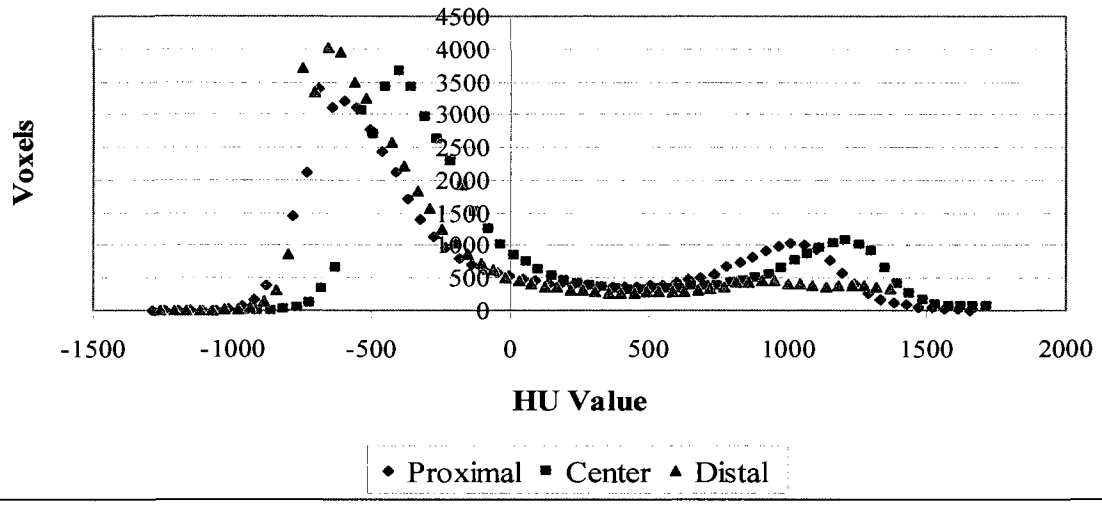
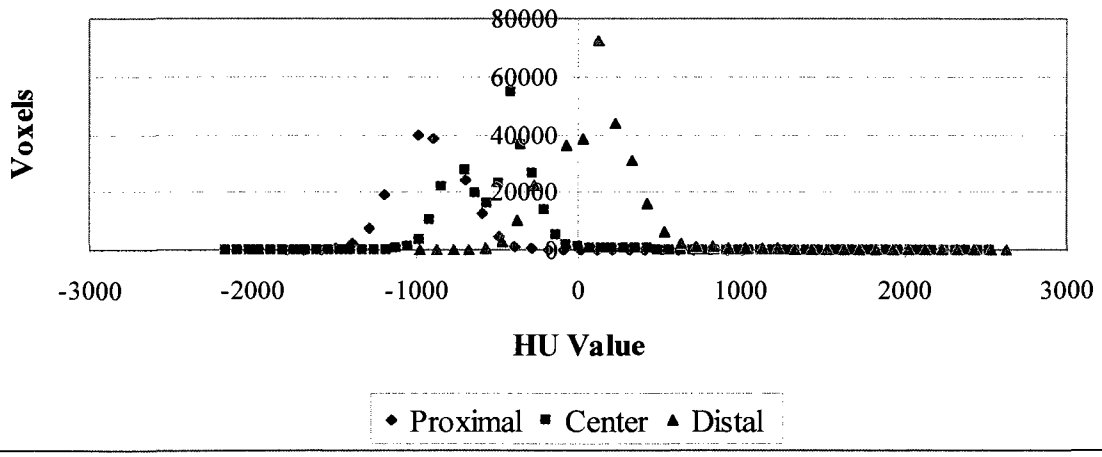
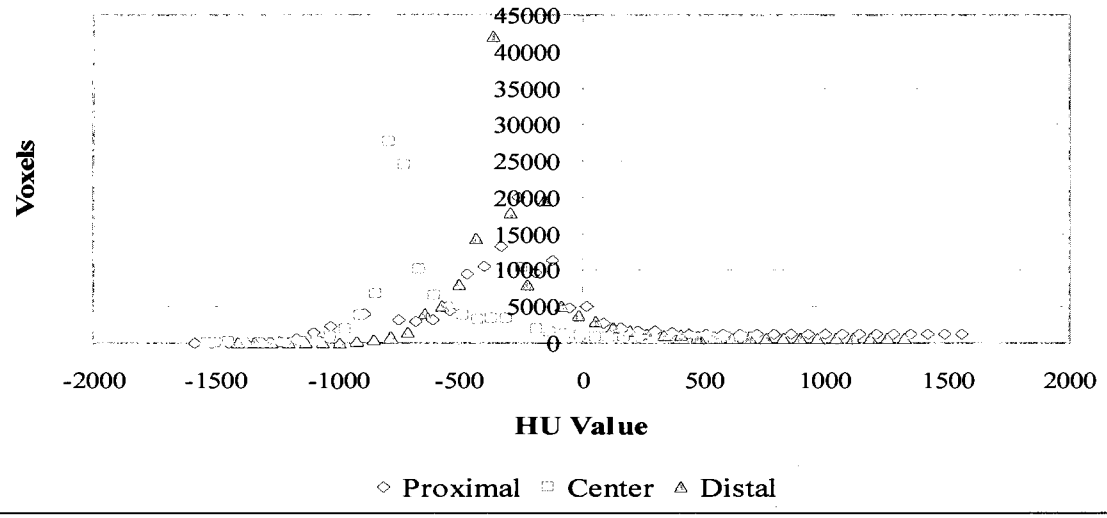


Figure 2.8D: Young Animals Regenerative Tissue Mineral Distribution (3 Month Implantation Time Period)



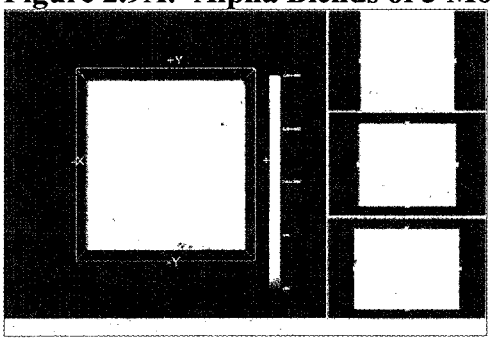
(C) The mineral distribution of bone voxels appears non uniform in regenerative specimens produced during a 4 month implantation time period in old animals. **(D)** The mineral distribution of bone voxels appears uniform in regenerative specimens produced during a 3 month implantation time period in young animals.

Figure 2.8E: Young Regenerative Tissue Mmineral Distribution (4 Month Implantation Period)

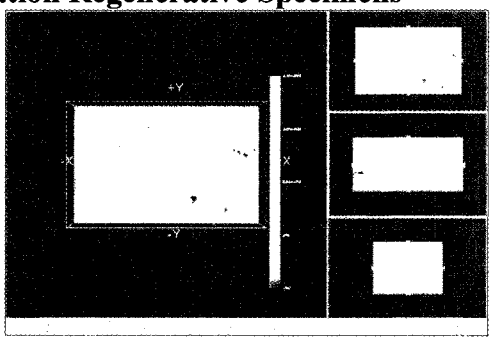


(E) The mineral distribution of bone voxels appears uniform in regenerative specimens produced during a 4 month implantation time period in young animals.

Figure 2.9A: Alpha Blends of 3 Month Implantation Regenerative Specimens



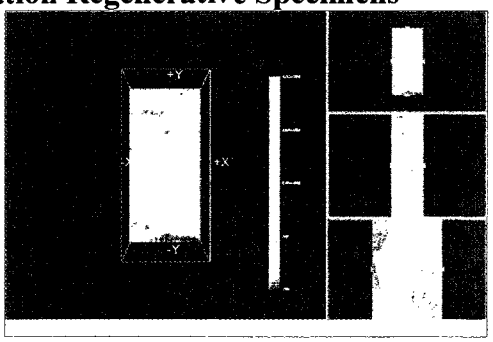
Old (n=6)



Young (n=3)

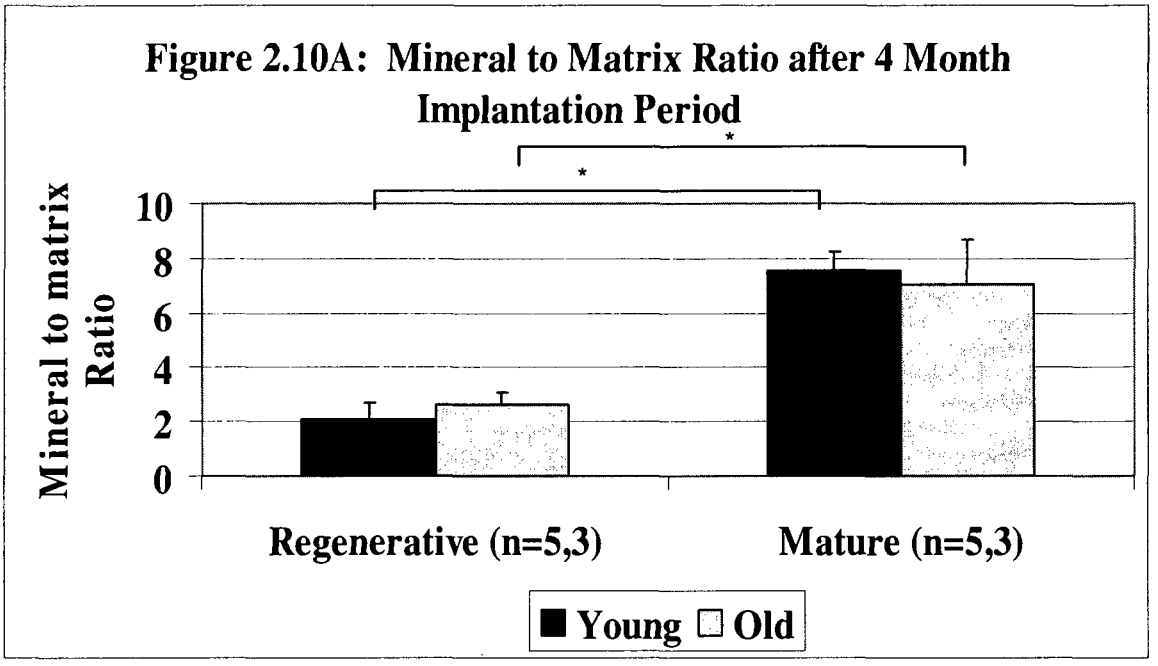
Figure 2.9B: Alpha Blends of 4 Month Implantation Regenerative Specimens

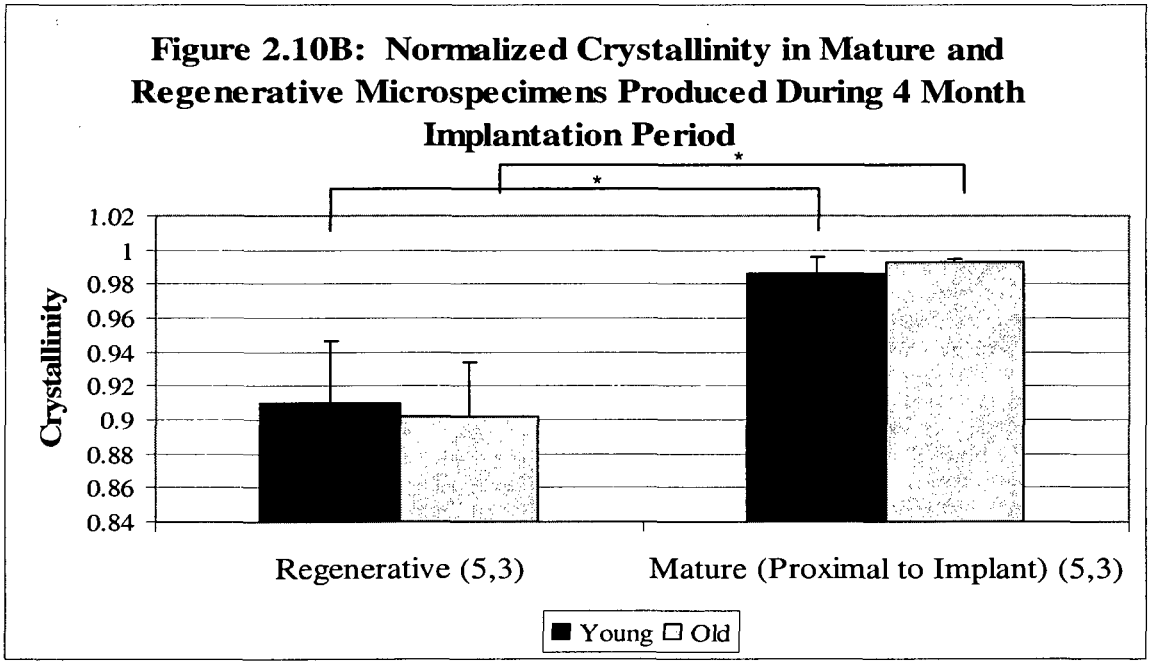
Old (n=6)



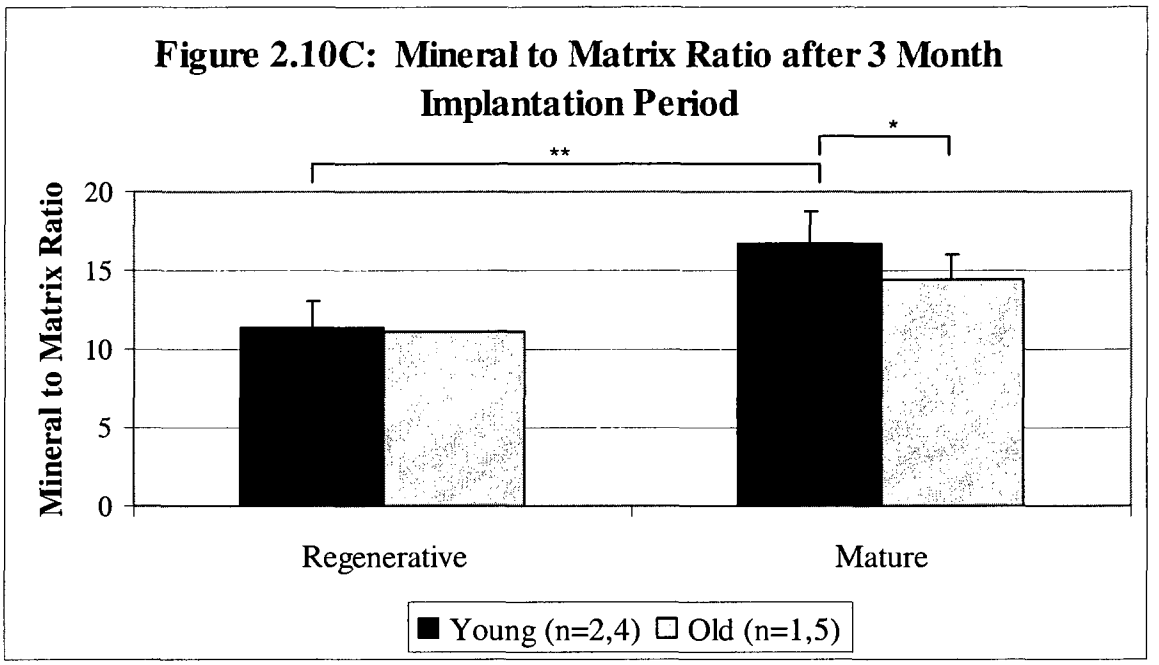
Young (n=7)

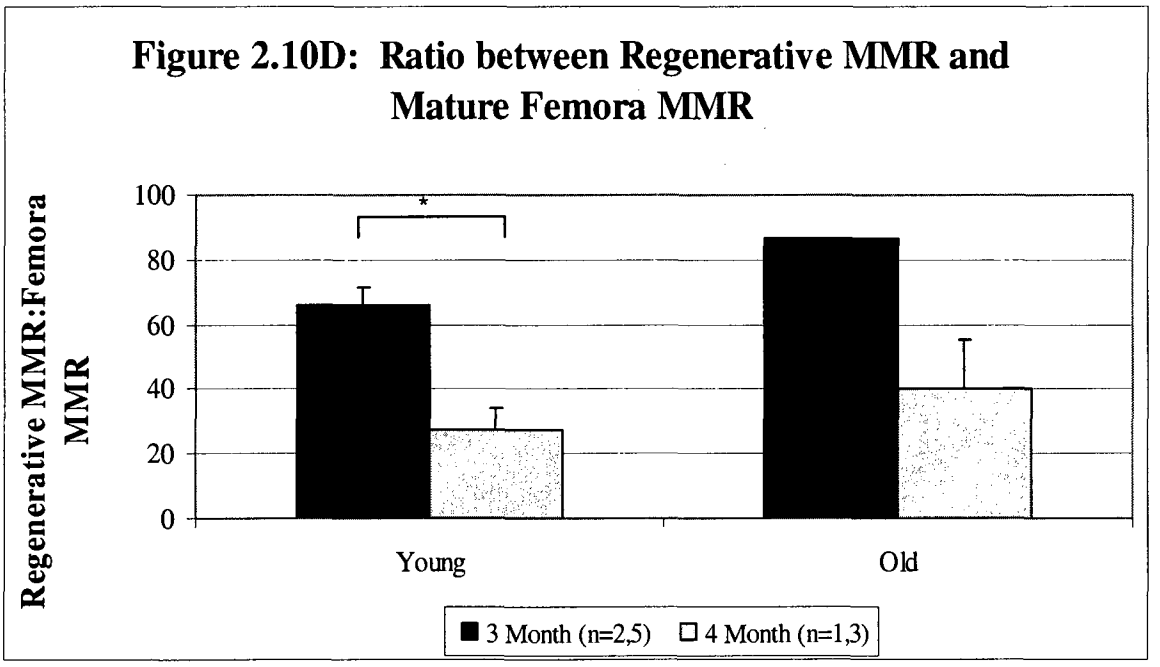
(A,B) There are more voxels at a higher HU in regenerative specimens produced in old animals. The distribution of similar voxels appears less uniform in regenerative specimens produced in old animals after a four month implantation period compared to those produced in the young animals for both implantation time periods.





(A) The MMR of mature specimens was significantly higher than the MMR of age respective regenerative specimens. There was no significant difference in MMR with age in either the regenerative or control mature bone tissue. **(B)** The crystallinity of control mature bone tissue was significantly higher than age respective regenerative tissue crystallinity. There was no significant difference with age in crystallinity of control mature or regenerative specimens.





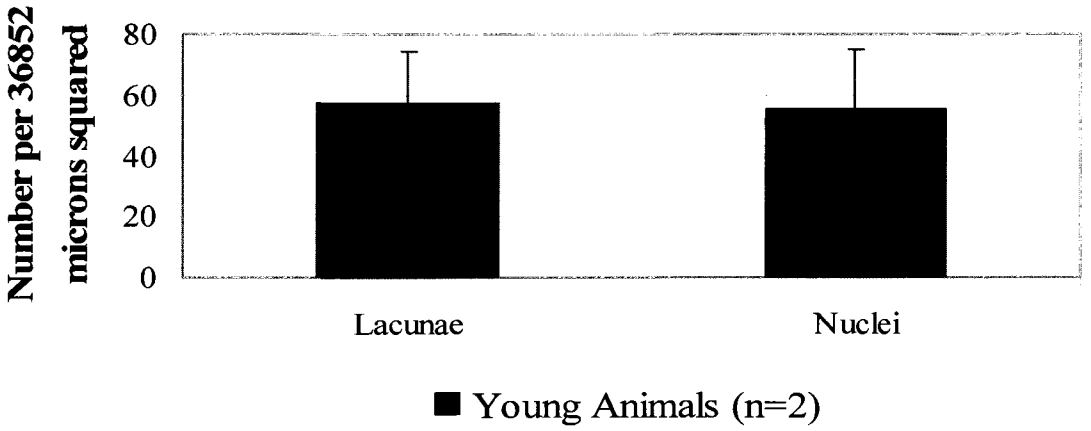
(C) The MMR of control mature specimens from young animals was significantly higher than the MMR of regenerative specimens produced in young animals. The MMR of control mature bone from young animals was significantly higher than the MMR of control mature bone from old animals. **(D)** The ratio between regenerative MMR and control mature femora MMR was higher in old animals compared to young for both implantation time periods. Regenerative MMR to control mature femora MMR was higher for both young and old animals after a 4 month implantation period when compared to a 3 month implantation time period.

Figure 2.11: Regenerative Bone Histology

Young **Old**

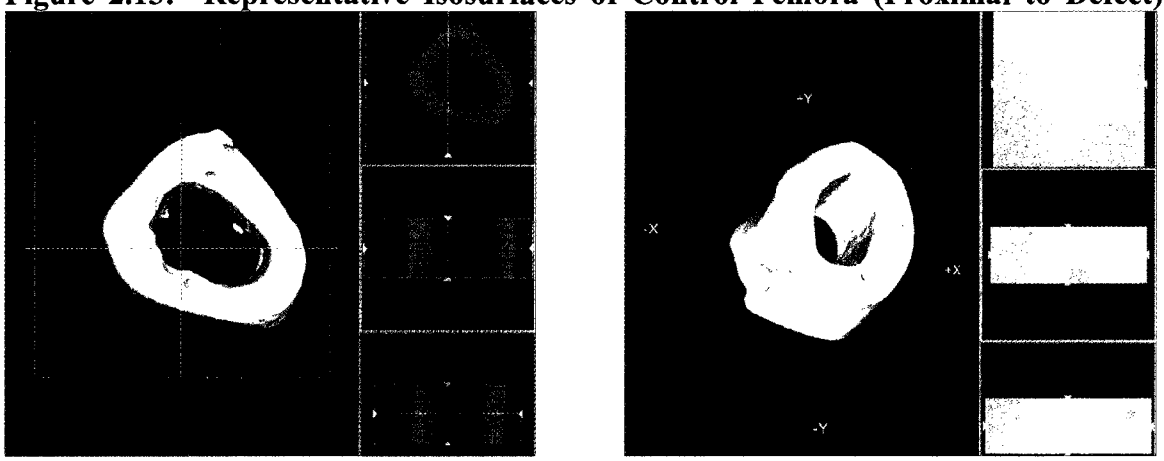
Osteocytes within lacunae are clearly visible in regenerative specimens produced in young animals. The regenerative tissue produced in old animals appears fibrous.

Figure 2.12: Number of Lacunae and Nuclei in Regenerative Specimens from Young Animals



There was no significant difference between the number of nuclei and the number of lacunae in regenerative specimens produced in young animals.

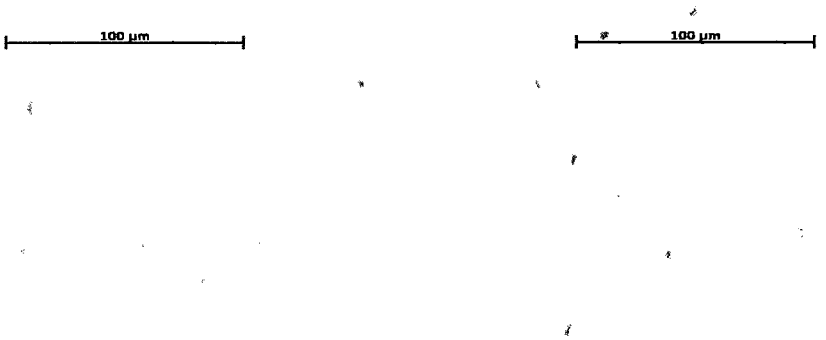
Figure 2.13: Representative Isosurfaces of Control Femora (Proximal to Defect)



Young (n=5) **Old (n=5)**

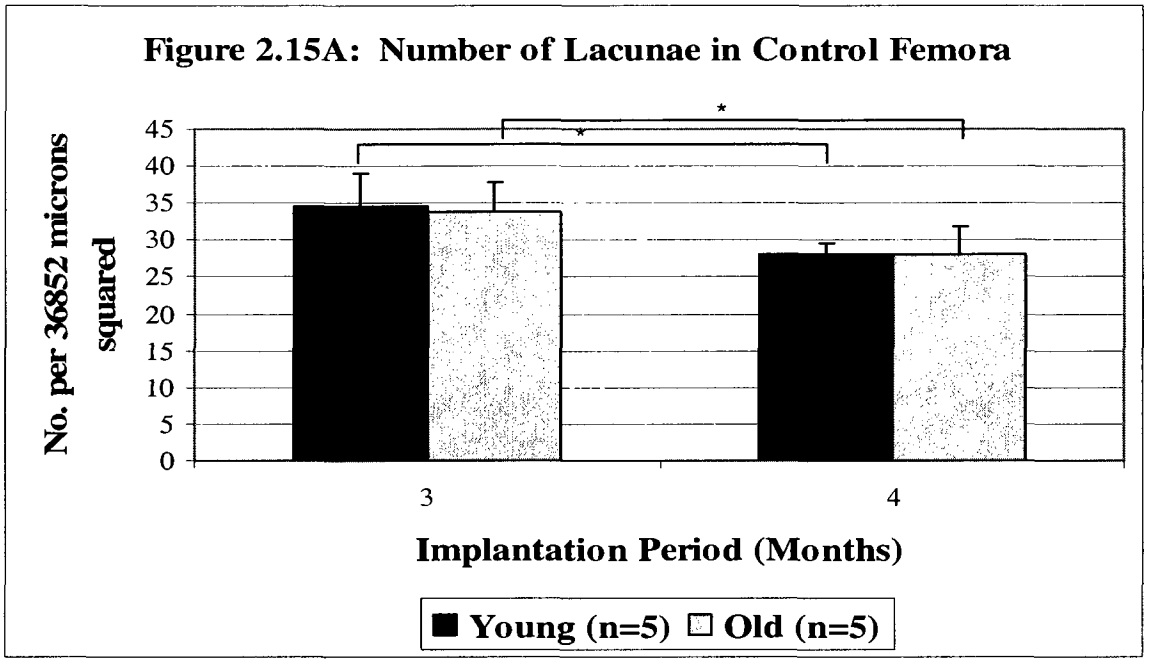
The region of interest proximal to the defect in mature control femora bone.

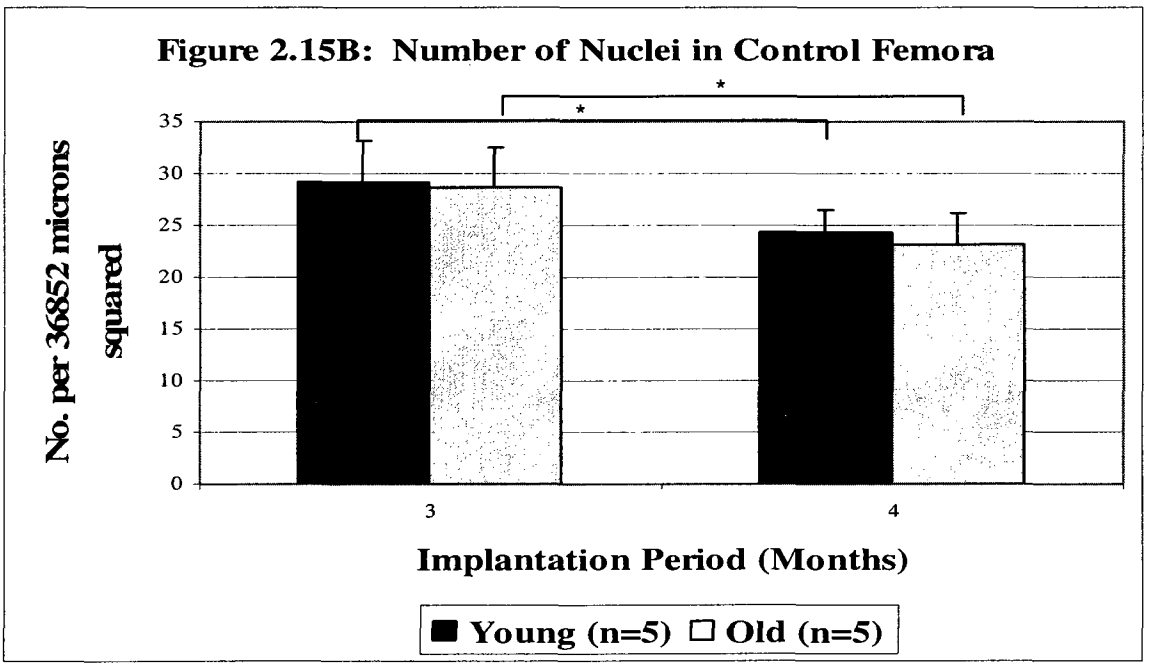
Figure 2.14: Control Bone Histology



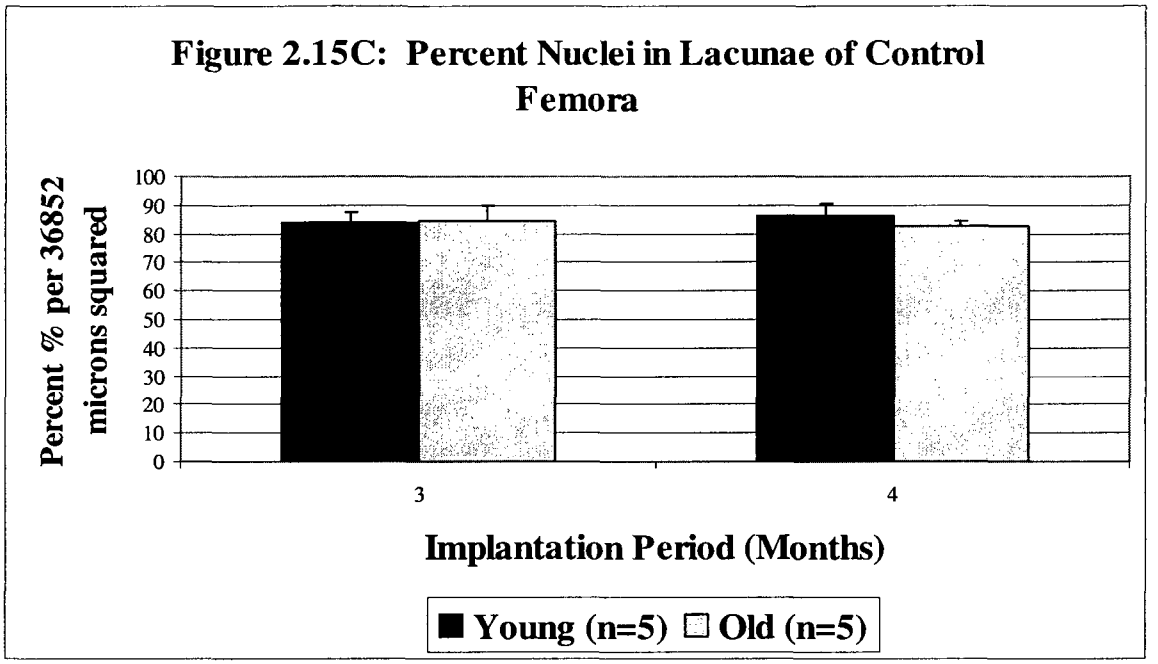
Young (n=5) **Old (n=5)**

The cellular characteristics of control mature femoral bone tissue appears be similar with age.

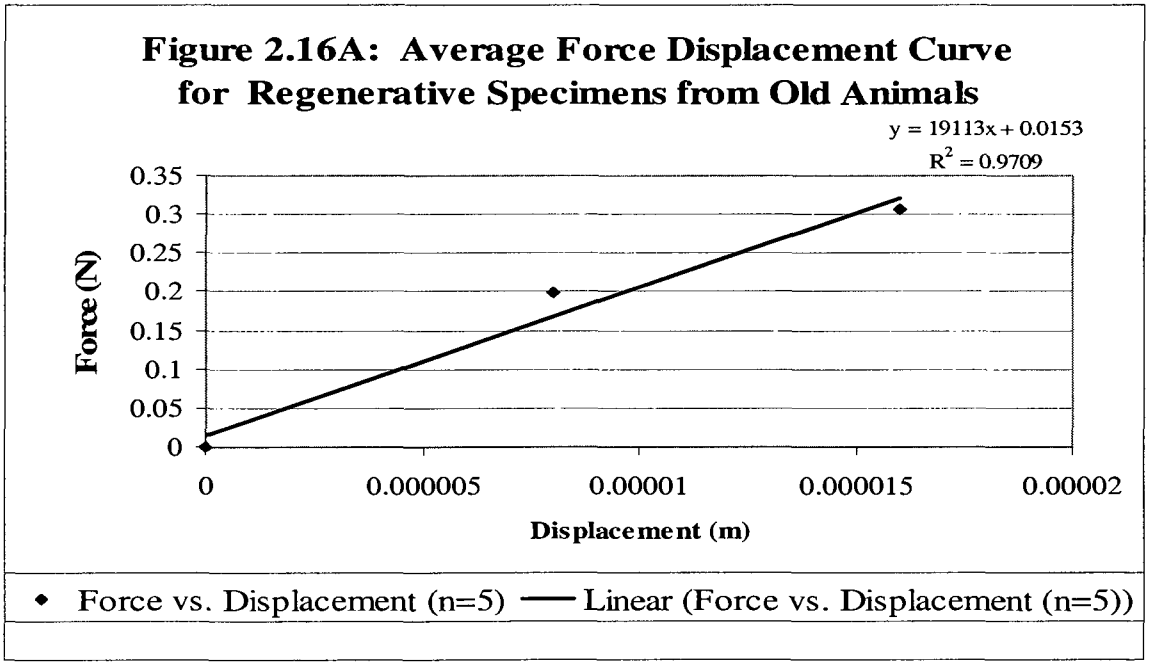




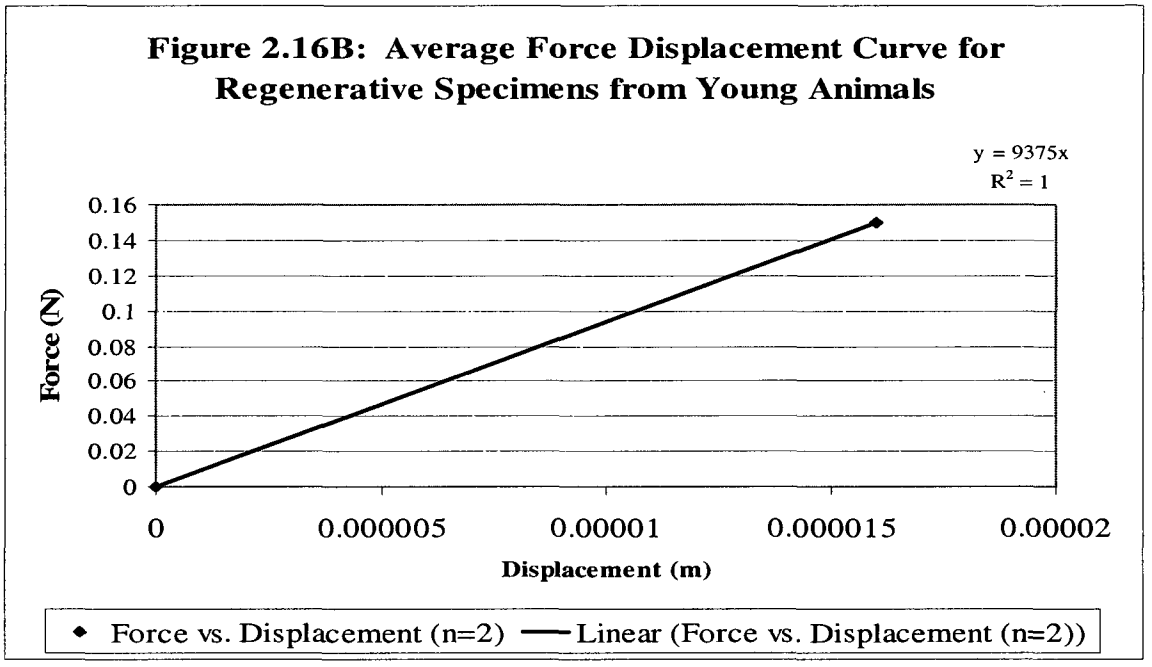
(A) There is no significant difference with age for either implantation period in the number of lacunae in control femora. However there is a significant decrease in number of nuclei with increasing implantation time period for both age groups.
(B) There is no significant difference with age for either implantation period in the number of nuclei in control femora. However there is a significant decrease in number of nuclei with increasing implantation time period for both age groups.



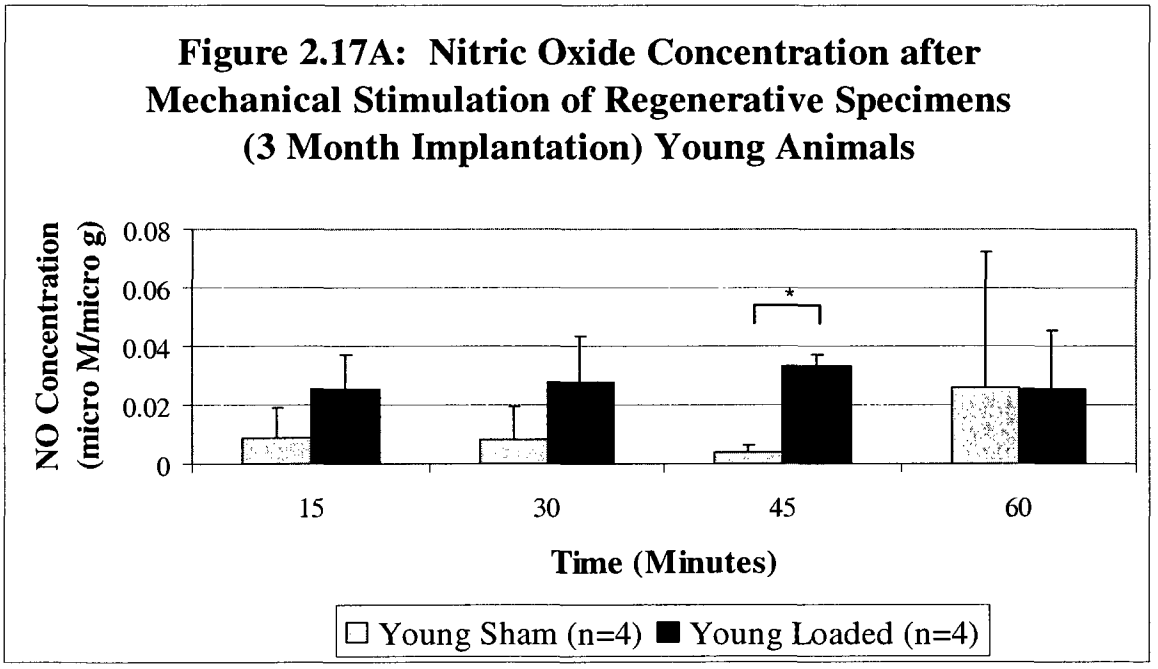
(C) There is no significant difference with age for either implantation period in percentage of occupied lacunae in control femora.



(A) The stiffness of old regenerative specimens was 19113N/m.



(B) The stiffness for young regenerative specimens (adapted from data by Hoffler, C. E. et al.) was 9375N/m and is less than that for old regenerative specimens.



(A) NO increased after all time points of mechanical loading for regenerative specimens from young animals produced in three months.

Figure 2.17B: Nitric Oxide Concentration after Mechanical Stimulation of Regenerative Specimens (3 Month Implantation) Old Animals

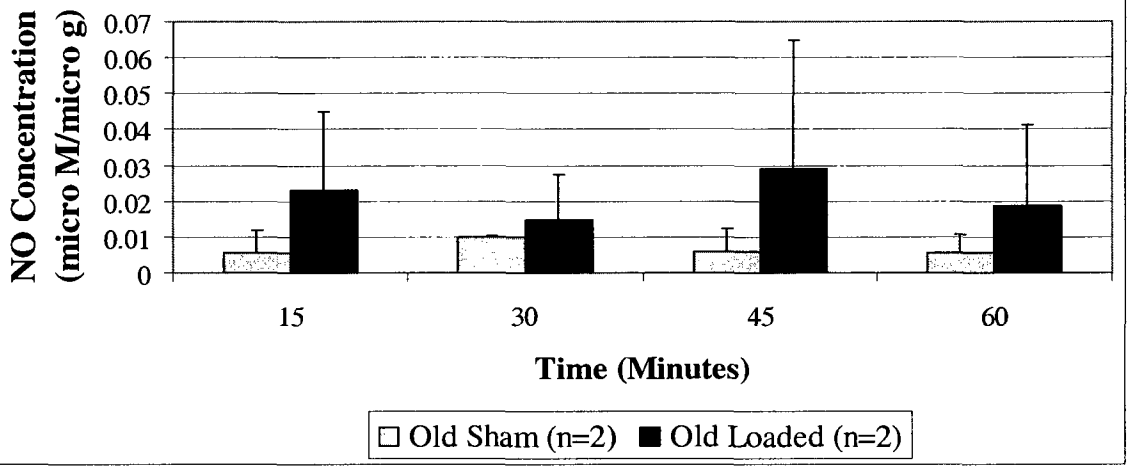
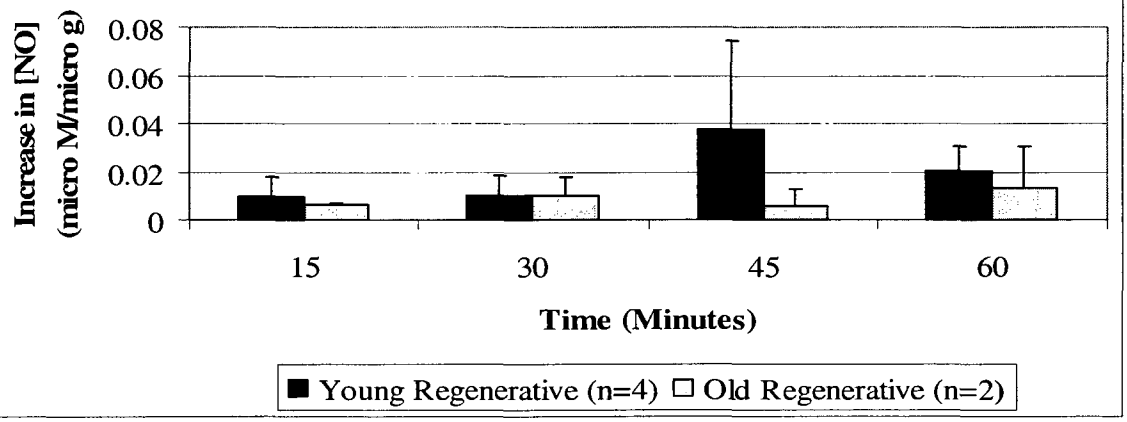


Figure 2.17C: Average Change per Animal in Nitric Oxide Concentration after Mechanical Stimulation (Three Month Implantation)



(B) NO increased after all time points of mechanical loading for regenerative specimens from old animals produced in three months. **(C)** The average change in NO was higher in specimens produced in young animals during a three month implantation time period for all loading time points except 30 minutes.

Figure 2.17D: Nitric Oxide Concentration after Mechanical Stimulation of Regenerative Specimens (4 Month Implantation) Young Animals

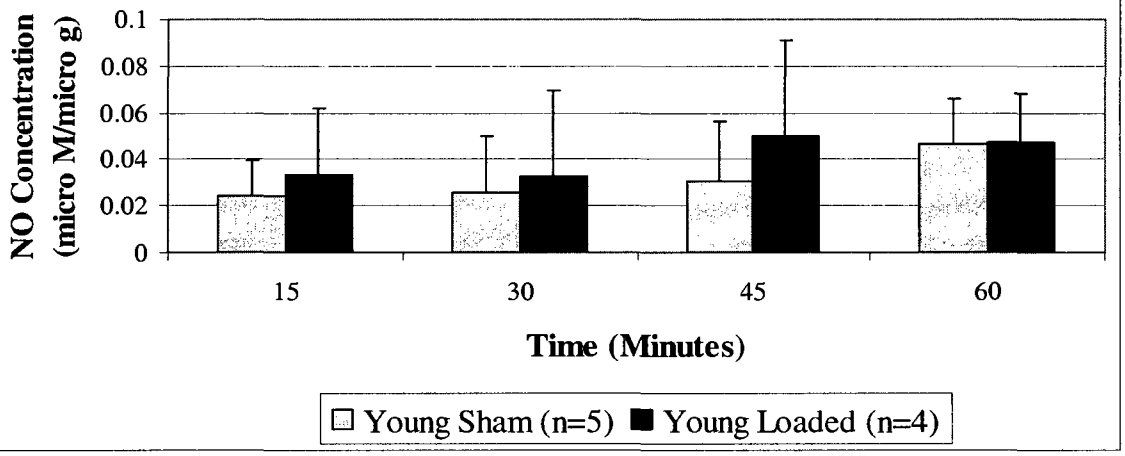
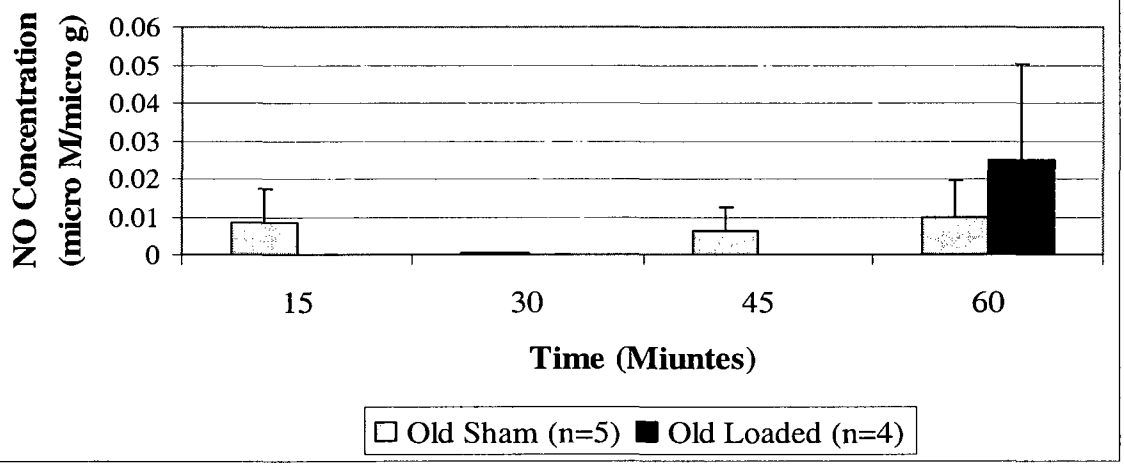
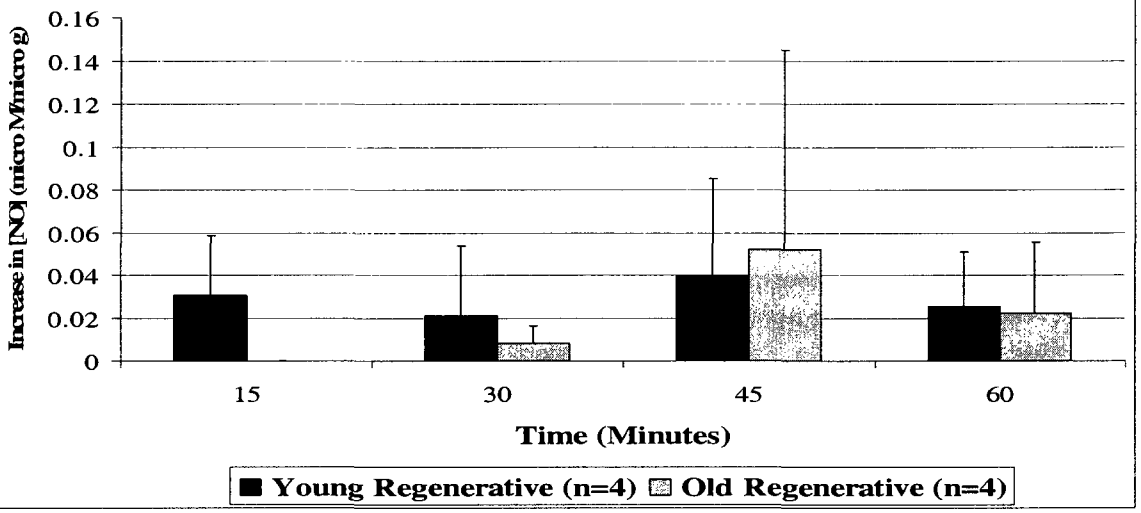


Figure 2.17E: Nitric Oxide Concentration after Mechanical Stimulation of Regenerative Specimens (4 Month Implantation) Old Animals



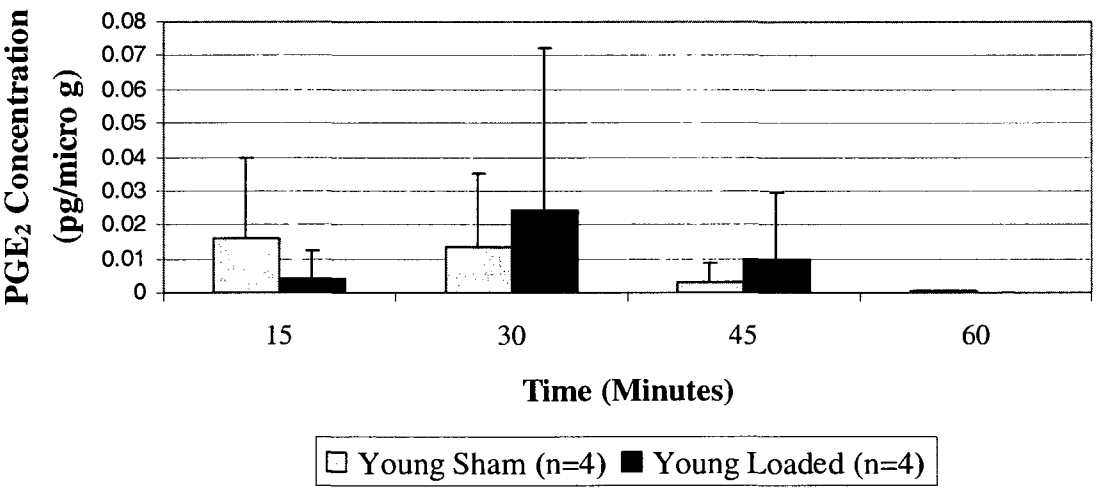
(D) NO increased after all time points of mechanical loading for regenerative specimens from young animals produced in four months. **(E)** NO increased after 60 minutes of mechanical loading for regenerative specimens from old animals produced in four months.

Figure 2.17F: Average Increase in Nitric Oxide per Animal after Mechanical Stimulation (Four Month Implantation)

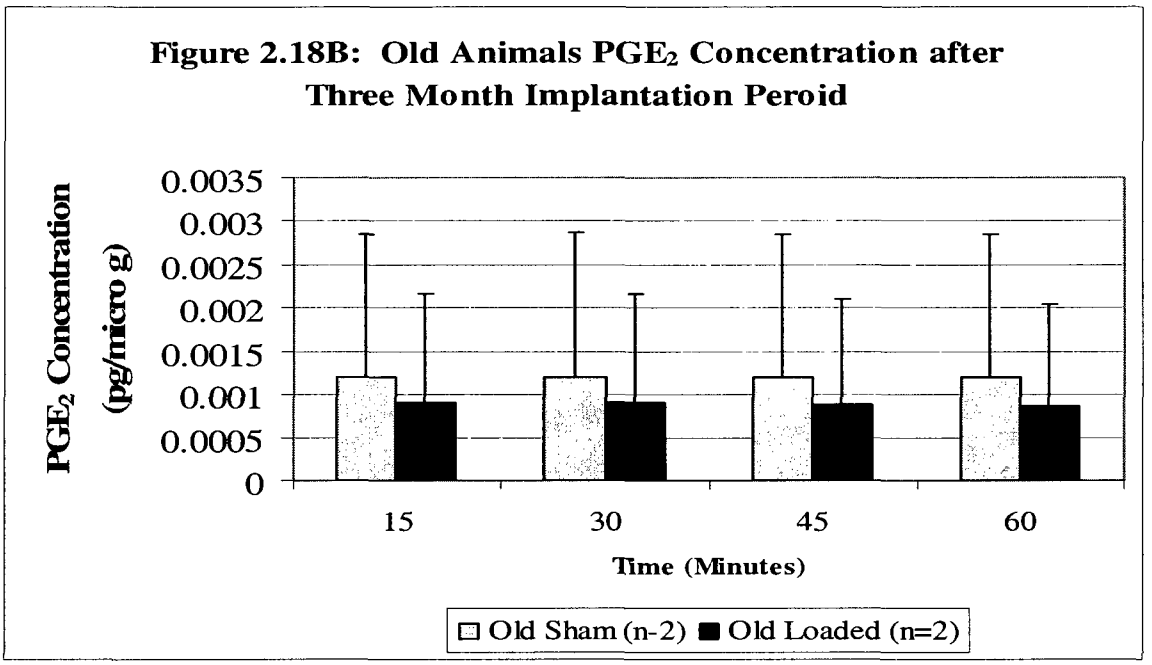


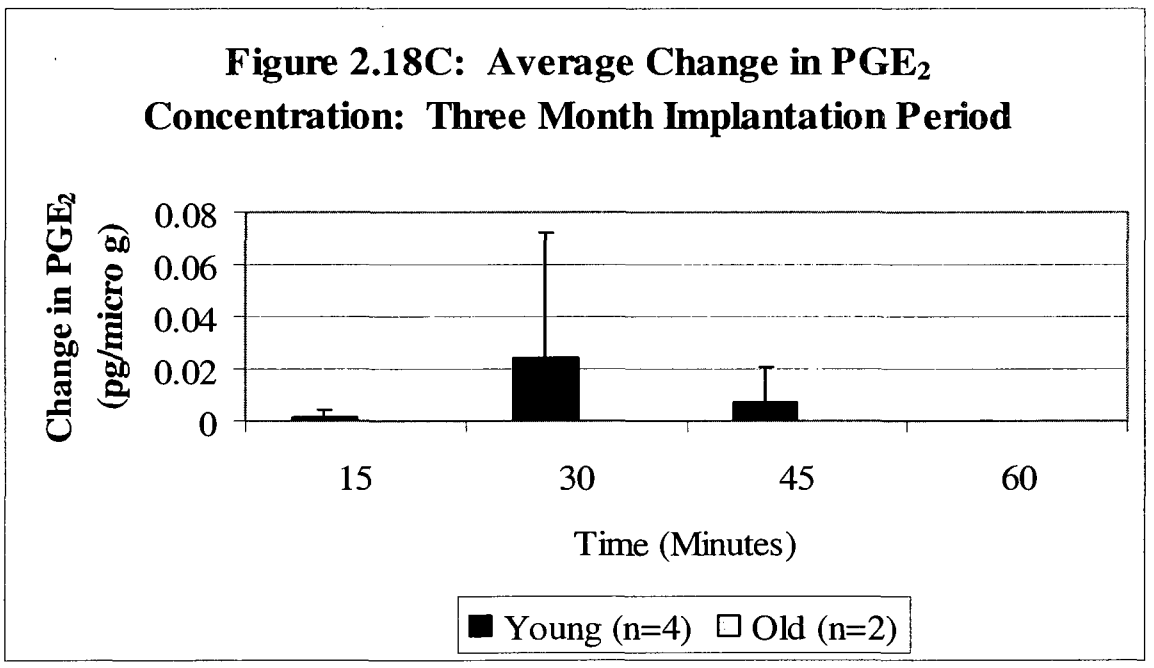
(F) The average change in NO was higher in specimens produced in young animals during a four month implantation time period for all loading time points except 45 minutes.

Figure 2.18A: Young Animals PGE_2 Concentration after Three Month Implantation Period



(A) PGE_2 increased after 30 and 45 minutes of mechanical loading for regenerative specimens from young animals produced in three months.





(B) PGE_2 did not increase after any time points of mechanical loading for regenerative specimens from old animals produced in three months. **(C)** The average change in PGE_2 was higher in specimens produced in young animals during a three month implantation time period for all loading time points except 60 minutes.

Figure 2.18D: PGE_2 Concentration after Mechanical Stimulation of Regenerative Specimens (4 Month Implantation) Young Animals

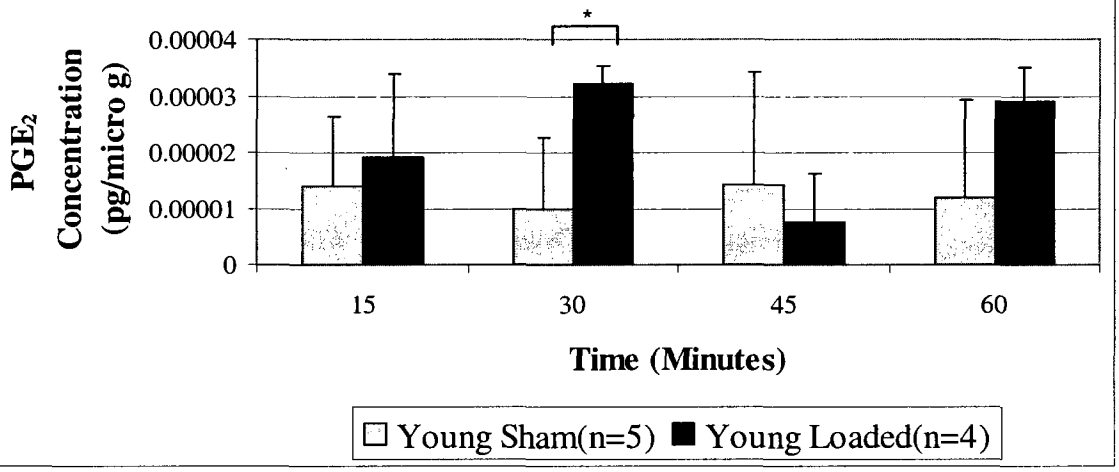
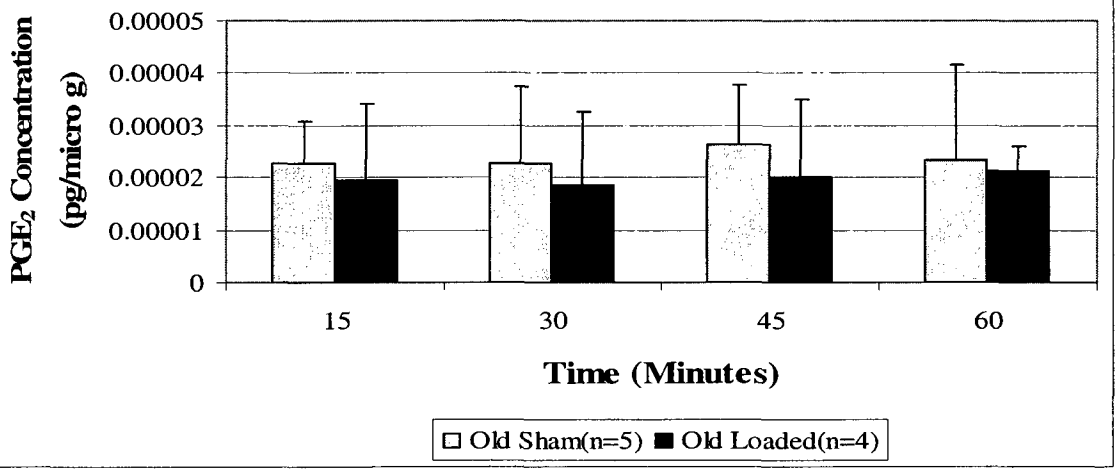
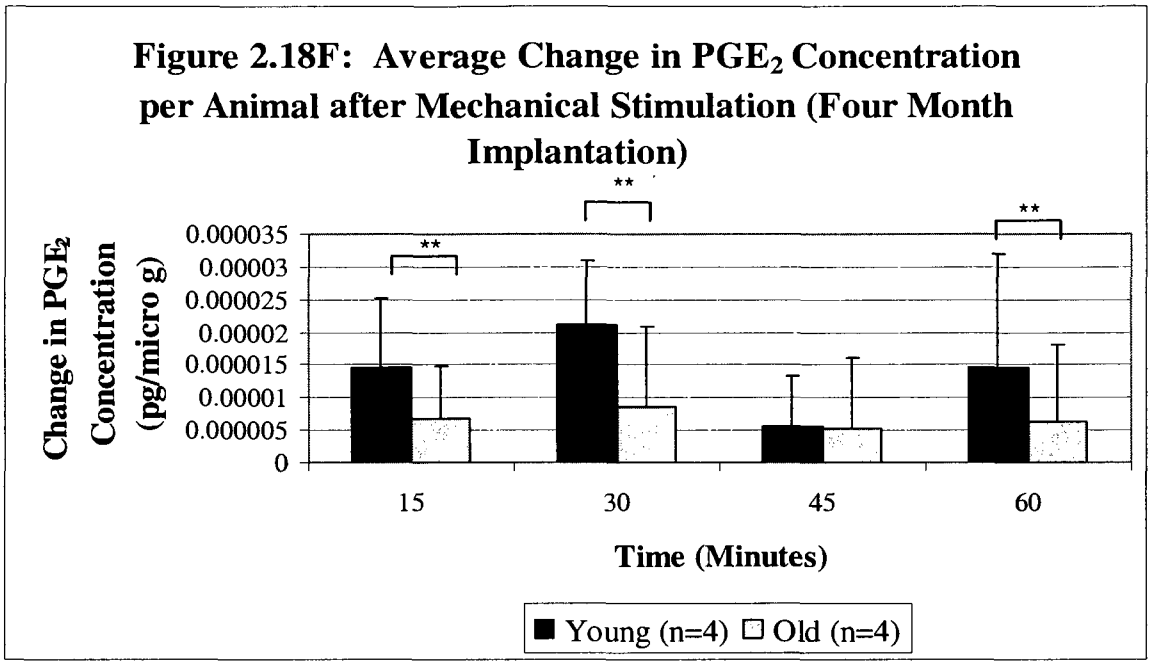


Figure 2.18E: PGE_2 Concentration after Mechanical Stimulation of Regenerative Specimens (4 Month Implantation) Old Animals



(D) PGE_2 increased after fifteen, thirty, and sixty minutes of mechanical loading for regenerative specimens from young animals produced in four months. **(E)** PGE_2 did not increase after any time points of loading for regenerative specimens from old animals produced in four months.



(F) The average change in PGE_2 was higher in specimens produced in young animals during a four month implantation time period for all loading time points except 45 minutes.

Figure 2.19A Connexin 43 Western Blot

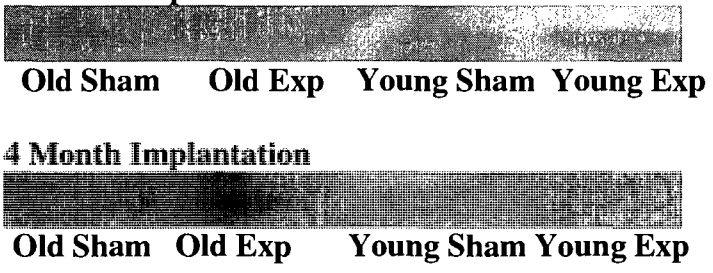
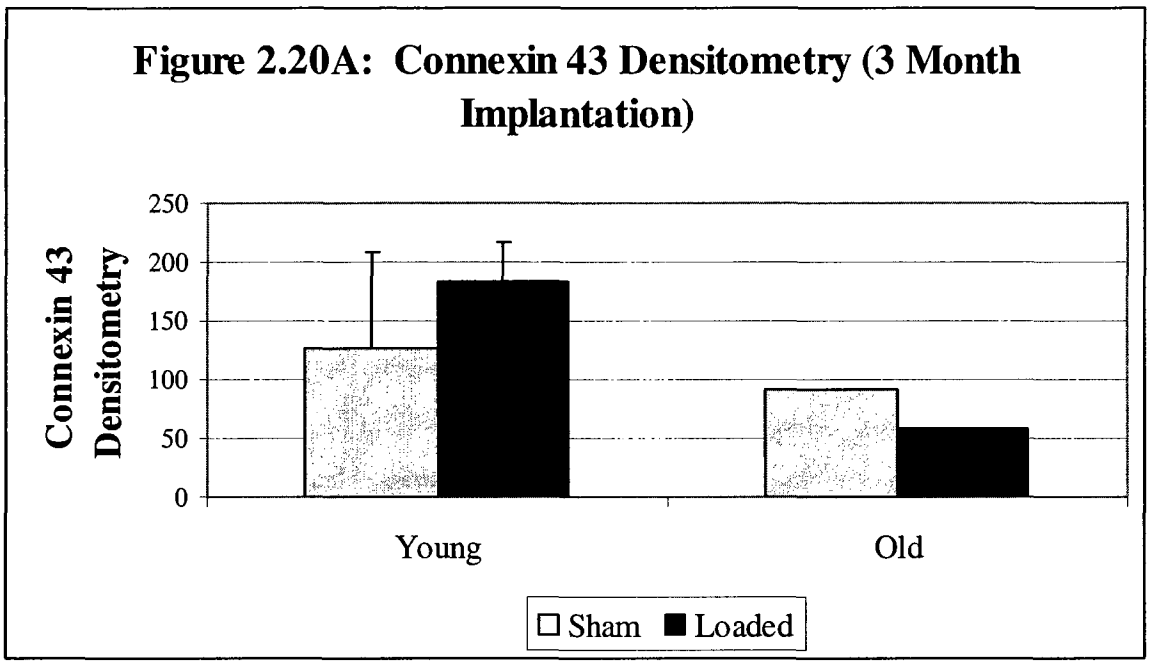
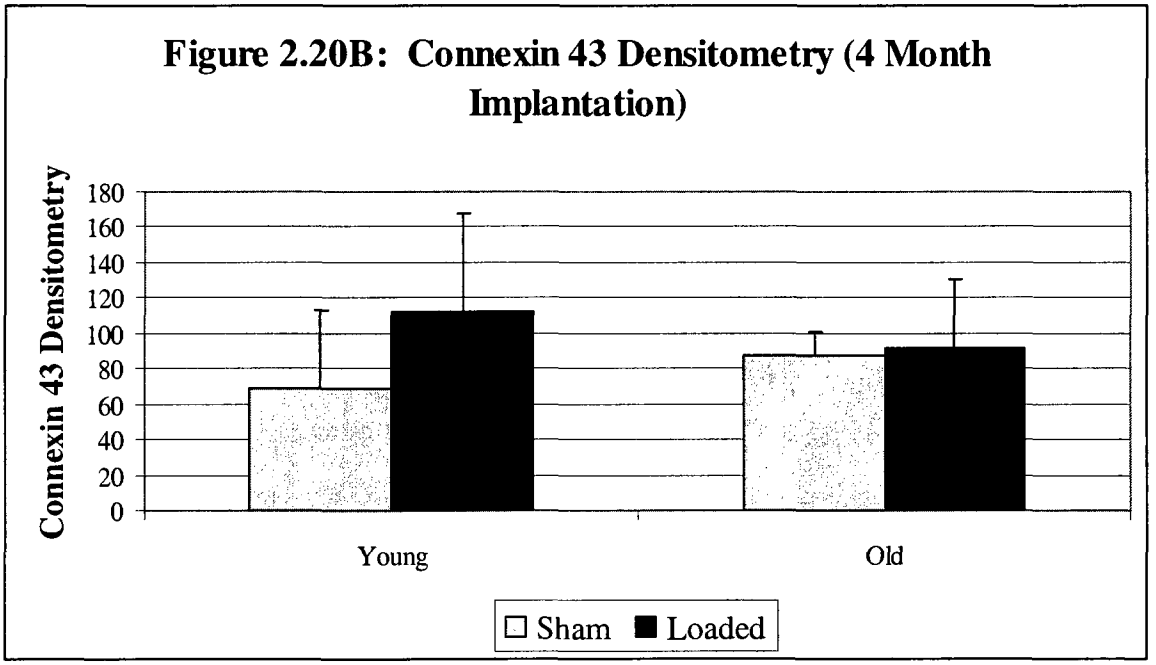


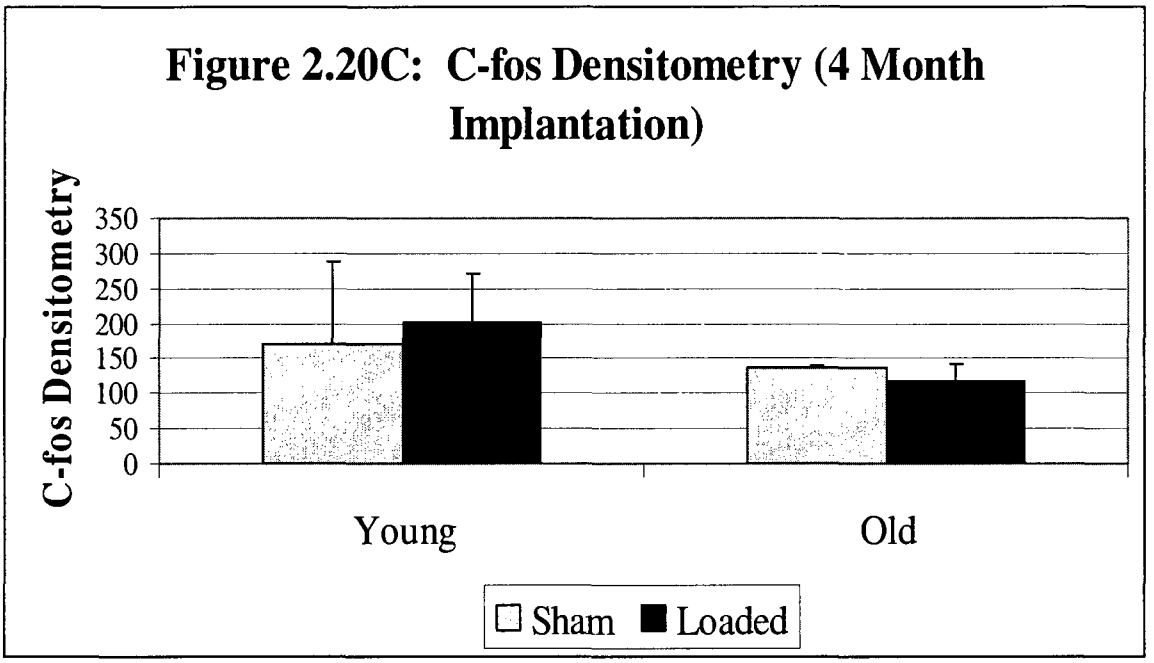
Figure 2.19B: C-fos Western Blot 4 Month Implantation

(A) Connexin 43 protein was detected in regenerative specimens from young and old animals produced during a three and four month implantation time period. This increase was higher in specimens from young animals. **(B)** C-fos protein was detected in regenerative specimens from young and old animals produced during a four month implantation time period and a slight increase with load was observed in specimens from young animals.

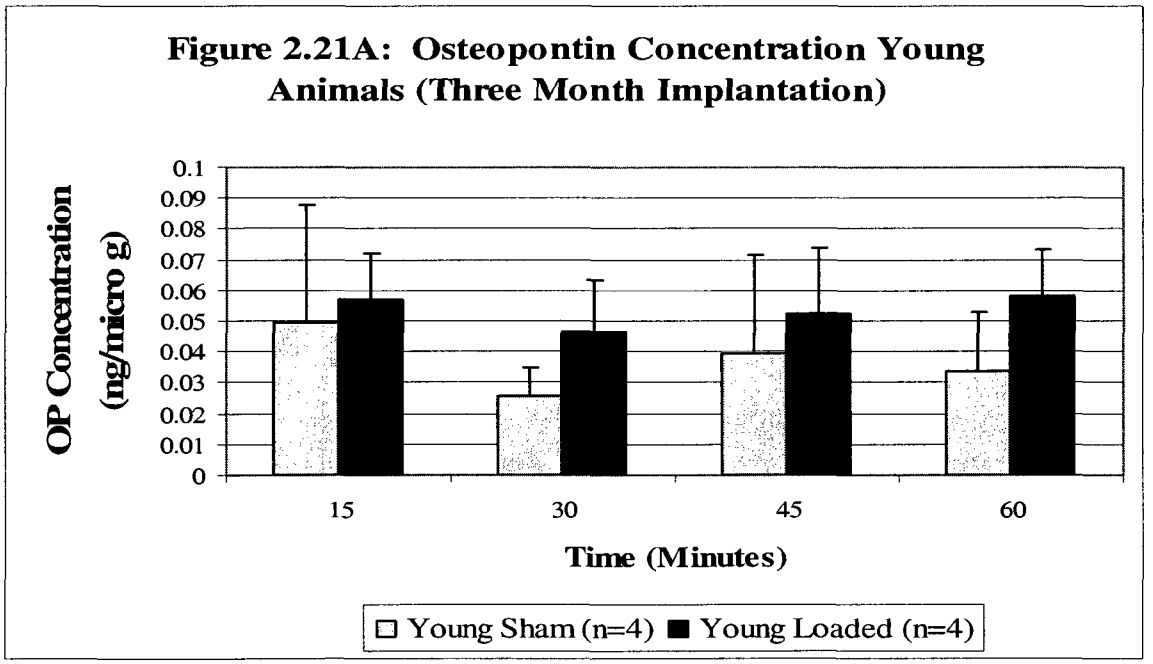




(A) There was an increase in connexin 43 protein expression in regenerative specimens produced in three months in young animals. **(B)** There was an increase in connexin 43 protein expression in regenerative specimens produced in four months in young and old animals.



(C) There was an increase in c-fos protein expression in regenerative specimens produced in four months in young animals.



(A) There was an increase in OP concentration after all time points of mechanical loading in specimens produced in young animals during a three month implantation time period.

Figure 2.21B: Osteopontin Concentration Old Animals (Three Month Implantation)

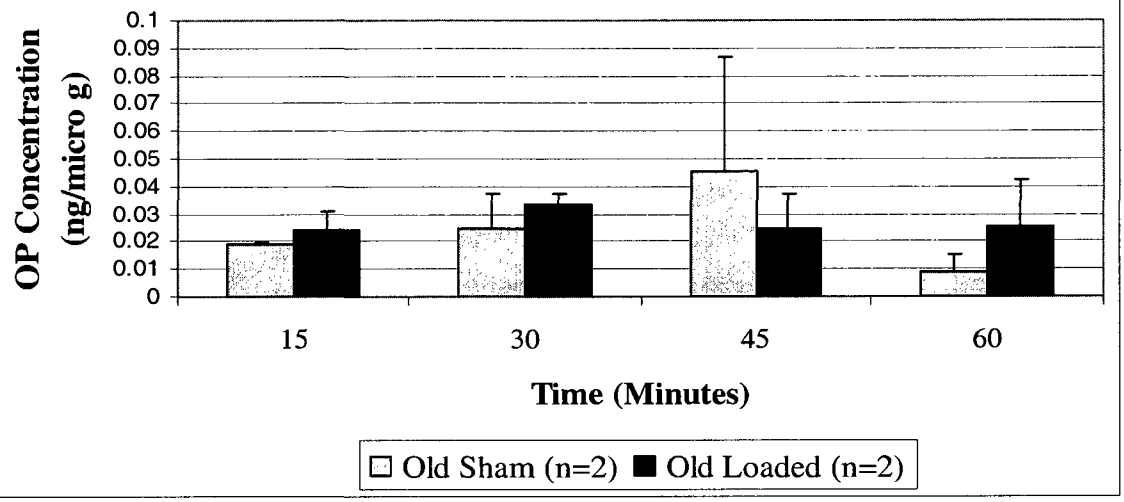
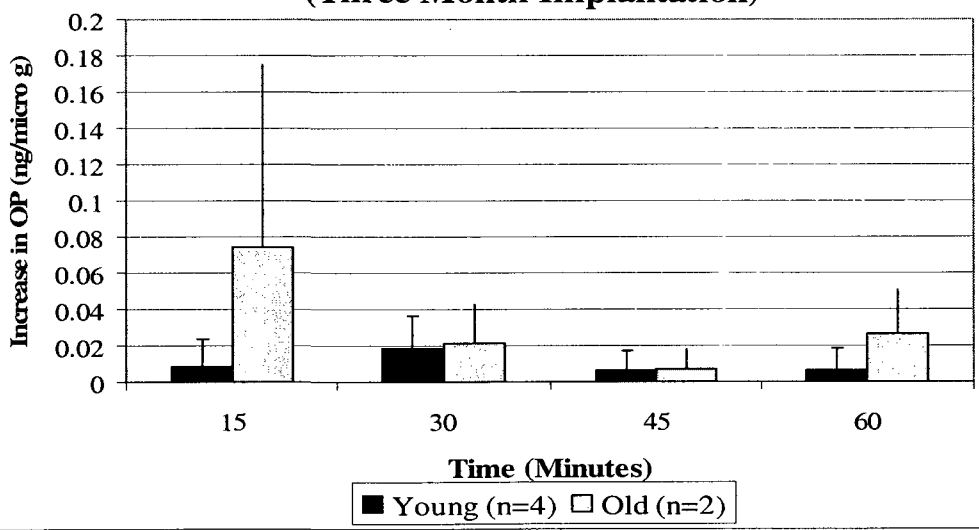


Figure 2.21C: Average Change in Osteopontin Concentration per Animal after Mechanical Stimulation (Three Month Implantation)



(B) There was an increase in OP concentration after all time points except 45 minutes of mechanical loading in specimens produced in old animals during a three month implantation time period. **(C)** After three months the average change in OP concentration was higher in regenerative specimens from old animals after all time points of loading.

Figure 2.21D: Osteopontin Concentration Young Animals (Four Month Implantation)

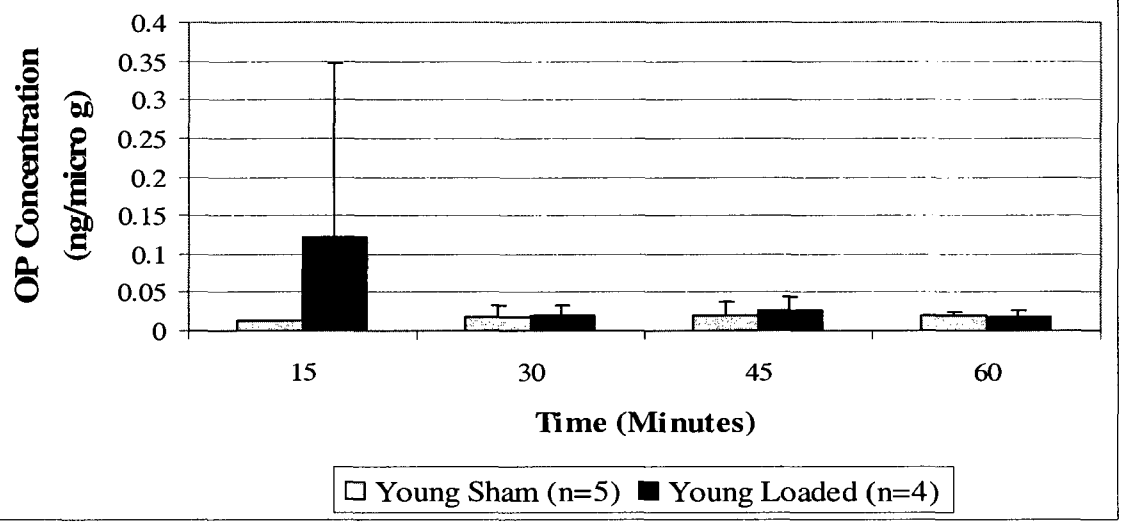
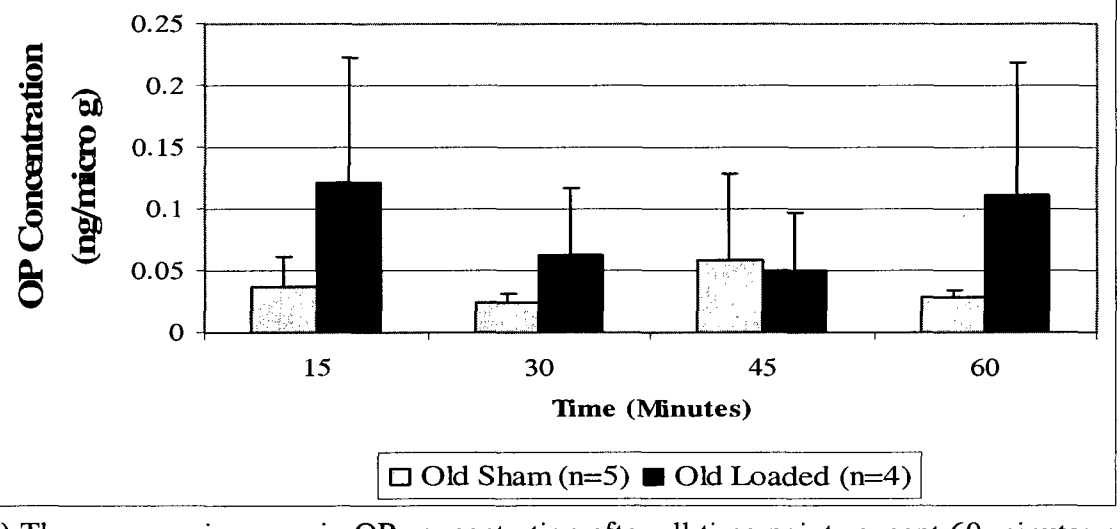
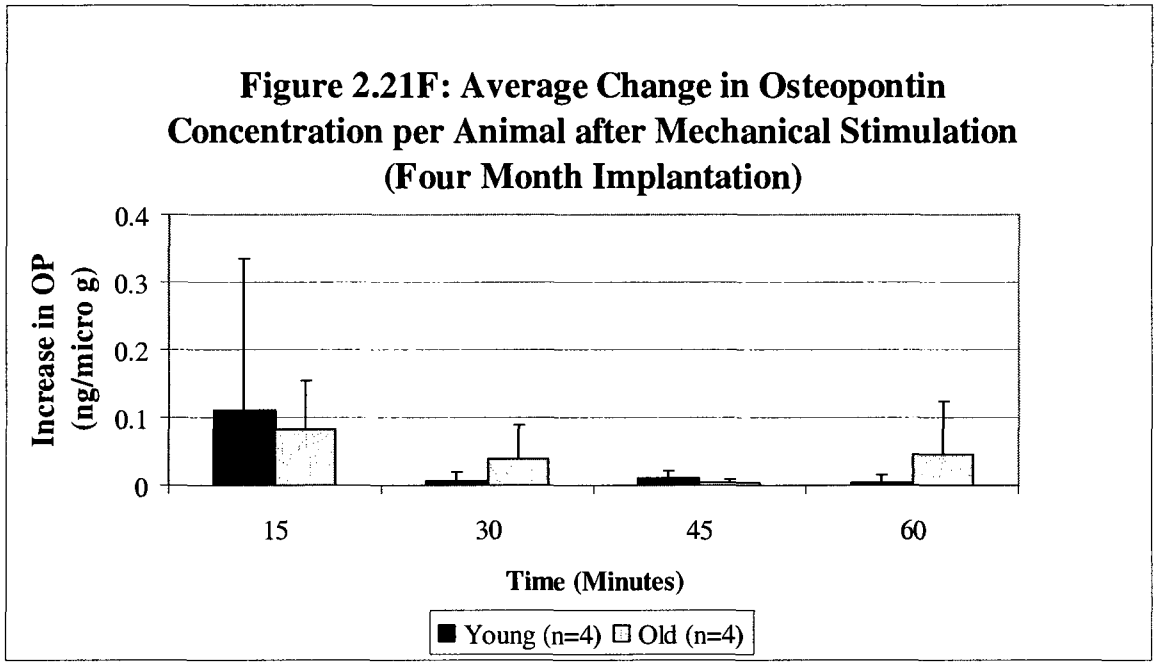


Figure 2.21E: Osteopontin Concentration Old Animals (Four Month Implantation)



(D) There was an increase in OP concentration after all time points except 60 minutes of mechanical loading in specimens produced in young animals during a four month implantation time period. **(E)** There was an increase in OP concentration after all time points of mechanical loading except 45 minutes in specimens produced in old animals during a four month implantation time period.



(F) After four months the average change in OP concentration was higher in regenerative specimens from young animals after 15 and 45 minutes of loading while the reverse was observed after 30 and 60 minutes of loading.

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CHAPTER III

THE EFFECT OF AGE ON MATURE BONE TISSUE AND ITS RESPONSE TO MECHANICAL STIMULATION

I'm not afraid of anything in this world. There's nothing you can throw at me that I haven't already heard

-Stuck in a Moment

3.1 Introduction

It is well established that mature bone can respond to the daily mechanical demands placed on it. Mechanical loading can stimulate bone formation and inhibit resorption as shown by reduced TRAP activity in periosteal cells in loaded limbs compared to non loaded controls (Hillam, R. R. et al., 1995). Previously experiments by Turner and colleagues suggested that there is a difference with age in the relative bone formation rate and bone forming surface of mature bone. Furthermore, there could be a difference with age in the threshold for lamellar bone formation (Turner, C. et al., 1995).

In the previous study the effect of age on regenerative bone and its ability to respond to mechanical stimulation was assessed. The data suggests that there is a difference with age in the degree of mineralization of regenerative specimens and possibly cellularity. Furthermore, there could be age related magnitude differences in the ways cells within regenerative bone tissue respond to mechanical stimulation through the secretion of nitric oxide, PGE_2, osteopontin, and the establishment of gap junctions.

The regeneration process may differ with age and it is well established that mechanical, geometrical, and BMD differences exist with age in trabecular and cortical bone (Majumdar, S. et al., 1997; Currey, J. D. et al., 1996, Jiang, Y. et al., 1996; Burstein, A. H. et al., 1976). In chapter two the mineralization of regenerative bone and its response to mechanical stimulation was assessed. In this study the mineralization, cellularity and mechanoresponsiveness of mature bone within the fibula was analyzed. Data obtained in chapter two for regenerative specimens was compared to that obtained for mature specimens. The fibula is deformed during load application between the knee and the ankle. Previously Moustafa and colleagues demonstrated that the mouse fibula responds to mechanical stimulation similar to the way in which the mouse tibia does. Dynamic axial loading was applied between the right flexed knee and ankle while the left limb served as a control. Cortical bone volume at the proximal and middle sites after mechanical stimulation and fluorochrome labeled images in the fibulae suggested the new bone formed in response to loading was both lamellar and woven. Twenty-four hours after loading there was also a reduction in the number of sclerostin positive osteocytes (Moustafa, A. et al., 2009).

3.2 Materials and Methods

3.2.1 Harvest of Mature Specimens

Specimens from the fibula were sterilely dissected from the tibia of animals post the regenerative tissue harvest. The harvested region is shown in **Figure 3.1**. Specimens were immediately placed in BGJb culture media. Tissue was then cultured in BGJb with 10% heat inactivated fetal bovine serum, penicillin (100U/ml), streptomycin (100μg/ml),

Amphotericin B (5μg/ml), and daily ascorbic acid (75μg/ml). Media was replenished every three days.

3.2.2 Micro CT

Specimens were scanned on a μCT system at a resolution of 18μm/voxel. Data was calibrated to air, water, and hyroxyappatite. The reconstructed three dimensional images were thresholded on a value based on the average minimum Hounsfield Unit (HU) value between two peaks on a graph of frequency (voxels) versus HU value for the specimens to separate bone voxels from non bone voxels. The region of interest was created with the cortical tool which selects an ROI corresponding to the cortical shell of the bone and uses a series of morphological operators to semi-automatically select cortical bone components. A grey level threshold value and 2 scaling size parameters were used to improve the accuracy of the ROI tool.

3.2.3 Histology

Specimens were fixed for 48 hours in 10% neutral buffered formalin (NBF) and then fixed in 70% ethanol until further processing. Specimens were decalcified in formic acid/sodium citrate for one week. Specimens were rinsed in water and stored in 70% ethanol until 24 hour processing through graded alcohols, xylene, and then embedded in Paraplast Plus wax. Specimens were processed for 7.5 hours and then embedded in paraffin. Specimens were sectioned at 7 microns and stained with alcian blue hematoxylin and acid fuchsin eosin. For quantitative histology an average of 23 sections were imaged at 40X with a Carl Zeiss (MicroImaging GmbH, Jena, Germany) microscope. Lacunae number and nuclei were counted for each slice and averaged. The amount of nuclei within lacunae was expressed as a percentage.

3.2.4 Raman Spectroscopy

Mineral to matrix ratios (MMR) were measured with a custom built Raman microscope as described in chapter 2.

3.2.5 Three Point Bending

Specimens were loaded in the medial lateral direction in cyclic three point bending as described in chapter 2.

3.2.6 Nitric Oxide and Prostaglandin E_2 Concentration

Nitric oxide and PGE_2 concentration secreted into the media were calculated as described previously in chapter 2. NO and PGE_2 values were normalized to total specimen protein.

3.2.7 Western Blot

After sham or mechanical stimulation treatment specimens were ground with a polytron in a total of 1mL RIPA lysis buffer, sodium orthovanadate, PMSF, and protease inhibitor cocktail (1000:1:1:2; Santa Cruz Biotechnology, Santa Cruz, CA), snap frozen in liquid nitrogen and stored at -80°C until further processing. Specimen protein concentration was determined with a BCA assay (Thermo Scientific, Rockford Il). Ten micro grams of protein were run on the lanes of a 10% Tris-HCL gel for ninety minutes at 150V and transferred to a PVDF membrane for forty minutes at 80V. Membranes were then blocked in 5% Blotto (5g dry milk:100mL 0.1% PBS-Tween) overnight and probed for connexin 43(1:8000 AbCam Cambridge, AM).

3.2.8 Statistical Analysis

A two sided unpaired t test was used to determine statistical significance within the data set. P values less than 0.05 are expressed with * and p values less than 0.01 are

expressed with **.

3.3 Results

Representative isosurfaces of mature specimens are shown in **Figure 3.2**. The average thickness of mature fibula specimens was not significantly different with age **(Figure 3.3)**; however the thickness of mature fibula specimens from young and old animals were significantly different from the thickness of regenerative femoral specimens from young and old animals respectively. Additionally the dimensions of mature specimens in the anterior-posterior and medial-lateral directions were not significantly different between age groups **(Figure 3.4)**. Data from an aging rat study shows a higher average degree of mineralization in mature bone specimens from old animals compared to young **(Figure 3.5A)**. Femoral regenerative TMD to mature bone TMD ratio was higher in young animals than old after a three month implantation period, however the reverse was observed after a four month implantation period **(Figure 3.5B)**. Mineral to matrix ratio was significantly higher in mature fibula specimens from old animals compared to young **(Figure 3.5C)**. However, the ratio between regenerative femoral MMR and mature fibula MMR was higher in young animals **(Figure 3.5D)**.

The cellularity of mature specimens from young and old animals was similar **(Figure 3.6)**. The distribution of mineral is illustrated with alpha blends **Figure 3.7A** which show a higher proportion of voxels mapped to a higher radiodensity value in mature specimens from old animals when compared to those from young. However this distribution of highly mineralized bone voxels is not uniform throughout the bone tissue. In **Figure 3.7B,C** it is apparent that there is a higher proportion of highly mineralized voxels in proximal and distal regions of the specimens compared to the center.

Furthermore, the distal and proximal portions of specimens from old animals have a higher frequency of voxels at 2000HU (the typical threshold for cortical bone) compared to distal and proximal portions from young animals.

There was no statistically significant difference with age between the number of lacunae, the number of nuclei, or the percent of nuclei in lacunae between mature specimens **(Figures 3.8A-C)**. It appears that with increased implantation time the regenerative specimens from young animals have a closer percentage of occupied lacunae to the percentage value of mature controls **(Figure 3.8D)**. With advancing age the bending stiffness of mature femoral bone appears to significantly increase and then gradually decrease after 28 months of age **(Figure 3.9A)**. A similar trend was also observed in compression stiffness of vertebrae **(Figure 3.9A)**. The ratio between regenerative and mature femora stiffness was higher in old animals compared to young **(Figure 3.9B)**.

There is an increase in NO concentration in mature specimens from young animals after thirty and forty-five minutes of loading **(Figure 3.10A)**. There is an increase in NO concentration in mature specimens from old animals after fifteen minutes of loading **(Figure 3.10B)**. The average increase in NO concentration for mature specimens per animals was higher for old animals after fifteen and sixty minutes of loading while the reverse was observed after thirty and forty-five minutes of loading **(Figure 3.10C)**. Among specimens from old animals regenerative specimens produced in four months had the highest increase in NO concentration most notably at later time points **(Figure 3.10D)**. Among specimens from young animals regenerative specimens produced in four months had the highest increase in NO concentration at all time points

(Figure 3.10E). There were considerably high NO increases for specimens produced in three months in that age group when compared to implantation time period matched old animals. After 15 and 30 minutes of loading regenerative specimens from young and old animals produced a greater concentration of nitric oxide when normalized to NO production from mature bone **(Figure 3.10F)**.

There was an increase in PGE_2 expression after mechanical stimulation of mature bone specimens from young animals after 15, 30, and 60 minutes of loading; however these changes were not statistically significant **(Figure 3.11A)**. There was an increase in PGE_2 expression after mechanical stimulation of mature bone specimens from old animals after 15 minutes of loading; however this change was not statistically significant **(Figure 3.11B)**. The average increase in PGE_2 expression per animal was higher in mature bone specimens from old animals after 15, 45, and 60 minutes of mechanical stimulation; however these changes were not statistically significant **(Figure 3.11C)**. The average change in PGE_2 concentration after mechanical stimulation was highest in mature bone specimens when compared to regenerative bone specimens produced during both a three month and four month implantation period for both young and old animals at all time points **(Figure 3.11D,E)**.

There is an increase in OP after fifteen minutes of loading in mature specimens from young animals **(Figure 3.12A)**. There was an increase in OP concentration after all time points of loading in mature specimens from old animals **(Figure 3.12B)**. The average increase in OP concentration per animal was only highest in specimens from young animals after fifteen minutes of loading **(Figure 3.12C)**. Among specimens from old animals the increase in OP concentration was highest for regenerative specimens

produced over four months after all loading time points except forty-five minutes **(Figure 3.12D)**. Among specimens from young animals the increase in OP concentration was highest for regenerative specimens produced over four months after fifteen and forty-five minutes of loading **(Figure 3.12E)**.

Connexin 43 protein was successfully measured in mature bone specimens from young and old animals which were both sham and experimentally loaded **(Figure 3.13A)**. There appears to be an increase in connexin 43 protein with mechanical loading in mature specimens from young and old animals, however this was not statistically significant **(Figure 3.313B)**.

3.4 Discussion:

In this study there was no significant difference in the cellularity of mature specimens with age. TMD was higher in regenerative and mature specimens from old animals compared to young; however the proportion of regenerative specimens TMD to mature fibula TMD was higher in young animals after a 3 month implantation time period and the reverse was observed after a 4 month implantation time period. The data suggests that while TMD measures may be lower in regenerative specimens from young animals, these specimens are closer to the value of mature tissue mineral density for their respective age group after a 3 month implantation time period unlike during a 4 month implantation time period. The ratio between regenerative MMR and mature fibula MMR was higher for young animals compared to old. This is likely the result of an effect of age on the bone matrix. Studies have found a greater increase in osteoid in old animals compared to young during regeneration (Sumner, D. et al., 2003). High TMD could make the bone more brittle and if the bone exceeds the upper limit of the life span of

osteocytes, which is estimated to be twenty-five years, the cells would die which could lead to hypermineralization of perilacunar bone and filling of lacunae and canaliculi with mineralized connective tissue which could lead to additional brittleness (Parfitt, A. M. et al., 1993)

Alpha blends of mature bone specimens suggest that the distribution of highly mineralized bone voxels is not uniform throughout specimens from old animals. This was also observed in regenerative bone tissue produced over a four month implantation time period in old animals. This could result in a highly non uniform strain distribution in the specimen during mechanical stimulation. Mechanical loading of regenerative specimens produced during a four month implantation period resulted in very high changes in NO concentration for both young and old animals when compared to mature bone tissue. Perhaps regenerative bone tissue which is less mineralized than mature bone tissue is deformed more during loading which places greater strain on the cells and results in increased release of nitric oxide. Interestingly, the loading of regenerative specimens from young animals produced during a three month implantation time period also resulted in a large release of nitric oxide. This trend was not observed in regenerative specimens produced over three months in old animals which could be the result of the aforementioned differences in strain. The degree of mineralization was lower for regenerative specimens produced in four months in young animals when compared to those produced in three months. The local strain placed on the cells could be higher and thus lead to a higher net release of nitric oxide from specimens produced during four months.

Similar to what was observed in the nitric oxide data regenerative specimens produced during four months released higher net values of osteopontin in both age groups when compared to regenerative specimens produced during three months and mature bone specimens. This could also be the results of larger strains placed on the regenerative bone tissue. Interestingly the net increase in PGE_2 was greatest in mature bone specimens from young and old animals. Perhaps PGE_2 is a signal molecule that is vital to transduction of mechanical cues in mature bone tissue for both age groups. Klein-Nulend and others have observed no change in COX2 mRNA or PGE_2 expression post mechanical stimulation of primary cells with age (Bakker, A. D. et al., 2006). Results from this study suggest that there could be a difference in PGE_2 expression with age from mature bone in response to mechanical stimulation.

Connexin 43 protein expression was minimally increased in response to mechanical stimulation. Perhaps the alterations observed in NO, PGE_2, and OP expression were independent of alterations in the number of gap junctions established after one hour or loading. There was no age related difference in the cellularity of mature bone specimens although there may be more baseline connexin 43 protein present in mature specimens from young animals. There could be differences in cellular sensitivity to mechanical stimulation and perhaps there is a compensation for this in mature bone through establishment of gap junctions independent of mechanical stimulation.

In an age related study of the human femoral cortex at age 80-85 years there was an increase in the highest density bone. Chemical studies, however, revealed no change in Ca, P, $Ca+PO_4$, or C/P molar ratio with respect to age (Simmons, E. et al., 1991). With age there is a decrease in collagen content and an increase in mean tissue

mineralization. In this study mature specimens from old animals had a higher crystallinity than mature specimens from young animals. Spectrometry studies suggest larger crystals are present in the bone of older, osteoporotic women and this increased crystallinity could permit earlier crack initiation by decreasing the amount of plastic deformation that can occur before ultimate failure. In this study mature bone from old animals had a higher degree of mineralization than mature bone from young animals. When bone tissue becomes too highly mineralized it tends to become brittle which reduces its toughness and makes it more prone to fracture from repeated loads and accumulated micro-cracking.

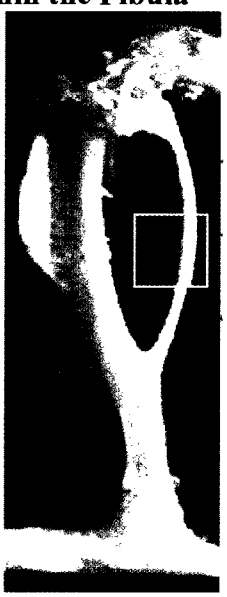
In this study no significant difference was found between ages groups in the number of lacunae or nuclei in mature cortical bone. Other studies have found that the number of osteocytes, total lacunae, and occupied lacunae decreased with advancing age primarily in deep cancellous bone (Qiu, S. et al., 2002). In the mouse calveria a decrease in the osteocyte population in relation to the increasing calverial thickness was observed with increasing age and the number of osteocyte lacunae per bone area and the number of actively remodeling osteons per bone area per year declined exponentially with age in canine bone (Aaron, J. et al., 1973; Frank, J. D. et al., 2002). Mullender and colleagues found that osteocyte death in trabecular bone was not related to age or increased in osteoporosis when compared with controls; however lacunar number per bone area decreased and bone mass (Mullender, M. G. et al., 1996; Mullender, M. G. et al., 2006)

This study could be limited by variations in the cross sectional dimensions of mature specimens. A consistent geometric template to produce mature specimens comparable to the regenerative microspecimens produced in chapter two would provide a

better comparison between regenerative and mature bone tissue and its response to mechanical stimulation. This study is also limited by the small sample size, which in conjunction with high variation in some parameters makes it difficult to reach statistical significance. In this study only one loading regimen was used to assess response to mechanical stimulation. Srinivasan and colleagues have found that the insertion of rest periods between mechanical loading may enhance osteogenesis in aged animals (Srinivasan, S. et al., 2003).

In conclusion mature bone specimens harvested from young and old animals had a higher degree of mineralization than regenerative bone tissue from their respective age groups. These specimens are most likely stiffer and the application of mechanical stimulation produces less local deformation on the cells. Therefore there was less of an increase in nitric oxide concentration from loaded mature specimens when compared to their age respective regenerative specimens. Interestingly mechanically loaded mature specimens from young and old animals released a higher concentration of PGE_2 than their age respective regenerative bone specimens. Notable age differences in NO and PGE_2 expression after mechanical stimulation were not observed in mature specimens as was the case in regenerative specimens, perhaps due to similarities in cellularity .

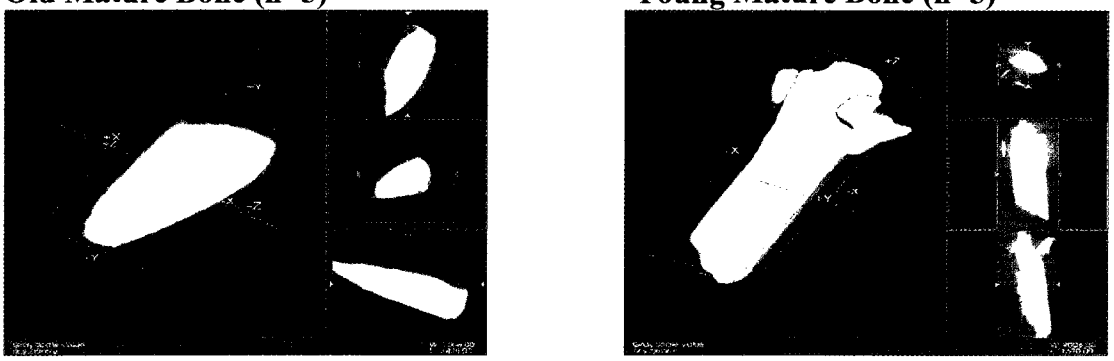
Figure 3.1: Region of Interest within the Fibula



Region of fibula dissected for mature bone analysis Moustafa, A. et al., (2009) *Bone*

Figure 3.2: Representative Isosurfaces of Mature Bone

Old Mature Bone (n=5) **Young Mature Bone (n=5)**

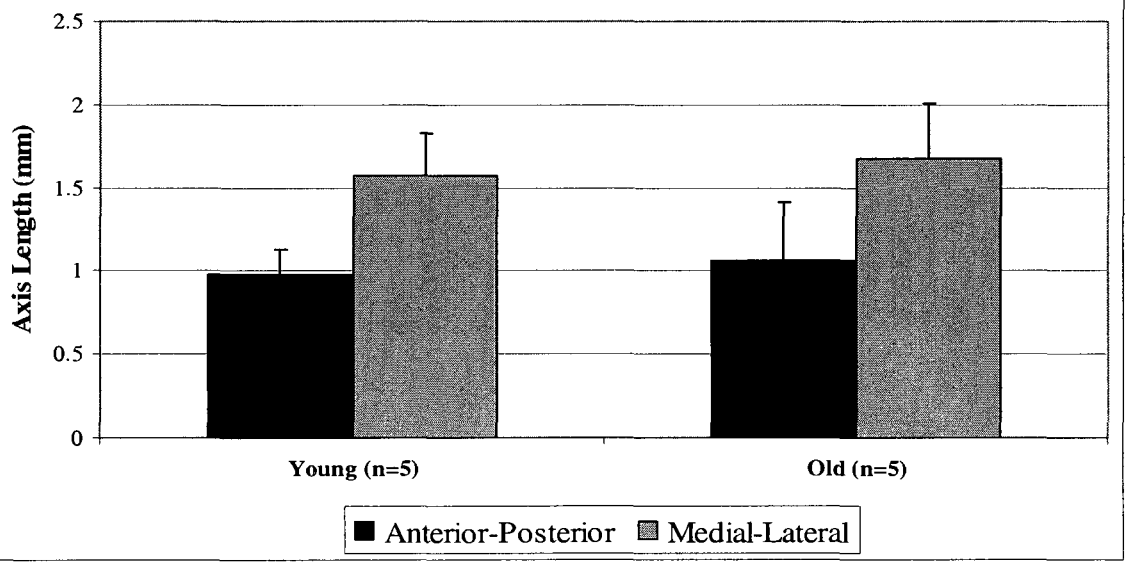


The geometry of mature specimens is shown in representative isosurfaces.

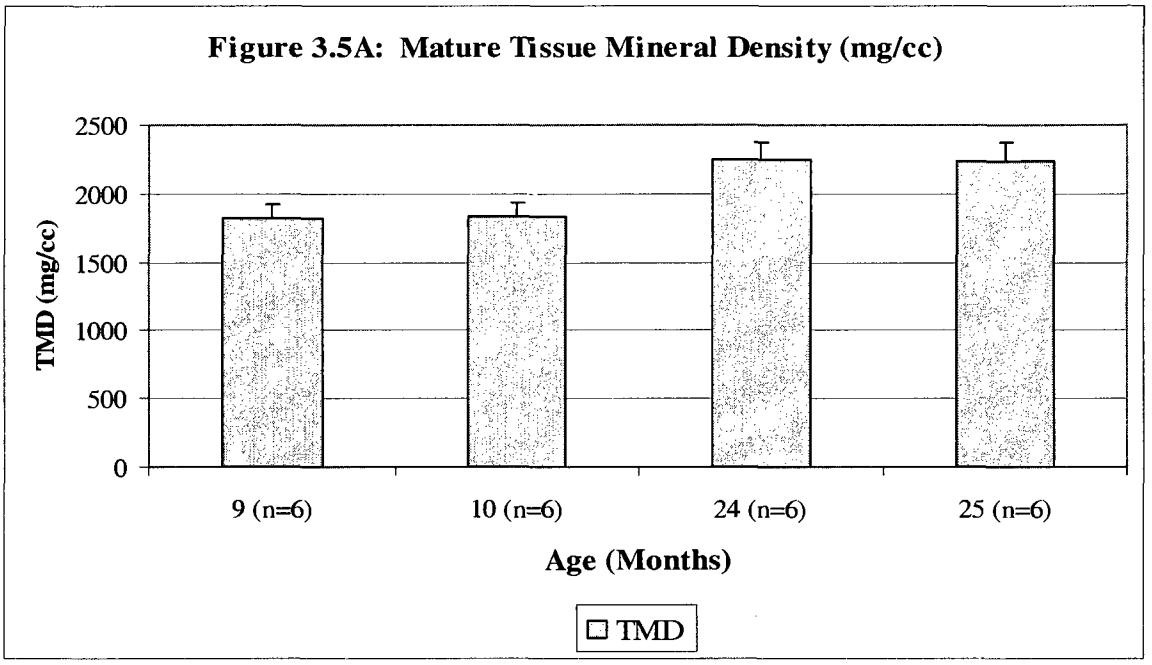
Figure 3.3: Mature Specimen Average Thickness

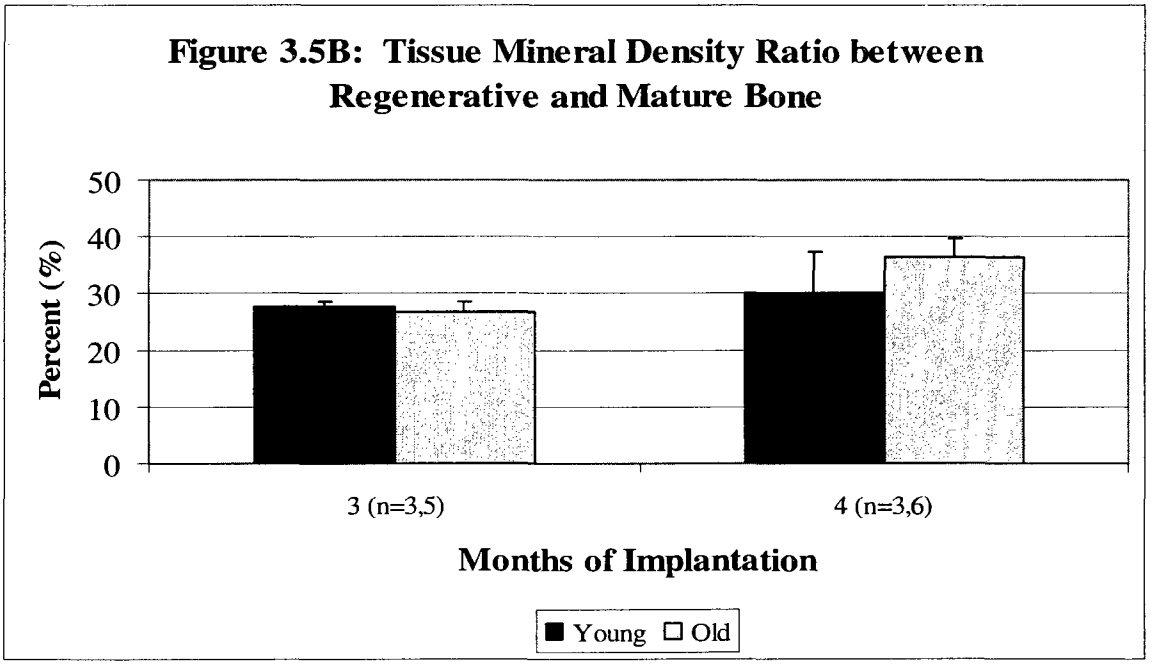
The average thickness of mature specimens from young and old animals was not significantly different.

Figure 3.4: Mature Specimen Cross Section Dimensions (mm)

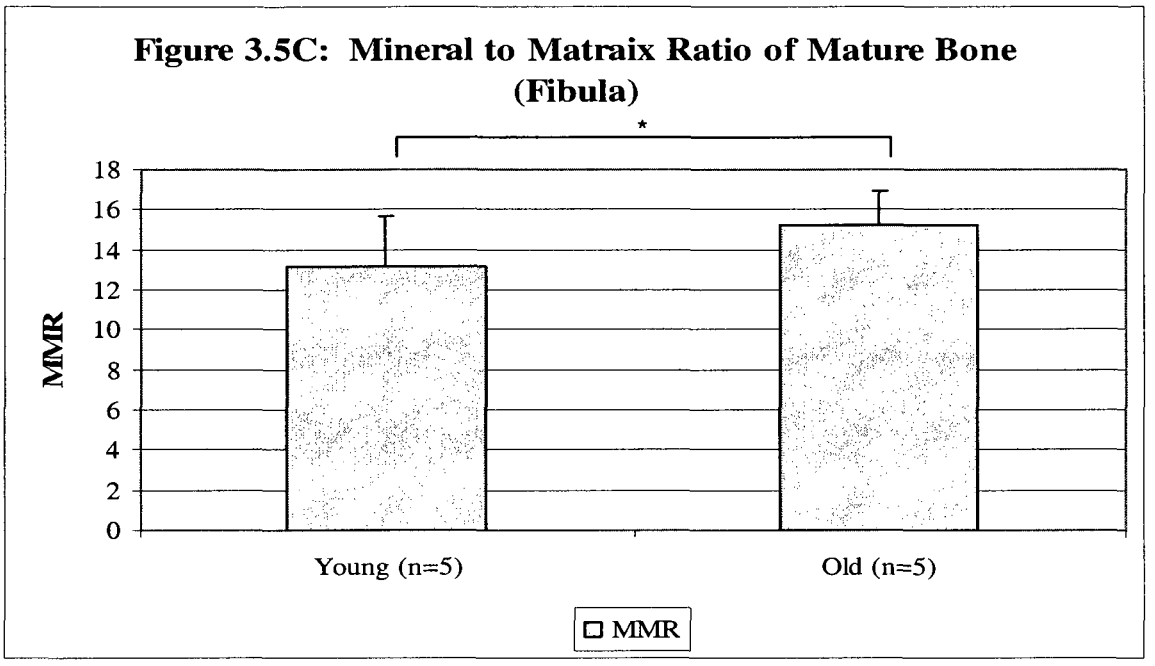


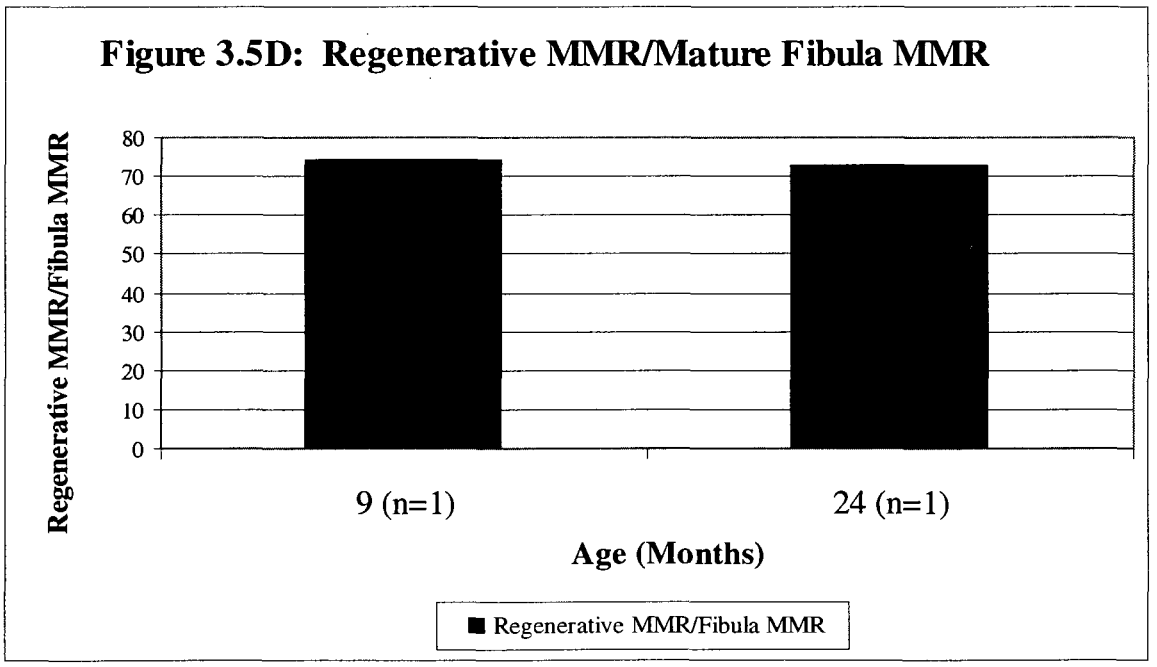
The cross sectional dimensions of mature specimens were not significantly different.





(A) The degree of mineralization appears to increase with advancing age. **(B)**The ratio of regenerative TMD to mature TMD was higher in specimens from young animals after a 3 month implantation period and the reverse was observed after 4 months.





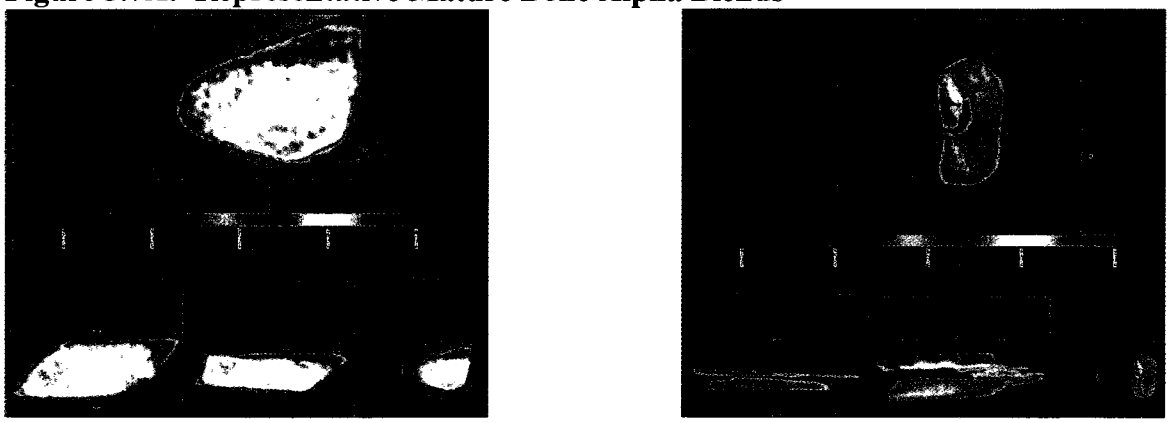
(C) The mineral to matrix ratio was higher in mature specimens from old animals. **(D)** The ratio of regenerative to mature fibula MMR was higher in specimens from young animals.

Figure 3.6: Mature Bone Alcian Blue Hematoxylin and Acid Fuchsin Eosin Stain

Old (n=5) **Young (n=5)**

The structure and cellularity of maure bone from the fibula apprears similar with age.

Figure 3.7A: Representative Mature Bone Alpha Blends



Old (n=5) **Young (n=5)**

(A) There appears to be a higher proportion of voxels mapped to a higher HU value in mature specimens from old animals when compared to mature specimnes from young animals.

Figure 3.7B: Young Animals Mature Bone Histograms

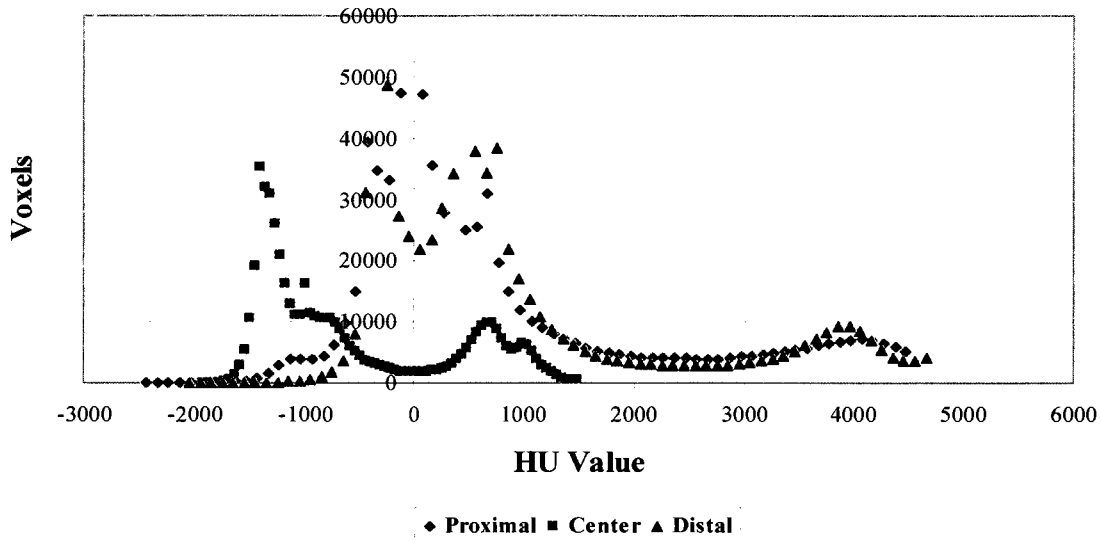
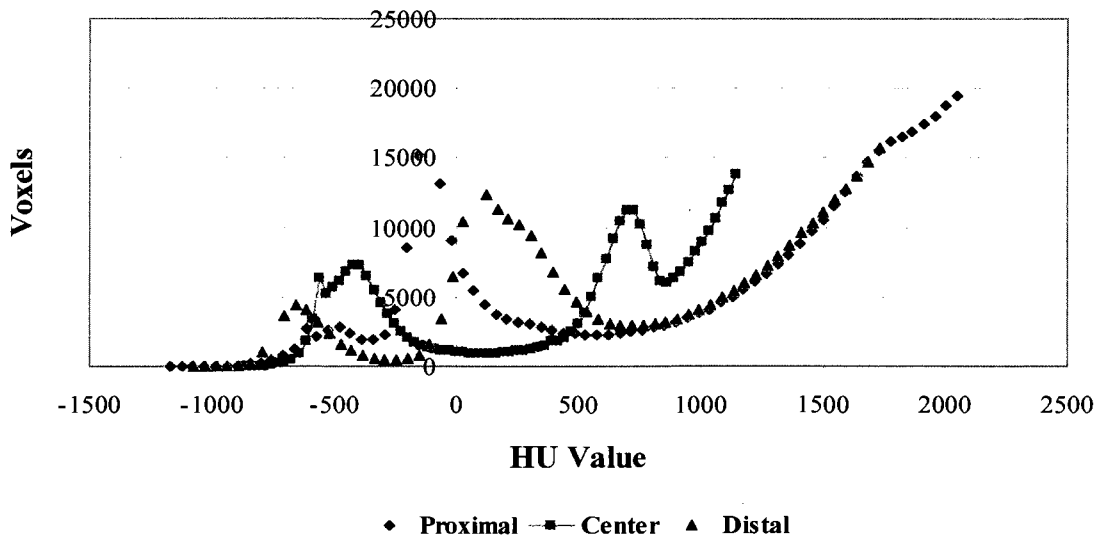
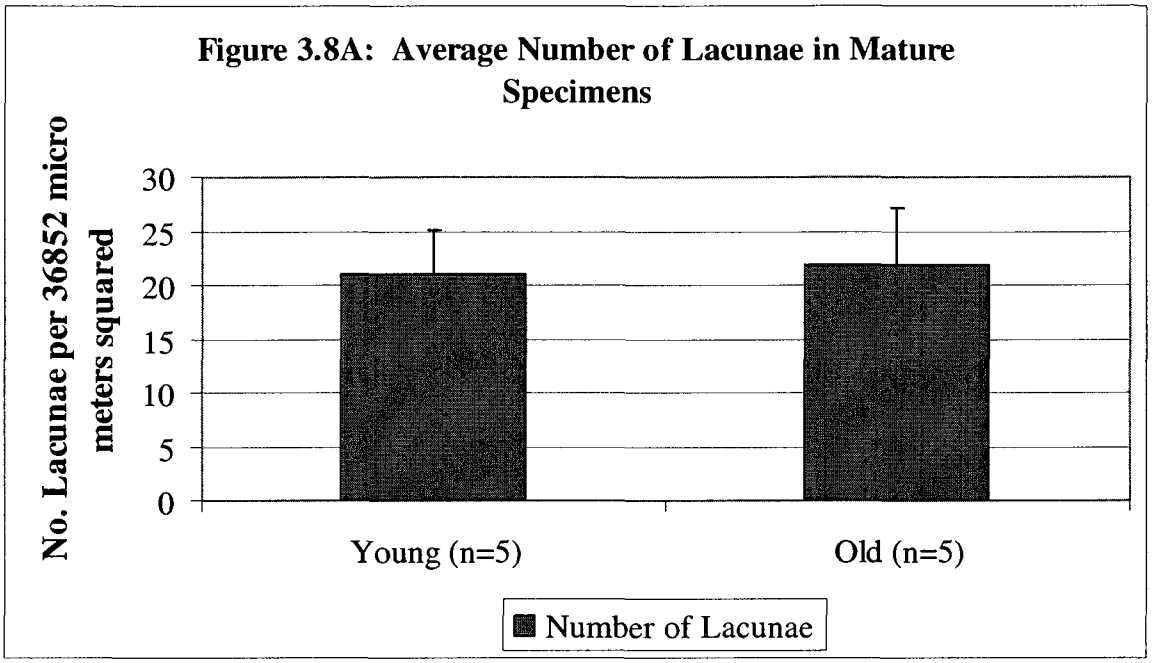
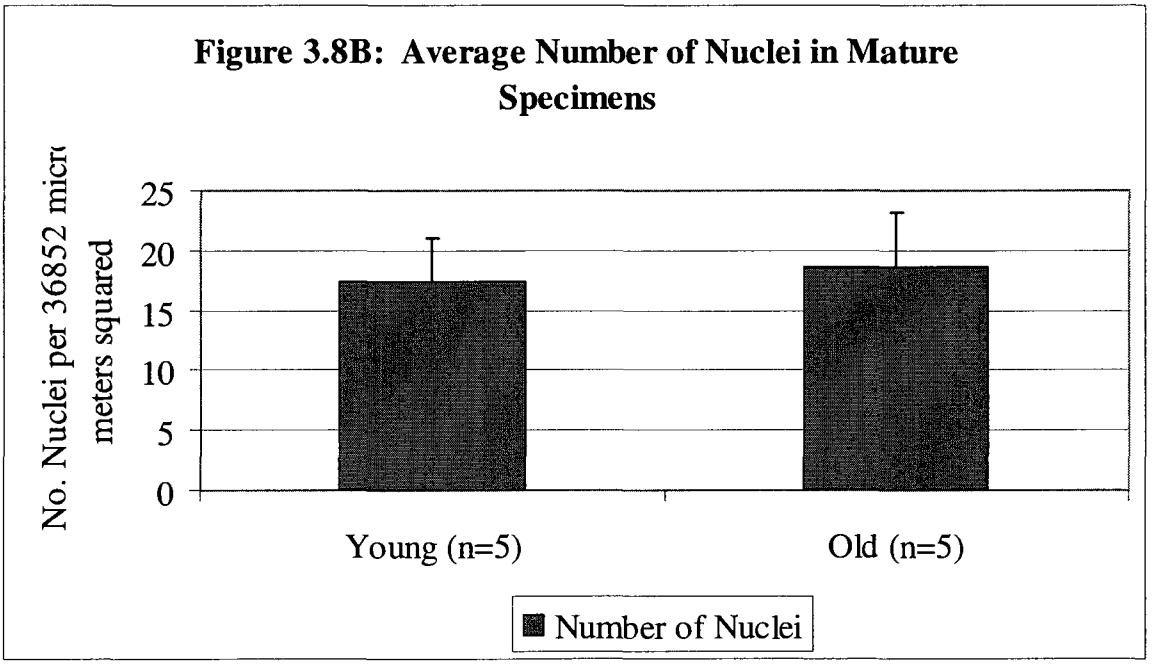


Figure 3.7C: Old Animals Mature Bone Histograms

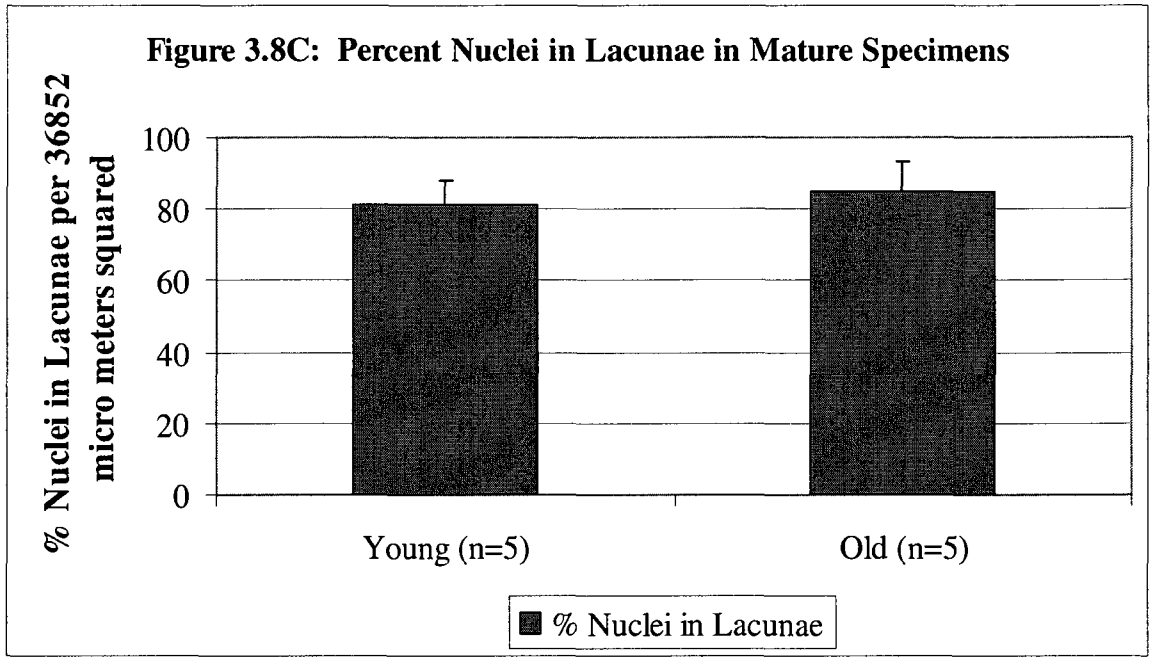


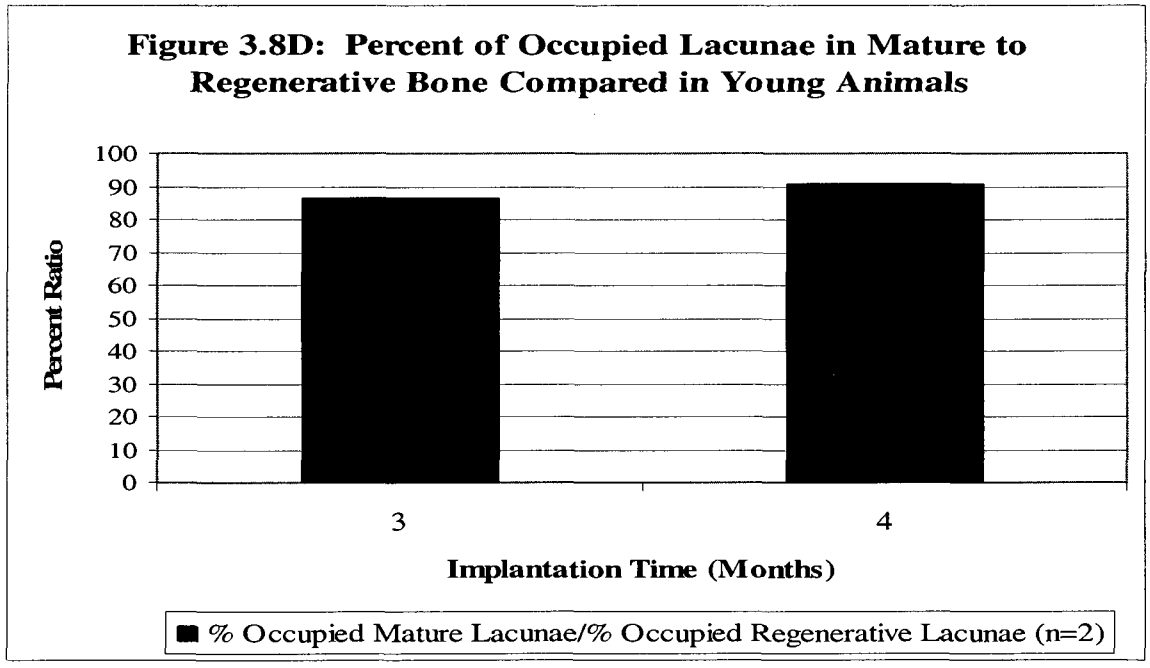
(B) The center portion of mature specimens from young animals has a lower number of voxels at high HU values compared to the proximal and distal ends. **(C)** Distal and proximal portions of specimens from old animals have a higher frequency of voxels at 2000HU (the typical threshold for cortical bone) compared to distal and proximal portions from young animals.



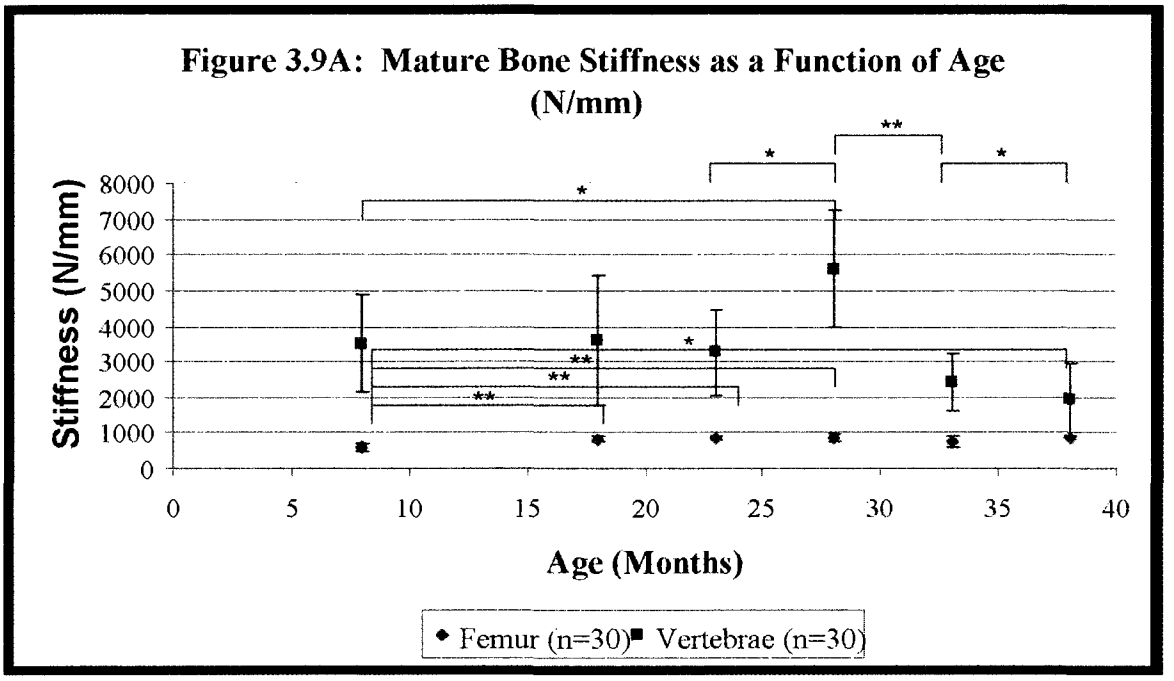


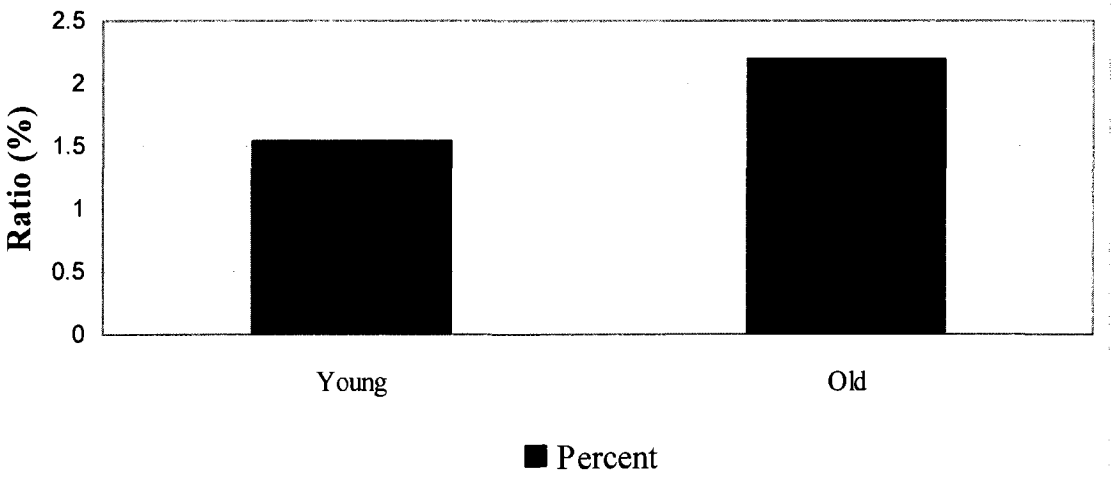
(A) There was no significant difference with age between the number of lacunae in mature specimens. **(B)** There was no significant difference with age between the number of nuclei in mature specimens.





(C) There was no significant difference with age between the percentage of occupied lacunae in mature specimens. **(D)** In young animals the percent occupied lacunae in regenerative bone produced over 4 month period is closer to percent occupied lacunae in mature bone when compared to specimens produced in 3 months.





(A) Femoral bending stiffness and vertebral compression stiffness increases and then decreases with advancing age. **(B)** The stiffness ratio between regenerative and mature femora bone tissue is higher in old animals compared to young.

Figure 3.10A: Nitric Oxide Concentration in Mature Specimens from Young Animals

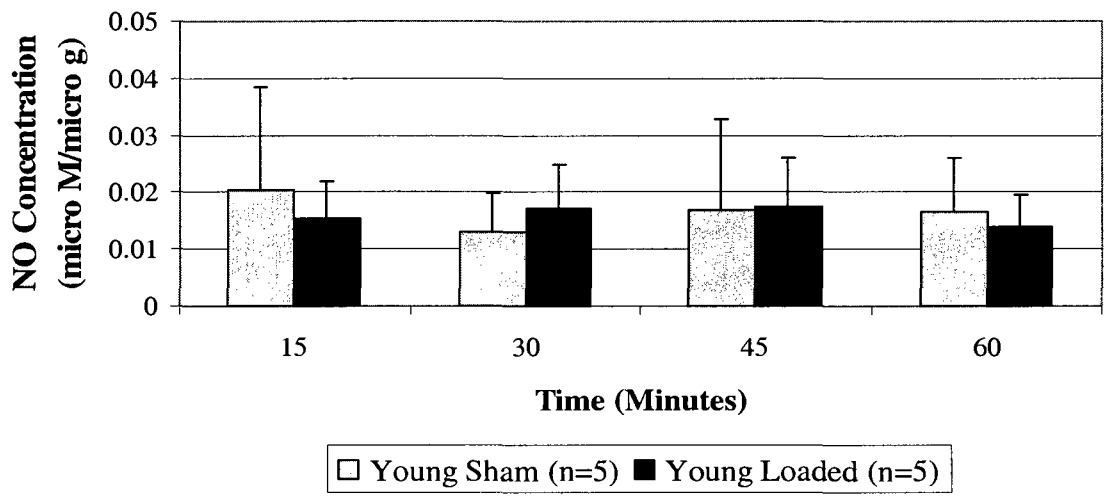
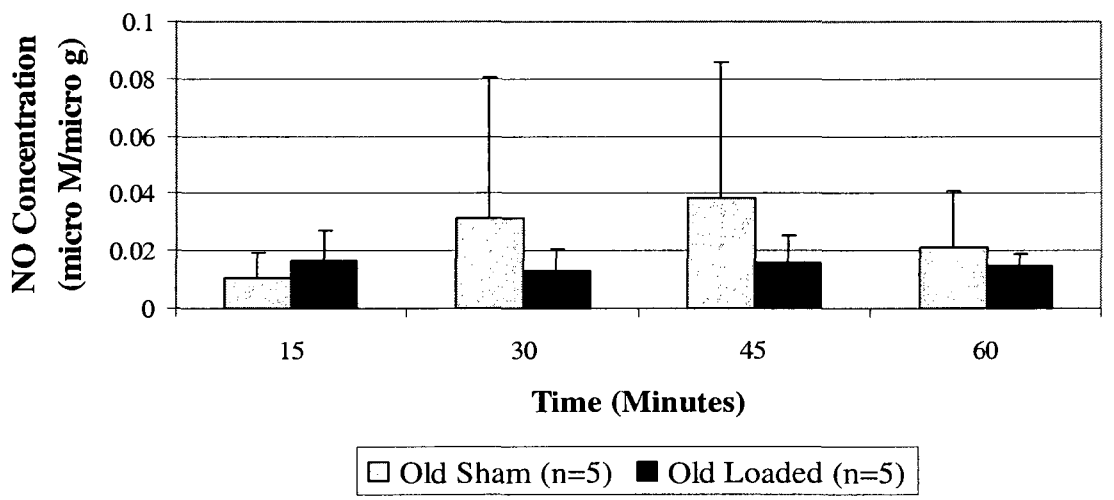


Figure 3.10B: Nitric Oxide Production in Mature Specimens from Old Animals



(A) There is an increase in NO in mature specimens from young animals after 30 and 45 minutes of loading. **(B)** There is an increase in NO in mature specimens from old animals after 15 minutes of loading.

Figure 3.10C: Average Change per Animal in Nitric Oxide Production in Mature Specimens

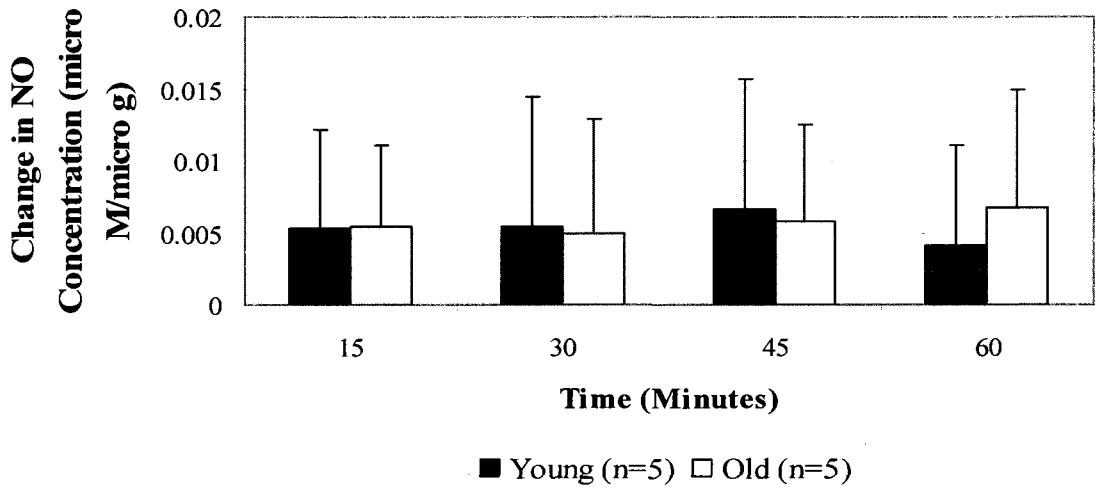
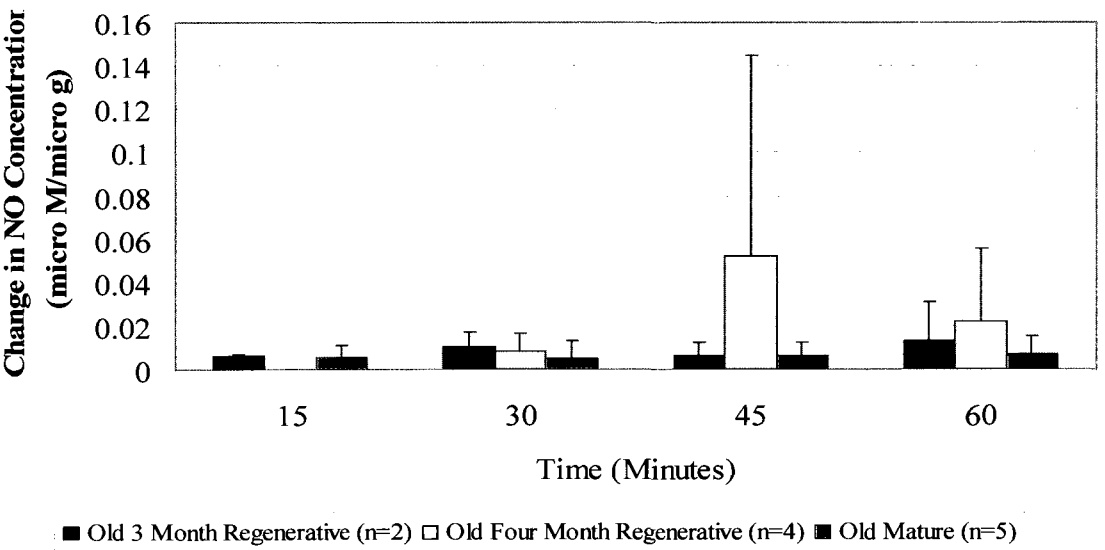


Figure 3.10D: All Changes in NO Concentration for Old Animals



(C) The average change in NO per animal is higher for mature specimens from youmg animals after 30 and 45 minutes of loading and the reverse is observed after 15 and 60 minutes of loading. **(D)** The highest increase in NO for old animals is observed in regenerative specimens produced in four months at later loading time points.

Figure 3.10E: All Changes in NO Concentration for Young Animals

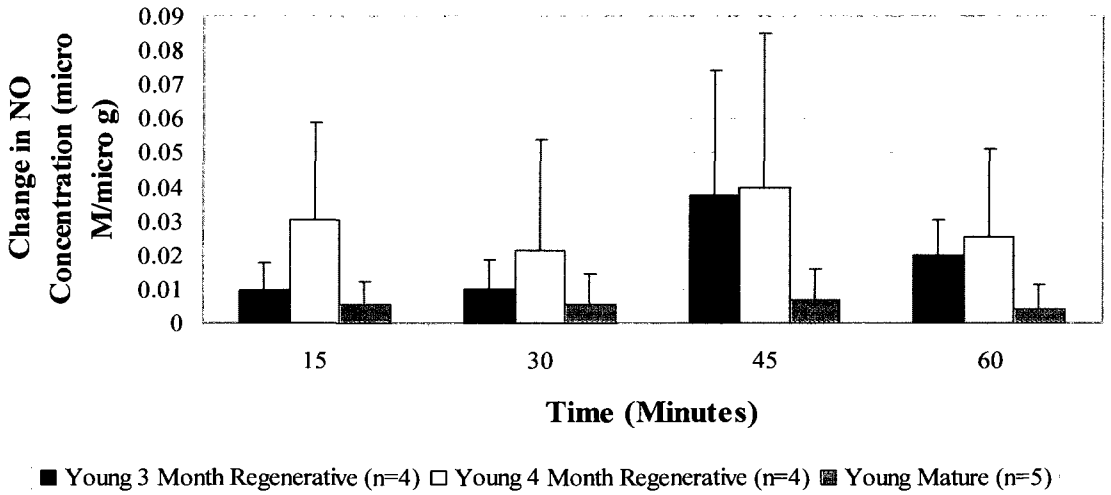
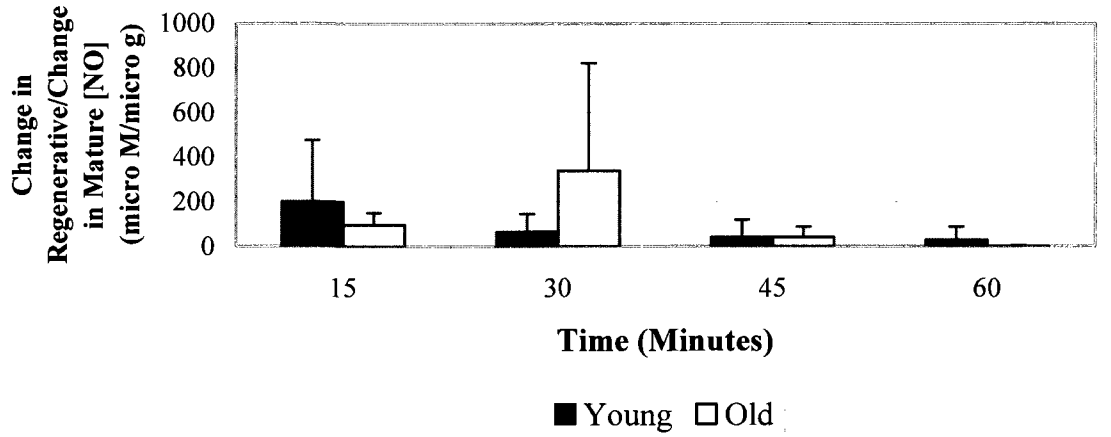


Figure 3.10F: Average Change in Regenerative NO Compared to Average Change in Mature NO per Animal



(E) The highest increase in NO for young animals is observed in regenerative specimens produced in four months at all loading time points. **(F)** After 15 and 30 minutes of loading regenerative specimens from young and old animals produced a greater concentration of NO normalized to mature bone.

Figure 3.11A: PGE_2 Concentration in Mature Specimens from Young Animals

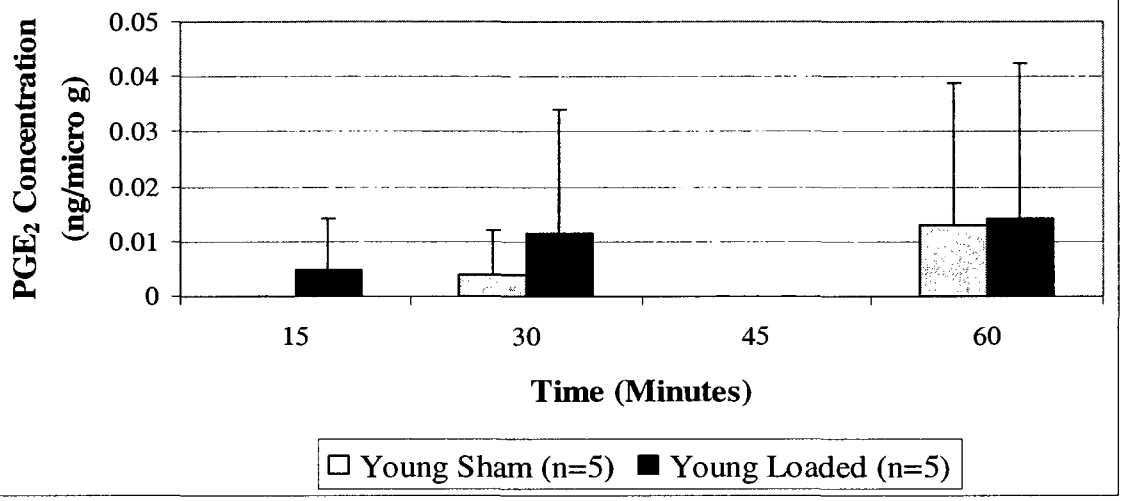
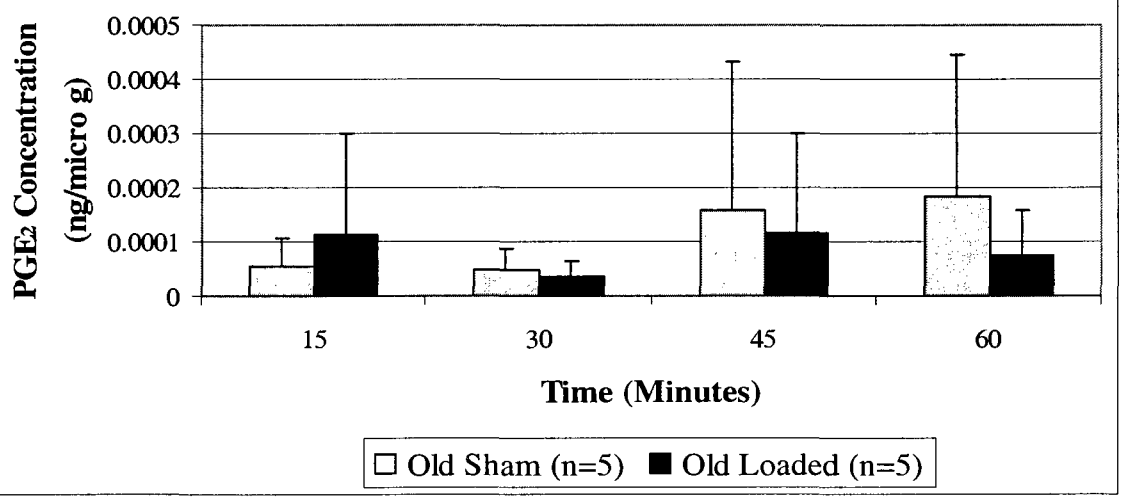


Figure 3.11B: PGE_2 Concentration in Mature Specimens from Old Animals



(A) There was an increase in PGE_2 concentration in mature specimens from young animals after 15, 30, and 45 minutes of loading. **(B)** There was an increase in PGE_2 concentration for mature specimens from old animals after 15 minutes of loading.

Figure 3.11C: Average Change per Animal in PGE_2 Production in Mature Specimens

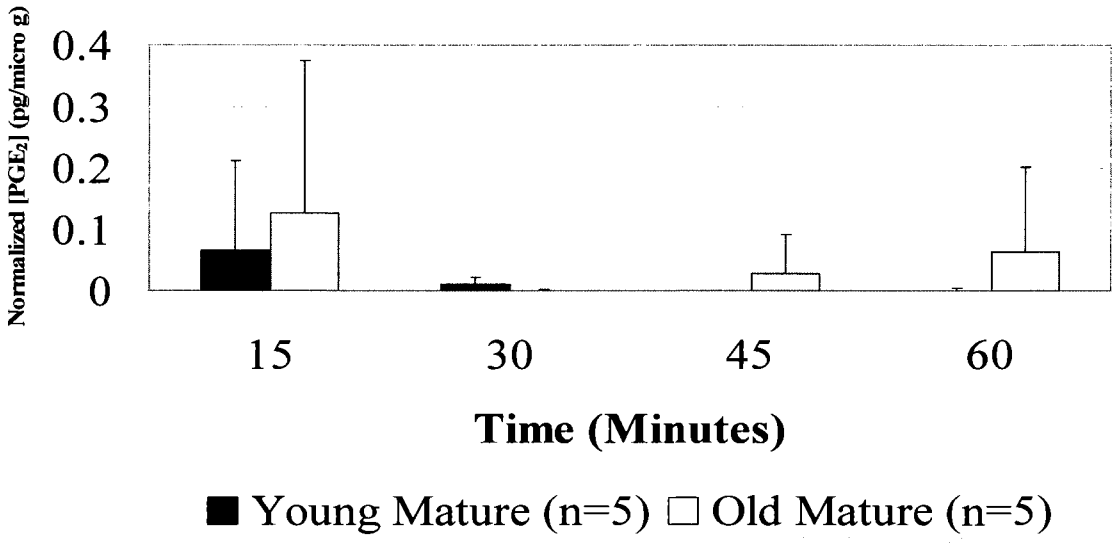
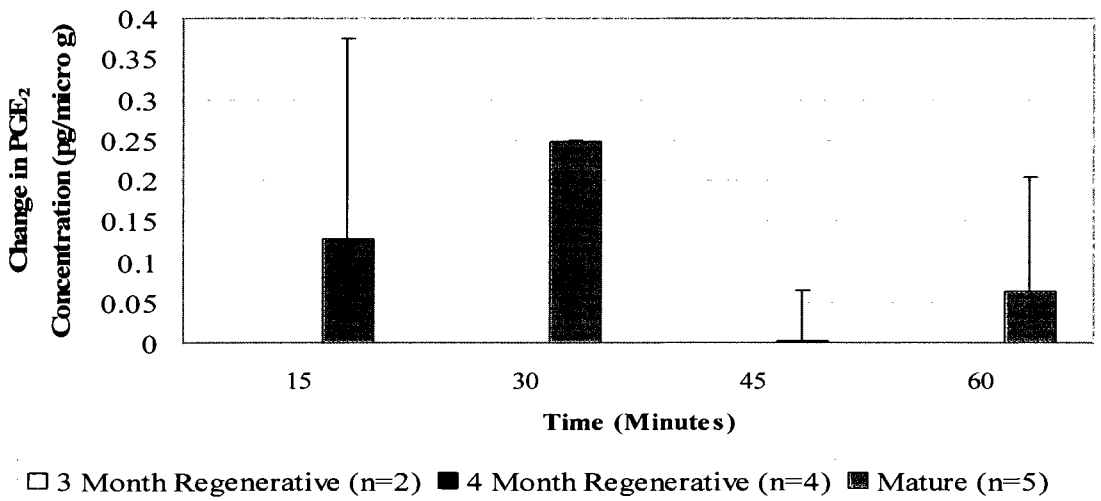
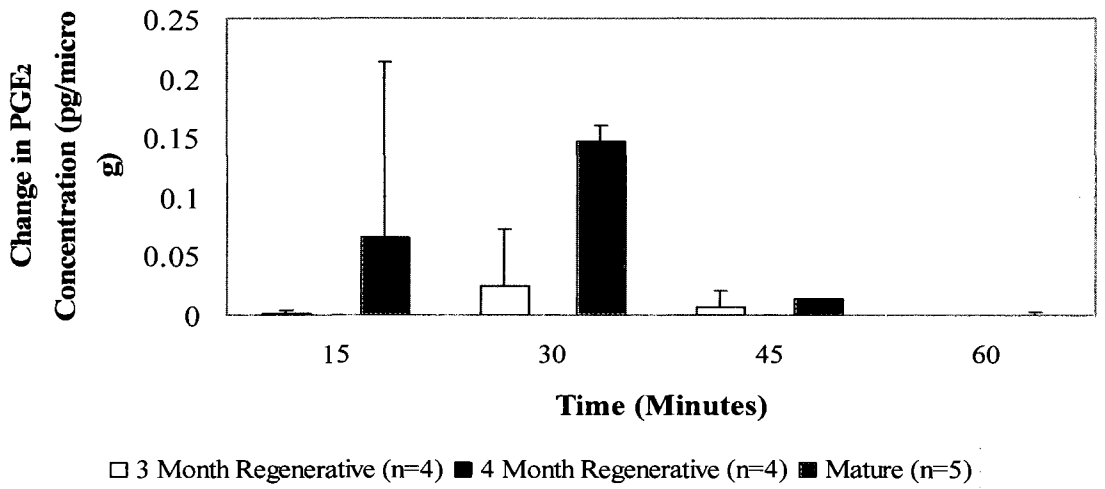


Figure 3.11D: All Changes in PGE_2 Concentration for Old Animals



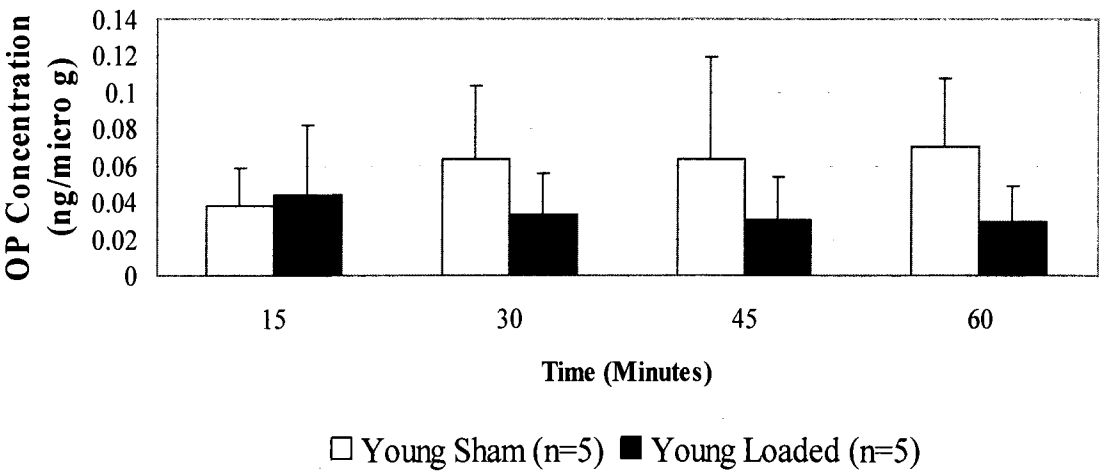
(C) The average increase in PGE_2 concentration per animal for mature specimens was highest for those from old animals after 15, 45, and 60 minutes of loading. **(D)** The highest increase in PGE_2 for old animals is observed in mature specimens at all loading time points.

Figure 3.11E: All Changes in PGE_2 Concentration for Young Animals



(E) The highest increase in PGE_2 for young animals is observed in mature specimens at all loading time points.

Figure 3.12A Osteopontin Production in Mature Specimens from Young Animals



(A) There is an increase in OP concentration after 15 minutes of loading in mature specimens from young animals.

Figure 3.12B Osteopontin Production in Mature Specimens from Old Animals

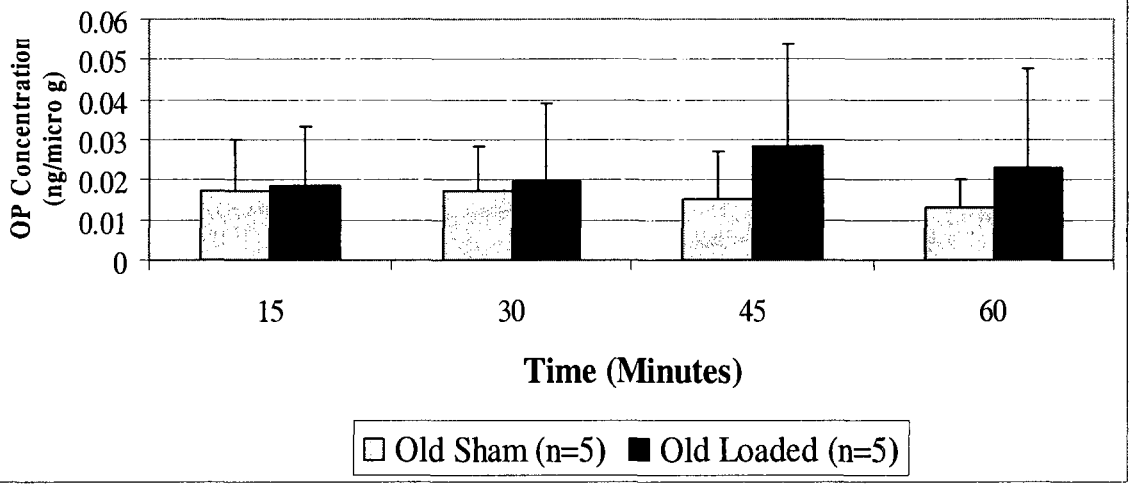
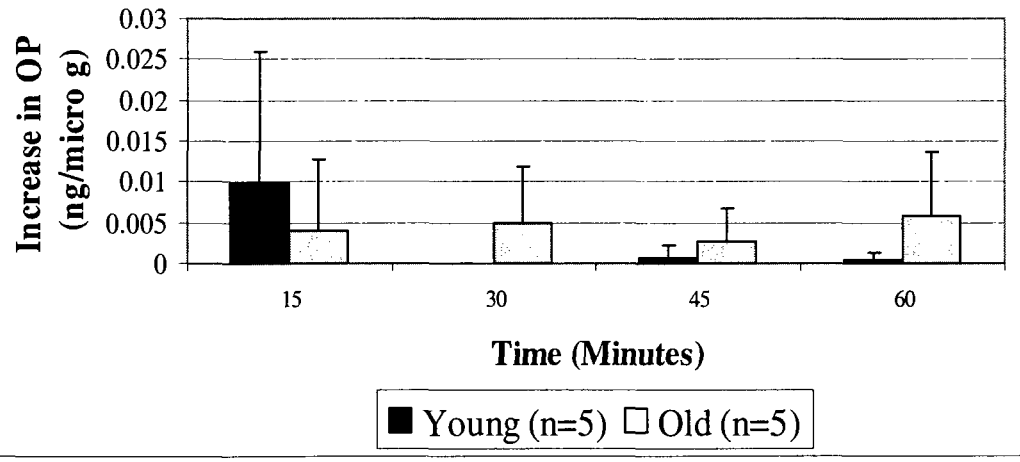


Figure 3.12C: Average Change per Animal in Osteopontin Production in Mature Specimens



(B) There is an increase in OP concentration after all time points of loading in mature specimens from old animals. **(C)** The average change in OP per animal was higher in young animals only after 15 minutes of loading.

Figure 3.12D: All Changes in Osteopontin Concentration for Old Animals

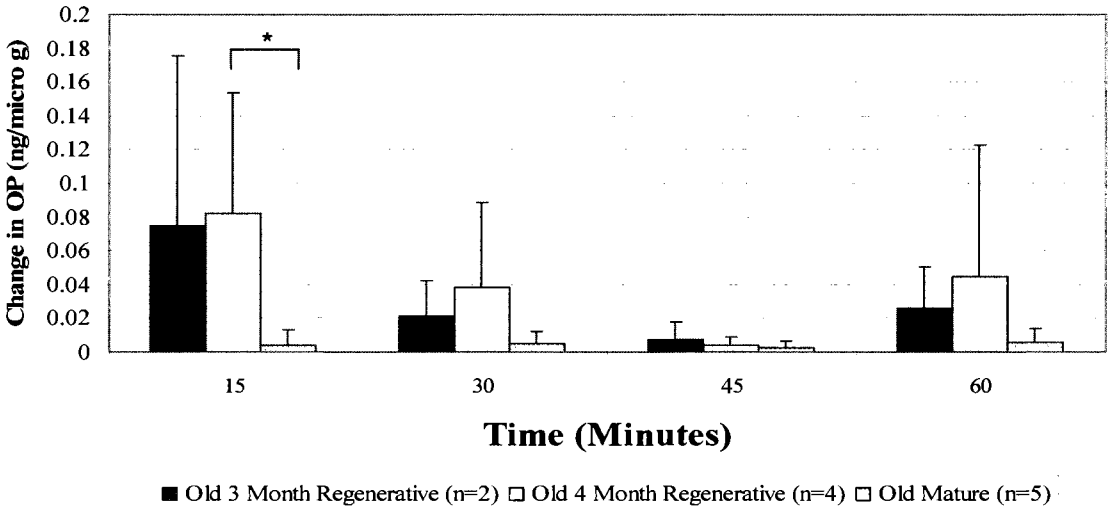
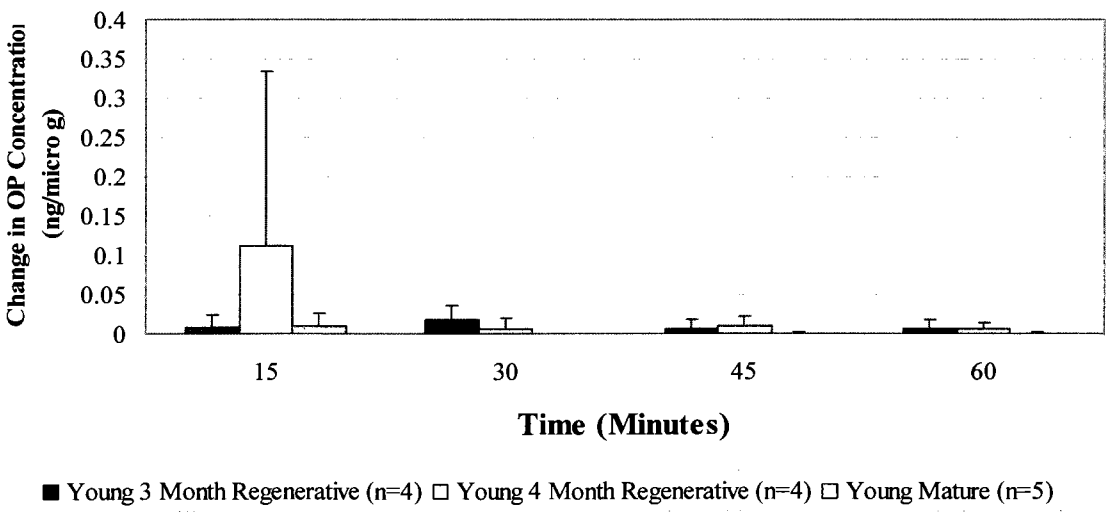
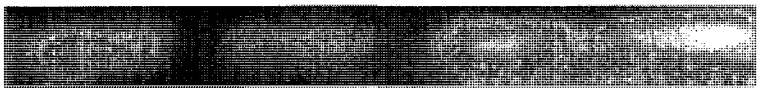


Figure 3.12E: All Changes in Osteopontin Concentration for Young Animals

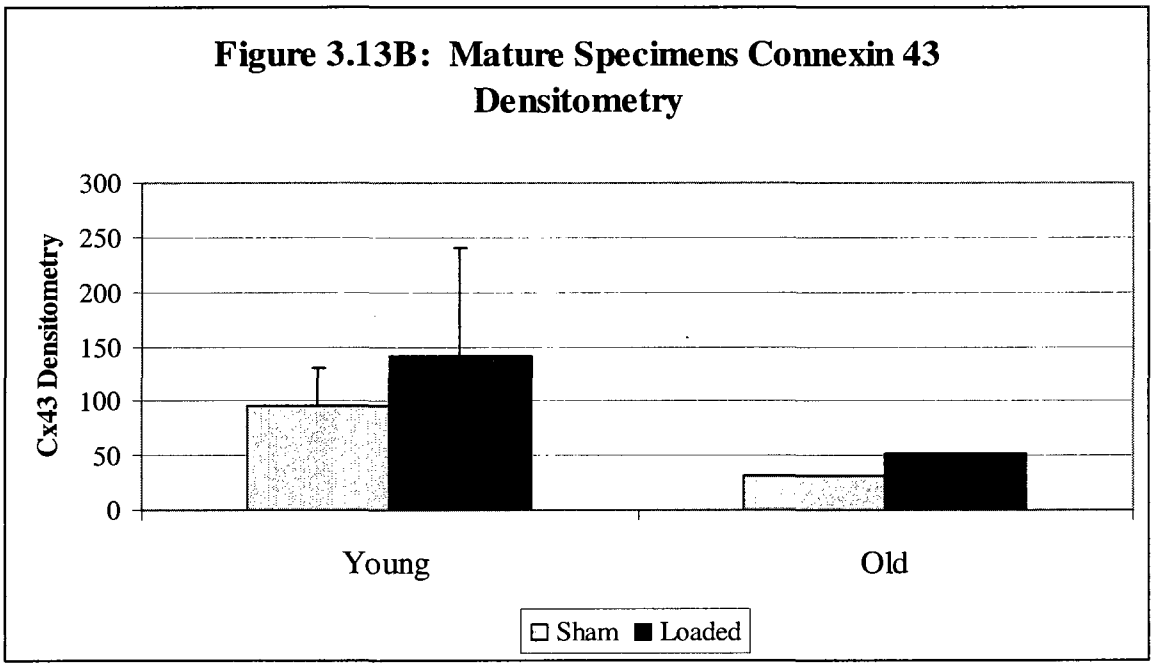


(D) Among specimens from old animals regenerative specimens produced in 4 mounts had the highest change in OP concentration at 15, 30, and 60 minutes of loading. **(E)** Among specimens from young animals regenerative specimens produced in 4 mounts had the highest change in OP concentration after 15 and 45 minutes of loading.

Figure 3.13A: Connexin 43 Mature Bone Western Blot



Young Sham Young Exp. Old Sham Old Exp.



(A) Sham represents sham treated and Exp. represents mechanically loaded specimens. Connexin 43 protein was detected in sham and mechanically loaded mature bone specimens harvested from young and old animals. **(B)** There appears to be an increase in connexin 43 protein with mechanical load in mature specimens from both old and young animals, however it was not statistically significant.

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CHAPTER IV

THE EFFECT OF AGE AND MATURARTION TIME ON MARROW STROMAL CELLS AND THEIR RESPONSE TO MECHANICAL LOAD

And it's you when I look in the mirror and it's you when
I don't pick up the phone sometimes you can't make it on your own

-Sometimes you Can't Make it on your Own

4.1 Introduction

The marrow stroma comprises the hematopoietic microenvironment which is involved in the maintenance and structural support of the marrow hemopoiesis. It is comprised of a network of fibroblastic cells, reticular cells, adipocytes, macrophages, endothelial, smooth muscle, and osteogenic cells. Studies suggest human bone marrow cells respond to the application of mechanical load similarly to the ways of the osteoblast and osteocyte and may be important in the proliferation and differentiation of bone marrow stromal cells (Riddle, R, et al., 2008). Human bone marrow stromal cells showed a significant increase in alkaline phosphatase gene expression, decreased type I collagen expression, and modified connexin 43 post fluid flow (Grellier, M. et al., 2009). Marrow stromal cells subjected to oscillatory fluid flow exhibit increased intracellular calcium mobilization, proliferation, and mRNA levels for osteopontin and osteocalcin genes (Li, Y. et al., 2004).

Typical strains in human bone tissue during vigorous exercise are < 2,000 micro strain or 0.2% deformation; however much larger strains (1%-10%) are necessary to activate bone cells (Burr, D. Bone and Riddle, R. et al., 2009). Studies suggest that interstitial fluid flow is an important part of the system by which tissue level strains are amplified in bone and that mechanical loads placed on the skeleton cause deformation of the tissue, pressurization of interstitial fluid, and its movement from the matrix into the Haversian system. It is thought that fluid flows outward from the cortex of long bones due to a hydrostatic pressure gradient as a result of medullary pressure. Studies showed that the movement of fluid produced streaming electric potentials. It is unknown whether cells respond to direct deformation of the extracellular matrix, fluid induced shear stress, pressure, streaming potentials, or some combination of these; however fluid makes up approximately 23% of the bone volume, can modulate bone cell function, and is a useful tool to observe the effect of mechanical forces on cells *in vitro* (Owan, I. et al., 1997).

Mechanical loading can enhance this flow by moving the fluid transcortically. Fluid shear stress depends on the cross section through which the flow travels, thus in the canuliculi (0.4μm in diameter) where the osteocyte processes (0.2μm) reside the fluid shear forces in bone are likely the highest. Shear stresses on the osteocyte processes are predicted to be 8-30 dynes/cm^2 based on theoretical models of bone matrix deformation (Weinbaum, S. et al., 1994). Marrow stromal cells could be exposed to fluid flow resulting from intramedullary pressure associated with mechanical loads or flow induced stresses as they migrate to sites of bone formation.

During bone development and remodeling osteoblast proliferation and differentiation are tightly regulated. With advancing age studies suggest that bone

marrow stromal cells have a diminished osteoblast differentiation capacity, however an increased adipocyte differentiation capacity. There is also evidence that the number of osteoprogenitor cells in human bone marrow markedly decreases after skeletal maturation (Nishida, S. et al., 1999; Chen, T. et al., 2004; Muschler, G. F. et al., 2001). In addition age may have an effect on bone formation within tissue engineered scaffolds. Inoue and colleagues found that the ratios of ALP activities of young bone marrow/hydroxy-apatite (HA) composites to old bone marrow/HA in young and old recipients were about five times and four times respectively (Inoue, K. et al., 1997). With advancing age bone marrow extracellular fluid may also inhibit bone cell proliferation (Egrise, D. et al., 1999). The ability of marrow stromal cells to proliferate and mineralize may be impaired with advancing age; however the effect of age on the ability of these cells to respond to mechanical load as they mature is unknown.

Interestingly, bone marrow stromal cells from experimental models of disuse form smaller and fewer osteogenic colonies thus they may suffer a deficit in proliferation potential and osteogenic capacity (Riddle, R. et al., 2007). Five days of hindlimb elevation lead to significant decreases in proliferation, alkaline phosphatase enzyme activity, and mineralization of BMSC cultures (Kostenuik, P. et al., 1997). In order to investigate the effect of age and maturation on marrow stromal cells and their response to mechanical loading cells were harvested from young, mature, and old animals and analyzed over a differentiation time course for osteoblastic maturation and response to mechanical loading.

4.2 Materials and Methods

4.2.1 Isolation of Marrow Stromal Cells

Tibia and femora were harvested from 2 (n=5), 9 (n=3), and 24 (n=5) month old animals under sterile conditions. Each end of the bone was removed and marrow was flushed from the bones into fresh Dulbecco's Modified Eagle Medium (DMEM) (Gibco), broken up with 18 and 21 gauge needles and plated in DMEM supplemented with 1% penstrep, 1% L-glutamine, and 15% Fetal Bovine Serum (FBS). After two weeks of culture to assess mineralization, cells were plated into 6-well tissue culture plates (BD Falcon) at a seeding density of 25,000 cells per 9.6cm^2 circular well. Two wells were maintained as controls and four wells were subjected to differentiation media consisting of 0.1% ascorbate and 0.1% beta glycerol phosphate (BGP). A separate population of cells was plated in 10 cm culture dishes differentiated for oscillatory fluid shear stress experiments.

4.2.2 Alizarin Red Stain

To determine the effect of age on MSC mineralization over time 25000 cells per well were seeded into six well plates, stained for Alizarin Red (1:100:10 Alizarin red, ddH_2O, and 0.1% Ammonium Hydroxide) and imaged with a Carl Zeiss (MicroImaging GmbH, Jena, Germany) microscope at 20x magnification after 3, 7, 10, 14, 21, and 28 days of differentiation. Alizarin red stain was solubilized with 0.1% Ammonium Hydroxide and its optical density read at 540nm (Spectra Max v5). Plates were then rinsed three times with 1X phosphate buffered saline (PBS) and wash buffer, incubated for 30 minutes in crystal violet (Sigma Aldrich), solubilized for one hour in 1% SDS, and

read at 550nm (Spectra Max v5 Molecular Devices, Sunnyvale, CA). Alizarin red optical densities were normalized to crystal violet optical densities.

4.2.3 Calcium Assay

25000 cells per well were seeded into six well plates and assayed for calcium after 3, 7, 10, 14, 21, and 28 days of differentiation. Cells were rinsed with 1X PBS, scraped in 1mL 0.5N HCL, and stored overnight at 4°C. Next samples were spun at 6000g for 10 minutes. The supernatant was assayed with a QuantiChrom Calcium Assay Kit according to manufacturer instructions (BioAssay Systems, Hayward, CA).

4.2.4 RNA Isolation

To determine the effect of age on markers of osteoblast maturation cells were plated at 25000 cells per well into six well plates and total RNA was harvested after 3, 7, 10, 14, 21, and 28 days of differentiation with an RNeasy Mini Kit following manufacturer's instructions (Qiagen, Valencia, CA). RNA was diluted in RNase free water and the concentration and purity was assessed with a spectrophotometer (Spectra Max v5). RNA was processed at the Affymetrix and Microarray Core Facility (University of Michigan, Ann Arbor, MI) to confirm its integrity.

4.2.5 RT-PCR

Oligonucleotide primers were designed to produce PCR fragments of the rat genes osteocalcin, alkaline phosphatase, and glyceraldehyde phosphate dehydrogenase (GAPHDH) based on published data (Kostenuik, P. et al., 1997). Each upper strand primer was designed to include a 5' *Eco*R restriction site and each lower strand primer contained a 3' *Hind* II site. The upper strand primer for Alkaline Phosphatase: 5'-TGCATGAATTCCCTGCCTTACCAACTCATTT-3'; lower strand primer for Alkaline

Phosphatase: 5'-TGCATAAGCTTGAGAGCCACAAAGGGGAACT-3'; upper strand primer for osteocalcin: 5'-TGCATGAATTCGACCTAGCAGACACCATGAG-3'; lower strand primer for osteocalcin: 5'-TGCATAAGCTTGTCATGAGCCCTT CCACGAT-3'; upper strand primer for GAPDH: 5'-TGCATGAATTCTGATTC TACCCACGGCAAGT-3', lower strand primer for GAPDH: 5'-TGCATAAGCTT GTCATGAGCCCTTCCACGAT-3'

4.2.6 Custom Oscillatory Fluid Shear Loading System

The loading device used in these experiments was adapted from the custom shear system developed by Ominsky (Ominsky, M. et al., 2003). Oscillatory fluid flow may be a more physiologic fluid flow profile than unidirectional fluid flow since *in vivo* loading of bone generates fluid shear stresses which are thought to be dynamic in nature. The device is based on the parallel plate flow chamber of Frangos, J. A. et al., 1985 and the oscillatory flow of You, J. et al., 2000. It can produce static, cyclic, or pulsatile fluid shear stress in the cell chambers with peak shears in excess of 4MPa. A custom designed, programmable syringe pump which produces intricate waveforms at higher frequencies was utilized. Laminar flow between the parallel plates produced fluid shear on the plate surface due to friction at the fluid-plate interfaces based on the laws of fluid dynamics. The equation $\tau=6\mu Q/bh^2$ governs this theory and is derived from the Navier-Stokes momentum equation of 2-D flow where μ=mean viscosity, Q=flow rate, b=channel width, and h=channel height. It is assumed that the media is a Newtonian fluid and media viscosity of α-MEM+2% FBS is assumed to be 0.00087Pa*s. To ensure that cells are exposed to laminar flow in the chamber the Reynolds number (Re), a number used to characterize flow, must be less than 2000. Since $R_e=\rho Qh/\mu$ if it is

assumed that Q=1m/s (high flow rate), and $\rho=1g/cm^3$ then $R_e=14.6<<1000$ which satisfies laminar flow criteria. As the fluid enters the chamber from a larger channel there is an entrance length (EL) over which the pressure must drop and the boundary layers merge for fully developed laminar flow. In this chamber $E_L=0.6Re_{max}h=0.11mm$ thus cells were cultured>1mm from the chamber walls to avoid this area of undeveloped flow.

Specimens were plated at a seeding density of $300x10^3$ cells on 38x75x1mm glass slides (Fisherbrand) and attached via an aluminum compression plate to a polycarbonate manifold. The manifold contained milled grooves for the glass slide (1mm deep), an o-ring to seal the slide to the manifold, and the flow area (0.13-0.26 mm deep). Slides were covalently modified with fibronectin and rectangular silicon rings (Sylgard 184, Dow-Corning), 3/8" thick creating a $14.2cm^2$ culture surface, were cast in an aluminum mold. A layer of silicon adhesive (RTV 108, GE Silicones) was applied to the bottom of the ring and cured on parafilm to enable an even and smooth surface. There rings were autoclave sterilized. The inlet and outlet ports were stainless steel made luer locks (McMaster-Carr), and the o-ring was autoclavable Aflas (sealsales.com, size AS586A-036). Two 10-32 screws were used to seal the chamber, and stainless steel Helicoil wire thread inserts were used to ease thread meshing and prevent excessive wear.

Flow rate in the chamber was controlled by a custom designed programmable syringe pump. Four glass syringe (10cc Perfekum with Luer Locks, Popper & Sons) connected to the chamber in which plated cells were placed. Autoclavable Teflon tubing (1/8" OD, 0.070"ID, Cole Palmer #064-7-42) was used to transport media from the syringes to the cell chambers and reservoir. Luer connectors made of polycarbonate

(Qosina) were bonded to the tube ends to allow stable seals with all components. The ends of the PTFE tubing were first sodium etched (Tetra-Etch Etchant, WL Gore), then sealed in the pocket-fit connectors with Loctite 4981 Medical Device Adhesive. To reduce air from tubing before shear loading media was filled from both the syringe and reservoir sides before the glass slide was sealed. Media from the reservoir filled by gravity and a one way stop cock (Qosina #99705) stopped flow once it had been evacuated. The syringe was directly connected to the input port on the shear chamber and the output port was connected to the lower media reservoir port. Media oscillated into and out of the reservoir. The media reservoir had wells which could hold up to 18mL and three stainless steel luer ports. Luer activated check valves (Qosina #80363) were used to retain media in the reservoir while disconnected from the pump outside the incubator during set up. Cells were exposed to 2Pa oscillatory fluid shear stress at 0.5Hz.

4.2.7 Quantification of Nitric Oxide and Prostaglandin E_2

Soluble nitric oxide and prostaglandin E_2 concentrations were calculated to assess response to mechanical stimulation as described in chapter two with colorimetric assay kits from Cayman Chemical (Ann Arbor, MI).

4.7.8 Calculation of Bone Morphogenetic Protein 2 (BMP-2)

Bone morphogenetic proteins are found in the bone matrix and can be released to interact with marrow stromal cells. Osteoblasts synthesize and secrete BMPs both *in vitro* and *in* vivo. Bone formation and resorption during remodeling may be linked through BMPs as BMPs are thought to regulate the transcription of many osteoblast specific transcription factors which modulate RANKL, an important signal for differentiation of hematopoietic cells into osteoclasts. With advancing age changes can

occur in the BMP levels and studies suggest that bone specific overexpression of BMP antagonists produce animals with an osteopenic phenotype (D'Ippolito, G. et al. 1999; Devlin, R. D. et al, 2003).

50μL of sample and standard were assayed in triplicate with a kit from R&D Systems (Minneapolis, MN). 100μL of assay dilutent RD1-19 was added to each well. The plate was incubated for two hours at room temperature on a horizontal orbital microplate shaker. Following incubation, each well was aspirated and washed with wash buffer a total of four times. 200μL of BMP-2 conjugate was added to each well, the plate covered and incubated for two hours on a shaker at room temperature. Following the incubation period, the plate was washed four times with wash buffer. 200μL of substrate solution was added to each well and the plate incubated for thirty minutes at room temperature in the dark. Next, 50μL of stop solution was added to each well and the optical density read on a plate reader (Spectra Max v5 Molecular Devices, Sunnyvale, CA) at 540nm and 450nm. The readings taken at 540nm were subtracted from the readings taken at 450nm. The unknown BMP-2 concentration of samples was determined by comparing these readings to a standard curve.

4.2.8 Western Blot

Cells were rinsed with 1X PBS and total protein was extracted as described in chapter 2. Protein was quantified with a BCA assay and 10μg of protein were boiled and loaded into the lanes of a 10% Tris-HCl gel with an equal volume of 2X sample buffer. The gel was run at 150V for 90 minutes and transferred to a nitrocellulose membrane for 40 minutes at 80V. Subsequent transfer blots were incubated with Ponceau Red for 30 minutes to determine the quality of the transfer. Blots were incubated at 4°C over night

with 5% bovine serum albumin (BSA) in 1X Tris Buffered Saline (TBS) with 0.05% Tween under gentle shaking. Blots were then incubated with primary antibodies for phosphorylated extracellular regulated kinase (pERK) (1:2000), extracellular regulated kinase (ERK) (1:1000), connexin 43 (1:500), and GAPDH (1:2500) overnight at 4°C under gentle shaking. Blots were then rinsed three times for 10 minutes each with 1X TBS with 0.05% Tween. Blots were then incubated for 120 minutes at room temperature with secondary antibody (1:25,000) Goat anti-rabbit IgG (Thermo Scientific, Rockford Il)

4.2.9 Statistical Analysis

A two sided unpaired t test was used to determine statistical significance within the data set. P values less than 0.05 are expressed with * and p values less than 0.01 are expressed with **.

4.3 Results

Marrow stromal cells harvested from 2 month, 9 month, and 24 month old animals differentiated over time. Alizarin data showed a delayed mineralization time for cells harvested from 24 month old animals when compared to 9 month and 2 month (**Figure 4.1**). The solubilized data confirms that alizarin red normalized to total DNA was less for cells harvested from 24 month old animals when compared to cells harvested from 9 month old animals at all differentiation time points; however most noticeably the earlier ones (**Figure 4.2**). Alizarin red data is supported by extracellular matrix calcium levels which increased in differentiated marrow stromal cells over time and was greatest in cells harvested from 9 month old donors when compared to 24 month old **(Figure 4.3)**. There was a significant difference in calcium concentration between cells harvested from 9 month old animals and cells harvested from 24 month old animals after 21 days of

differentiation **(Figure 4.3)**. Solubilized values of crystal violet indicate that cellular DNA increased in cells harvested from 2 month, 9 month, and 24 month old animals over time **(Figure 4.4)**.

Normalized BMP-2 expression per day increased in cultured primary marrow stromal cells for all age groups and reached its peak at day 10 **(Figure 4.5)**. This up-regulation was less in cells harvested from 24 month old animals when compared to 9 month old animals with the exception of day 14. By day ten normalized values of BMP-2 expression from cells harvested from 24 month old animals dramatically approach the same value as BMP-2 expression from cells harvested from 9 month old animals. Normalized *osteocalcin* and alkaline phosphatase mRNA was up-regulated in differentiated marrow stromal cells from 24 month and 9 month old donors, with the exception of 24 month old animals at day 3 **(Figure 4.6, 4.7)** when compared to control cells. The increase in *osteocalcin* and alkaline phosphatase gene expression was higher in cultured cells from 9 month old animals compared to 24 month old animals with the exception of alkaline phosphates measurements at day 10 and day 21 **(Figure 4.8, 4.9)**.

Representative western blots of phosphorylated ERK and total ERK are shown in **Figure 4.10** for 2 month, 9 month, and 24 month old animals over a 21 day differentiation time course. There was an increase in ERK phosphorylation for marrow stromal cells harvested from 2 month, 9 month, and 24 month old donors at all differentiation time points and after short and long periods of loading with the exception of 2 month animals day 10 after 120 minutes of loading, 9 month animals day 21 after 30 minutes of loading, and 24 month animals day 7 and day 10 after 30 and 120 minutes of loading **(Figure 4.11A-F)**. The increase was highest in cells from 9 month old animals

for all differentiation time points after thirty minutes of loading and again after 120 minutes of loading with the exception of day 10 where phosphorylated ERK was higher in loaded cells from 2 month animals **(Figure 4.11G,4.11H)**. There was a statistically significant increase in phosphorylated ERK for 9 month cells differentiated for 21 days and loaded for 120 minutes **(Figure 4.11D)**.

Oscillatory fluid flow induced an increase in normalized nitric oxide concentration in marrow stromal cells from each age group and for all time points of differentiation except 2 month old animals day 10 after 120 minutes of loading **(Figures 4.12A-F)**. With increased differentiation time cells from 9 month and 24 month old animals steadily increase their production of nitric oxide during both short and long periods of loading **(Figure 4.12C-F)**. There was a statistically significant increase in NO expression in cells harvested from 9 month old animals on day 3 loaded for 120 minutes **(Figure 4.12D)**. After a short and long period of loading this increase was greatest for cells harvested from 9 month old animals at all differentiation time points **(Figures 4.12G, 4.12H)**. There is a significant difference in increased nitric oxide expression between 2 month and 9 month old animals at day 7 after 30 minutes of loading, and approaching significance between the two age groups after 30 minutes of loading on day 3 ($p=0.7$) **(Figure 4.12G)**.

Prostaglandin E_2 concentration increased with loading for all age groups, loading time points, and differentiation days except in cells from 2 month animals at day 21 after 30 and 120 minutes of loading, 2 month animals day 10 after 120 minutes of loading, 9 month animals day 3 after 30 minutes of loading, and 9 month animals day 10 after 120 minutes of loading **(Figures 4.13A-F)**. There was a significant increase in PGE_2

expression in cells from 9 month old animals loaded for 30 minutes after 3 days of differentiation and in cells from 24 month old animals loaded for 30 minutes after 21 days of differentiation **(Figure 4.13C, 4.13E)**. There was an increase in PGE_2 concentration in cells from 24 month animals for all loading time points and days of differentiation, however only absolute values of PGE_2 comparable to sham PGE_2 values for 9 month and 2 month animals were observed after 30 minutes of loading on day 3 **(Figure 4.13E)**.

4.4 Discussion:

Marrow stromal cells have the ability to self renew and differentiate and are the source for replacing the cells lost on a daily basis during regeneration which spans the entire life of an organism. Marrow stromal cells are also a useful component of tissue engineering constructs as they can enhance tissue formation in regions that are difficult to heal. Previous studies have shown that bone marrow stromal cells have the capacity to respond to mechanical stimulation similar to osteoblasts. It is also known that fluid flow can enhance mineralization of marrow stromal cells in tissue engineering scaffolds. Bancroft and colleagues found that flow perfusion induced *de novo* tissue modeling with the formation of pore like structures in the scaffolds, enhanced the distribution of cells and matrix throughout the scaffold, and increased mineralized matrix production (Bancroft, G. et al., 2002).

With aging there are many changes that occur in the marrow stromal cell population. Zhou and colleagues found that there was no significant difference in the percentage of STRO-1+ cells with subject age, however there was a uniform prolongation of the duration of all phases of the cell cycle in cells from older subjects as well as slower

cell expansion. Telomere length in MSCs from young donors is significantly longer than in adult donors and the average loss *in vivo* could be approximately 17bp/year *(Roobrouck, V.* et al., 2008*)*. In this study the osteoblast differentiation capacity of marrow stromal cells appears to be decreased and delayed with age. This is consistent with other studies in which there was an age dependent decrease in the percent of sorted marrow stromal cells that differentiated into AlkP-positive osteoblastic cells.

With age more cells were apoptotic and had increased *p*53, *p*21, and *BAX* expression. Fourfold more human bone MSCs tested positive for senescence β-galactosidase in samples from older than young subjects, the doubling time for hMSCs was 1.7-fold longer in cells from older subjects, and there was an age dependent decrease in proliferation and osteoblast differentiation *(Zhou, S. et al., 2008l).* Solubilized alizarin red data appears to increase, plateau, and then decrease over time. This decrease in alizarin red is likely the result of normalizing the data to crystal violet optical density which increased dramatically over differentiation time.

The up-regulation of normalized *osteocalcin* and alkaline phosohatase mRNA in differentiated marrow stromal cells from 9 month old donors was higher than that observed in 24 month old donors for the majority of time points. This data is consistent with a previous study in which the relative mRNA values of Runx2 were lower in differentiated marrow stromal cells from old donors compared to young (Zhang, W. et al., 2008). In another study the yield of alkaline phosphatase-positive colonies decreased with age however did not correlate with the gender of the donor (Majors, A. K. et al., 1997).

Lu and colleagues found that by day 5 through 10 *osteocalcin* levels were significantly higher in fractures from young animals (Lu, C. et al., 2005). Similar to what was found in this study cells from 9 month old and 24 month old animals expressed peak values of osteocalcin at days 14-18. Cells from 9 month old and 24 month old donors have a similar up-regulation of osteocalcin and alkaline phosphatase mRNA after ten days of differentiation which is consistent with the increase in relative type I collagen mRNA levels measured in marrow stromal cells from mature and old animals (Zhang, W. et al., 2008). Data from this study suggests there is a decrease in both the up-regulation of early and late stage markers of osteoblast differentiation with advancing age and that this disparity continues over a twenty-eight day differentiation time course.

BMP-2 expression per day was higher in differentiating cells from 9 month old animals compared to 24 month old animals during days 1-10. This is consistent with data from a fracture healing study in which expression of BMP-2 was measured in the fracture callous. In that study fractures harvested from young animals had elevated BMP-2 expression early during the endochondral phase of fracture healing with peak expression during days 5 through 10 with a subsequent decrease during the bone formation phase of repair (Naik, A. et al., 2009). A similar decrease in BMP-2 expression was observed in cells from both age groups after 10 days of differentiation. By day 10 cells from 24 month old animals begin to express BMP-2 at a concentration very similar to cells harvested from 9 month old animals. Interestingly at day 10 and 21 the change in *osteocalcin* and alkaline phosphatase mRNA from differentiated cells compared to untouched control cells is similar for cells from 9 month and 24 month old animals.

Mitogen-activated protein kinase (MAPK) is involved in the differentiation and commitment of pluripotent mesenchymal cells towards the osteoblastic lineage. The suppression of MAPK can lead to adipogenesis while studies have shown that MAPK signaling is involved in the commitment and differentiation of primary bone marrow stromal cells (Chan, G. et al., 2002). Wadhwa et al. found that fluid shear stress transcriptionally induces COX-2 gene expression in osteoblasts, and the maximum induction requires new protein synthesis which is largely via the ERK signaling pathway (Wadhwa, S. JBMR). In addition, the inhibition of ERK can attenuate calcium deposition by 55% (Simmons, C. A. et al., 2003). Furthermore, the presence of both ERK and p38 inhibitors can abolish the effect of oscillatory fluid flow on steady state osteopontin mRNA levels (Gwendolen, J. et al., 2001). In this study cells harvested from 2 month, 9 month, and 24 month old donors were responsive to oscillatory fluid shear stress during all time points of differentiation and produced conformational changes in ERK. Normalized ERK phosphorylation was higher in loaded cells from 9 month old animals when compared to 24 month old animals for long and short periods of loading and at all differentiation time points. This difference in ERK phosphorylation could affect cell signaling and the commitment of marrow stromal cells. These cues could push surrounding marrow stromal cells down the adipocyte lineage in the older population.

The primary NOS isoform found in adult bone is endothelial (eNOS) and eNOS deficient transgenic mice (eNOS-/-) have significant abnormalities in bone formation and increased blood pressure. Young transgenic mice have reduced bone volume and bone formation rates, fewer osteoblasts in trabecular bone, and decreased mineralization capacity. Rahnert and colleagues found that prolonged mechanical strain can increase

nitric oxide generation and eNOS mRNA expression, however inhibit RANKL expression in stromal cells harvested from C57BL/6 wild type mice. When stromal cells were treated with a NOS inhibitor which blocks all three NOS isoforms mechanorepression of RANKL was prevented (Rahnert, J. et al., 2008). In this study there was an increase in nitric oxide expression in marrow stromal cells harvested from young, mature, and old animals exposed to oscillatory fluid shear stress after 3, 7, 10, and 21 days of differentiation. Differentiated marrow stromal cells from 24 month old donors had less of an increase in nitric oxide than cells from 9 month old animals after subjection to mechanical loading. A difference in RANKL signaling could result from this difference in nitric oxide production with age. Perhaps more RANKL is suppressed after the mechanical loading of cells from 9 month old donors and there is a reduction in osteoclastic bone resorption.

Osteocytes and osteoblasts increase expression of cyclooxygenase-s (Cox 2) and release of prostaglandin-E_2 (PGE_2) in response to either unidirectional or oscillatory fluid flow (Ponik, S. et al., 2007). Klein-Nulend and colleagues found no evidence of loss of mechanosensitivity to pulsatile fluid flow (PFF) with donor age with the measurement of PGE_2. Cell culture from old donors had a higher PGE_2 response than cultures from younger donors, however studies suggests that fluid flow forces in bone are dynamic and oscillatory in nature. Bakker and colleagues found that cells from osteoporotic and osteoarthritic donors were also able to respond to pulsating fluid flow with significant increases in PGE_2 and NO expression (Bakker, A. D. et al., 2006). In addition bone cells from the iliac crest of nine elderly women subjected to PFF treatment released a 3.5 fold increase in PGE_2 and 2.9 fold increase in COX-2 mRNA (Joldersma, M. et al., 2000).

Data from this study demonstrates the effect of oscillatory fluid flow on marrow stromal cells harvested from 2 month, 9 month, and 24 month old animals over a twenty-one day differentiation time course.

Data from this study suggests mineralization is delayed in cells from 24 month old animals, however with time their mineralization capacity is comparable to cells harvested from 9 month old animals. Cells from 9 month old animals appear to have a greater ability to respond to mechanical stimulation than cells from 24 month old animals as measured by nitric oxide expression, ERK phosphorylation, and PGE_2 concentration, despite this equilibrium of mineralization at later time points. Perhaps cells from 9 month old animals mineralize faster than those from 24 month old animals and acquire a different sensitivity to their mechanical environment over their differentiation time course. Mechanical sensitivity was not assessed in this study and it would be interesting to measure cellular mechanical response under varied load and deformation conditions. The distribution of cells differentiated among the cells harvested from mature and old animals is not known. This could affect the ability of the cells to respond to mechanical stimulation as a unit. Future work could use FAC sorting to determine how many cells from each population are in various osteoblast differentiation states. There could be a higher proportion of more mature osteoblasts in cells harvested from 9 month old animals and differentiated over time. This might enable the cells as a unit to have a greater mechanical response.

Although it is know that bone cells respond to oscillatory fluid shear stress the exact mechanisms of that transduction are not completely understood. Studies to examine the effect of fluid flow on osteoblasts suggest increased viscosity of the fluid

media causes an increase in the osteoblastic response even if the flow rate is held constant and others have found no relationship between convective current density and intracellular calcium changes induced by fluid flow (Owan, I. et al., 1997). This may suggest that the osteoblastic response is attributable to fluid shear stress which depends on the viscosity as opposed to convective currents and streaming potentials. In this study some markers used to measure mechanical response showed diminished load effects at later time periods of differentiation. This could be the result of a smaller mechanical stimulus to the cells due to their increased mineralization.

Cells in this study were plated on gelatin coated slides and subjected to laminar oscillatory fluid shear stress. However, the importance of a 3D ECM has been recognized for many cell types. For example, in epithelial cells, a 3D environment facilitates normal epithelial polarity and differentiation. The culture of fibroblast cells on flat 2D substrates introduces an artificial polarity between lower and upper surfaces of these normally non polar cells and their morphology and migration differ once they are suspended on collagen gels (Murshid, S. et al., 2007 and Hoffler, C.E. et al, 2006). The true biological environment of a bone cell is comprised of a dynamic interaction between responsively active cells experiencing mechanical forces and a continuously changing 3D matrix architecture. *In vitro* studies in 3D dramatically increase mineralized matrix production, total calcium content, and enhanced distribution of cells and matrix throughout scaffolds (Bancroft, G. et al., 2002).

Alizarin red and calcium data from this study suggest a difference in the time course of mineralization in marrow stromal cells harvested from 2 month, 9 month, and 24 month old donors. The amount of mineral present could affect the local deformation

of the cells. Further work should focus on the measurement of cellular strains over a differentiation time course. Future, more controlled experiments could subject the cells from different age groups to a local strain matched mechanical load and subsequently observe their response to mechanical stimulation. In this study nitric oxide, phosphorylated ERK, and PGE_2 were used to assess a mechanical response. A measurement of an anabolic response to mechanical stimulation such as the measurement of osteopontin is lacking. Future work should examine the effect if age and differentiation on cellular bone formation markers.

In this study marrow stromal cells were assessed for their ability to differentiate into osteoblasts, however studies suggest that marrow stromal cells are more likely to differentiate into adipocytes with advancing age. Total marrow fat increases with age and there is an inverse relationship between marrow adipocytes and osteoblasts with aging (D'Ippolito, G. et al., 1999). Aging can cause a decrease in the commitment to the osteoblast lineage and the expression of collagen and osteocalcin is decreased with age while the expression of adipocytes transcription factor PPARγ2 and fatty acid binding protein aP2 (Moerman, E. et al., 2004) increases. An interesting study would be to analyze the potential of marrow stromal cells from a young, mature, and old population to differentiate into osteoblasts and adipocytes. In addition studies suggest that in old mice there is an increase in the number of marrow cells capable of forming osteoclasts in co-culture and their responsiveness to growth factors thought to be in the monocyte and osteoclast series such as Il-3, GM-CSF, and M-CSF (Kahn, A. et al., 1993). The expression of marrow stromal cell mRNA transcripts of OPG, assessed with RT-PCR, suggests a decline with age (Makhluf, H. et al., 2000).

The expression of RANKL and OPG can also change with age. In 2003 studies showed that RANKL mRNA levels in whole bone were 2.1 fold and 4.4 fold higher in adult and old mice respectively when compared to young mice. The expression of RANKL was higher and OPG lower in cells from older animals early in culture and with cell maturation RANKL mRNA levels in cells from young and adult mice increased while levels in cells from old animals decreased. However by late days of culture (days 21 and 28) there were no differences with age in RANKL mRNA (Cao, J. et al., 2003). Future work should examine the ability of hemiatopoeic cells from young, mature, and old populations to differentiate into osteoclasts. Osteoclast formation can increase dramatically when stromal/osteoblastic cells from old compared with young donors were used to induce osteoclastogenesis (Cao, J. et al., 2005). Co-cultures of adipocytes, osteoblasts, osteoclasts, and marrow stromal cells from different aged animals could provide interesting data on the effect these cells have on cell proliferation and differentiation in a context more similar to what occurs *in vivo*. *In vivo* these cells could communicate with one another and studies suggest that mechanical stimulation can alter the fate of marrow stromal cells adjacent to the stimulus source and also those at a considerable distance as assessed by the upregulation of Runx2 (Leucht, P. et al., 2007).

Osteocytes are convincing candidates for sensing mechanical perturbations to their environment as they are ideally situated to sense mechanical load and communicate with each other and bone lining cells (Van der plas, A. et al., 1994; Burger, E. et al., 1999). In this study the effect of age on differentiating marrow stromal cells and their ability to respond to mechanical load was examined. Although osteocytes are difficult to isolate, the study of these cells harvested from young and old donors either *in vitro* in

three dimensional loaded scaffolds or *in vivo* through fluorescent tags labeled for specific osteocyte proteins would be useful. Kurpinski and colleagues found that mechanical sensing by MSCs may be anisotropic (Kurpinski, K. et al., 2006). Perhaps cell alignment or orientation to strain differs with aging and could results in a difference in their response to mechanical load. Future work could utilize micropatterning to align cells in specific directions to the applied mechanical strain.

In conclusion, mineralization appears to be delayed in marrow stromal cells harvested from 24 month old animals especially when compared to marrow stromal cells harvested from skeletally mature (9 month old) animals. This was supported by alizarin red staining for mineralized nodules, calcium concentration, BMP-2 expression, and the expression of the osteoblast genes alkaline phosphatase and osteocalcin. While mineralization appears delayed in marrow stromal cells from 24 month old animals the concentration of DNA, which indirectly provides a sense of proliferation, is not delayed when compared to cells harvested and cultured from 2 month old and 9 month old animals.

PGE_2 expression appears to be minimally affected by mechanical stimulation in cells harvested from 24 month old animals and loaded for 30 and 120 minutes over the differentiation time course. An increase and then decrease over the differentiation time course is observed in PGE_2 concentration in cells harvested from 9 month old animals which were mechanically loaded. This could be due to a decrease in locally applied strain, due to changes in mineralization, which may no longer be at the required threshold to induce a mechanical response through increased PGE_2 expression.

With increased differentiation time and mineralization there is an increase in nitric oxide concentration after mechanical stimulation for both a short and long period of loading in cells from 9 month and 24 month old animals. This increase in NO is less in mechanically loaded cells harvested from 24 month old animals compared to cells from 9 month old animals for all differentiation time points after 30 minutes of loading and after late differentiation time points for 120 minutes of loading. After days 3, 7, 10, and 21 of differentiation there is a greater increase in osteocalcin mRNA expression in cells from 9 month old animals compared to 24 month old animals. Perhaps the proportion of mature osteoblasts is larger in the cells harvested and cultured from 9 month old animals and thus they have a higher response to mechanical stimulation through NO production. NO has a relatively short half life so this age related increase difference in nitric oxide may be observed more often after a short period of mechanical loading.

Phosphorylated ERK normalized to total ERK has an early and late differentiation time point peak in cells harvested from 24 month old animals which were mechanically loaded for 30 and 120 minutes. This increase is less than the normalized proportion of phosphorylated ERK observed in cells from 9 month old animals after both loading time periods for all differentiation time periods. Since RT-PCR data suggests there may be more mature osteoblasts present in the cell population harvested from 9 month old animals compared to cells from 24 month old animals there could be a higher increase in phosphorylation of ERK. Phosphorylated ERK may peak and then decline with increased differentiation time in cells from 9 month old animals because the cells are more mineralized and the locally applied shear stress may not be enough to add the chemotransport effects that it might at a lower local shear stress during less mineralized

states. An early and late differentiation time point peak in pERK may be observed in cells from 24 month old animals because the proportion of mineral to cell is lower at these time points and could enable the cells to sense a greater local stress.

Figure 4.1A 2 Month Alizarin Red Stain

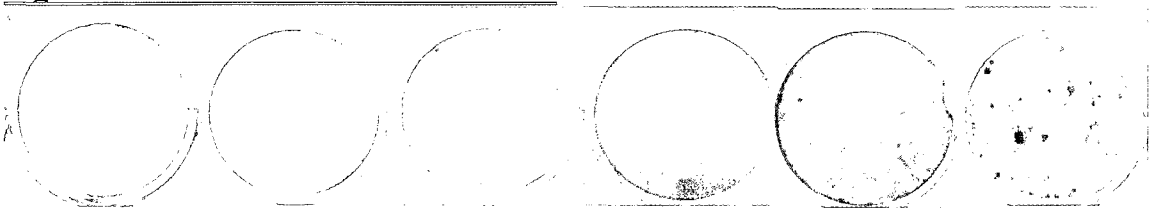


Figure 4.1B 9 Month Alizarin Red Stain

Figure 4.1C 24 Month Alizarin Red Stain

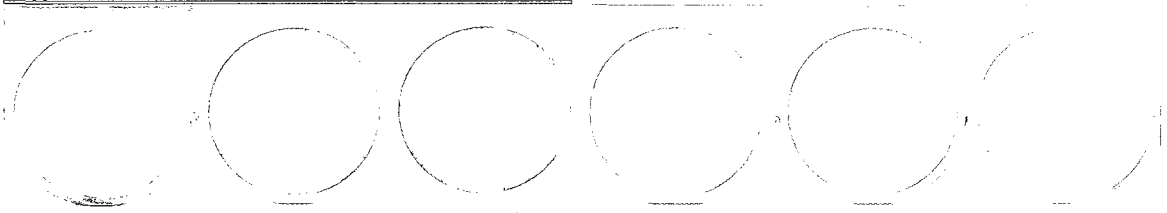
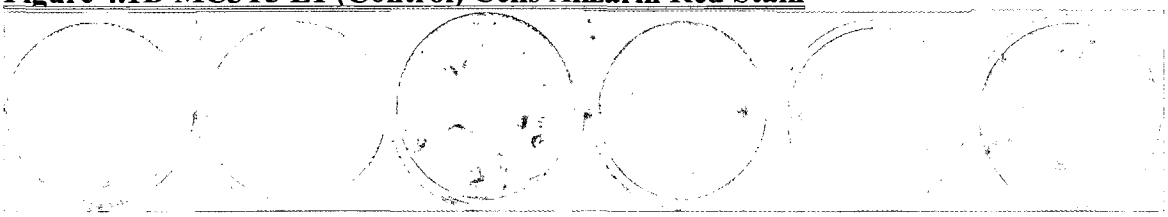
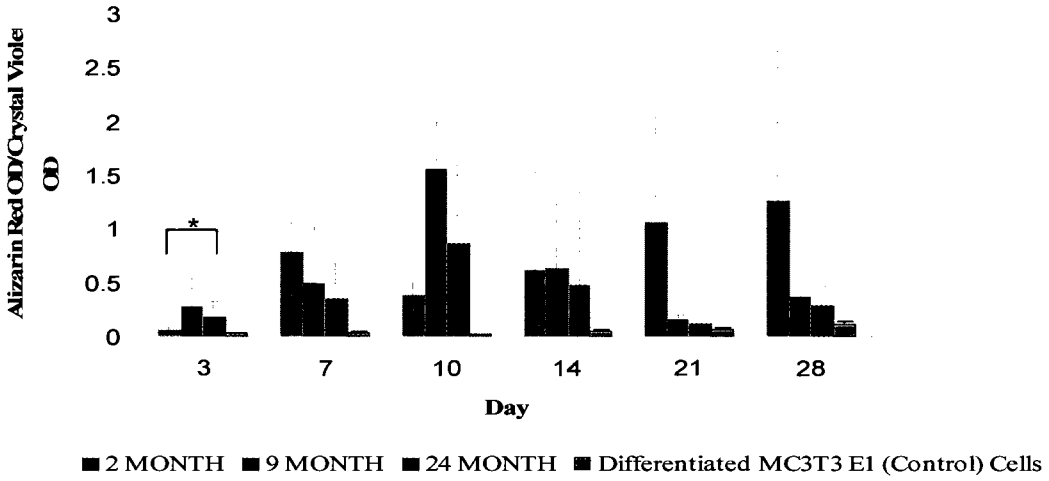


Figure 4.1D MC3T3 E1 (Control) Cells Alizarin Red Stain



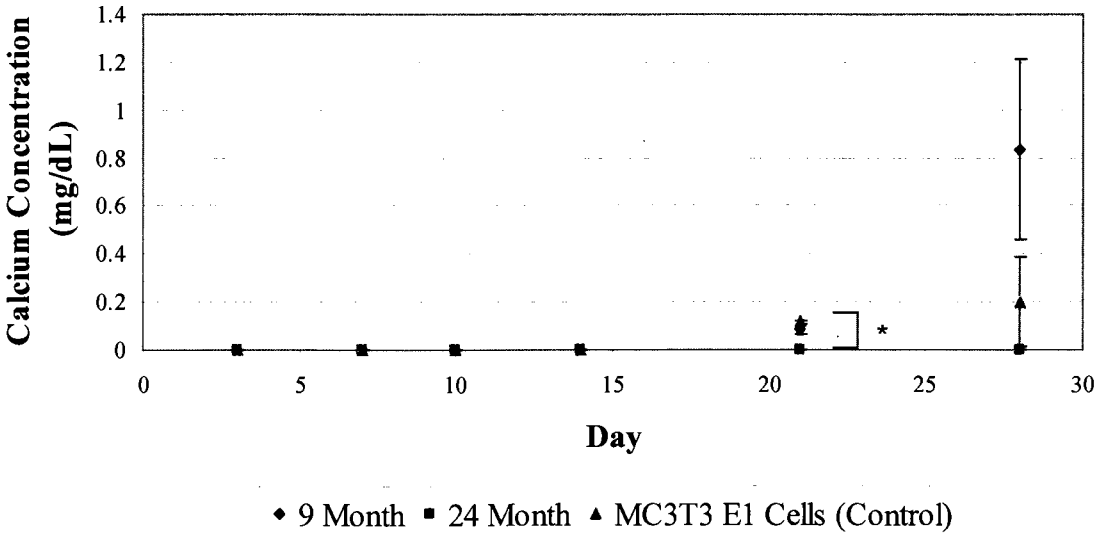
(A) Cells from 2 month old animals stain positive for alizarin red over the differentiation time course **(B)** Cells from 9 month old animals stain positive for alizarin red over the differentiation time course **(C)** Cells from 21 month old animals stain positive for alizarin red over the differentiation time course, however its onset is delayed. **(D)** MC3T3-E1 cells stain positive for alizarin red over the differentiation time course.

Figure 4.2: Normalized Alizarin Red Stain in Differentiated MSCs



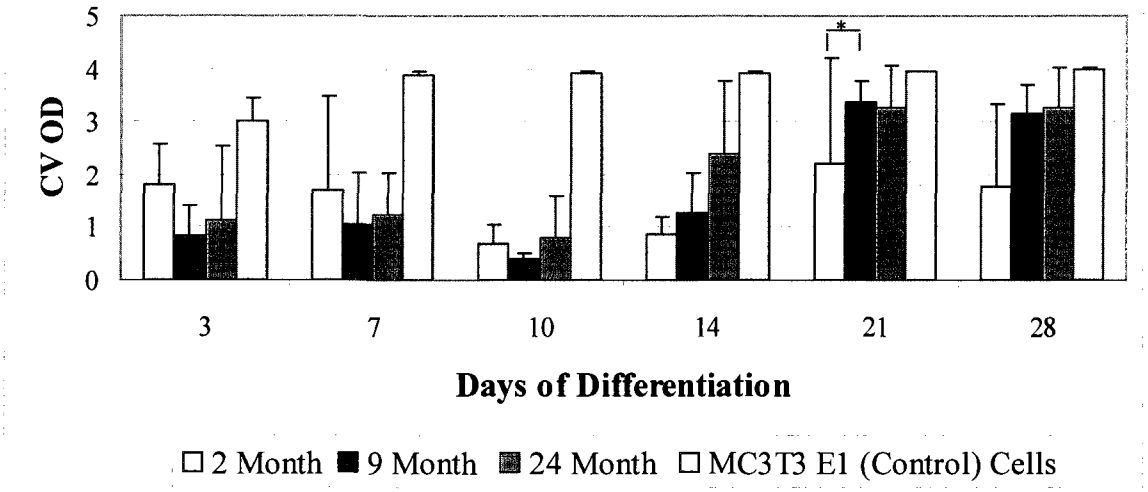
Normalized alizarin red stain increases over time up to day 10 and then decreases in cells harvested from 9 month old and 24 month old animals. The increase in normalized AR is higher for cells from 9 month old animals up through day 14 when compared to 24 month animals.

Figure 4.3: Temporal Calcium Concentration



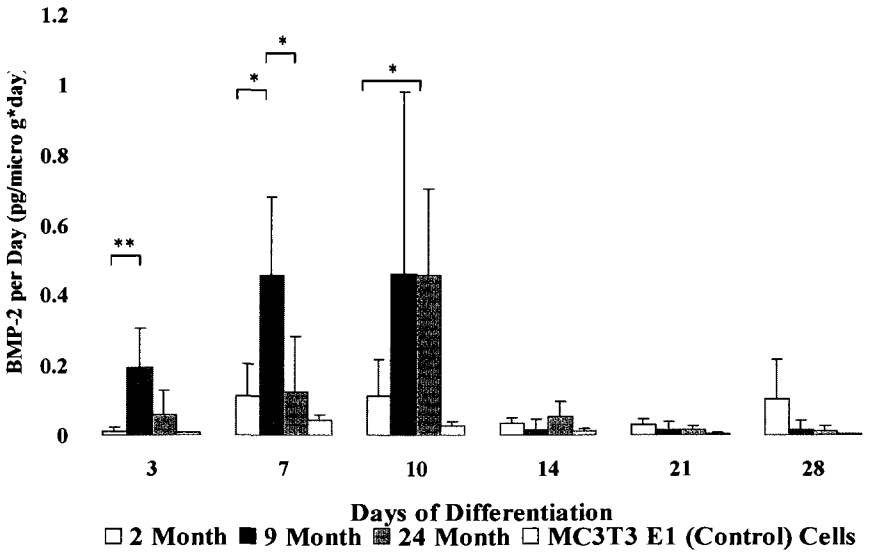
Calcium concentration increases with differentiation time in MSCs from 9 month old animals and MC3T3 E1 cells.

Figure 4.4: Marrow Stromal Cell Crystal Violet Optical Density



MC3T3 E1 cells and cells from 2 month old, 9 month old, and 24 month old animals increase DNA content over time.

Figure 4.5: MSC BMP-2 Expression



BMP-2 expression increases up through day and then decreases for differentiated MC3T3 E1 cells and cells harvested from 9 and 24 month old animals.

Figure 4.6A: Osteocalcin mRNA in Cells from 9 Month Old Animals

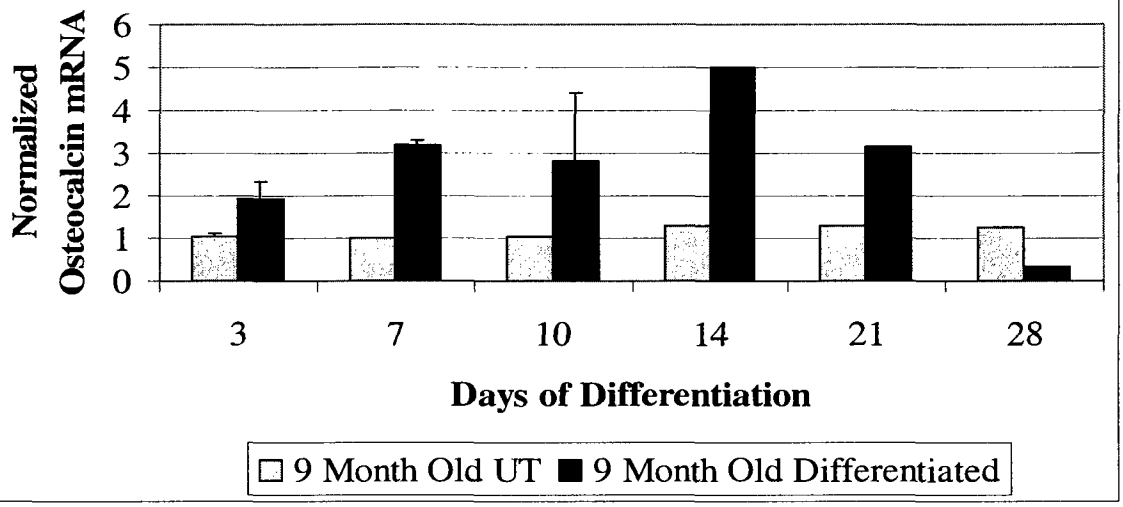
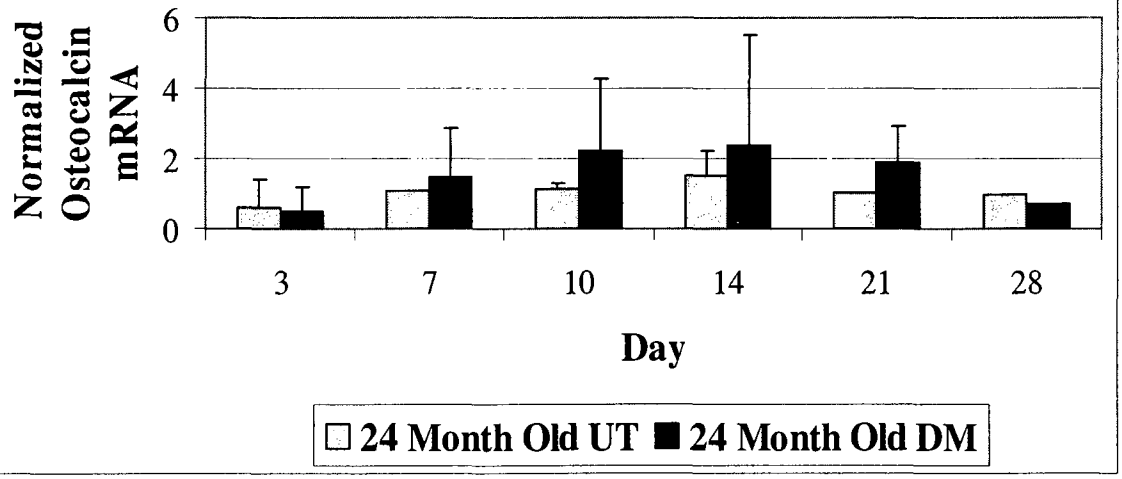
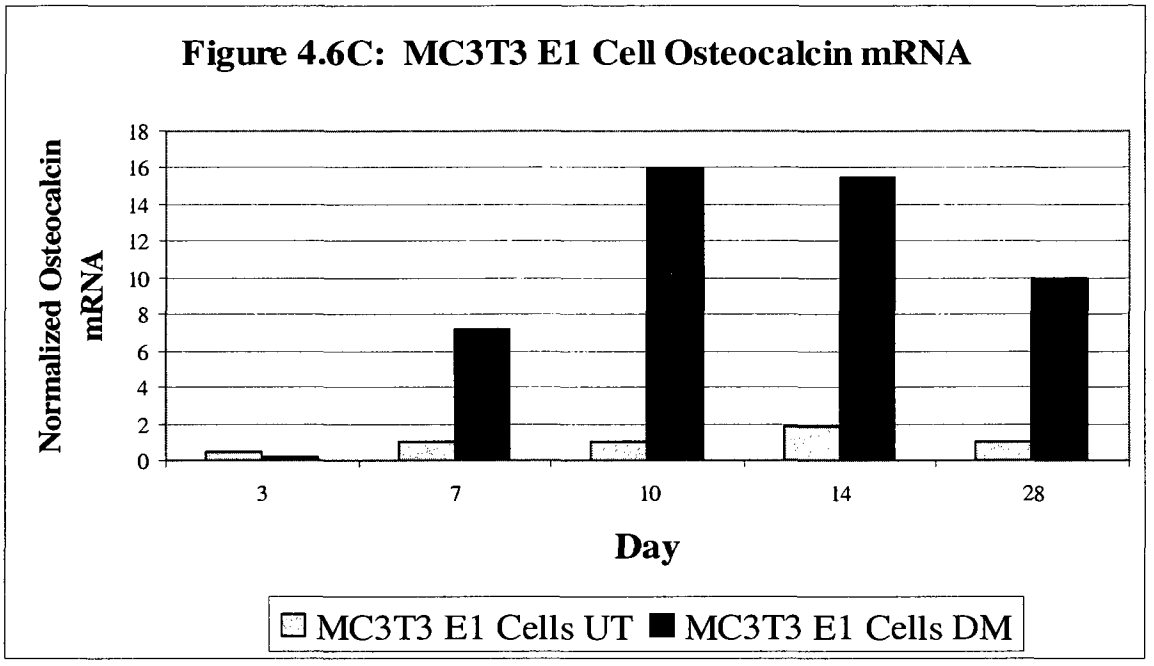


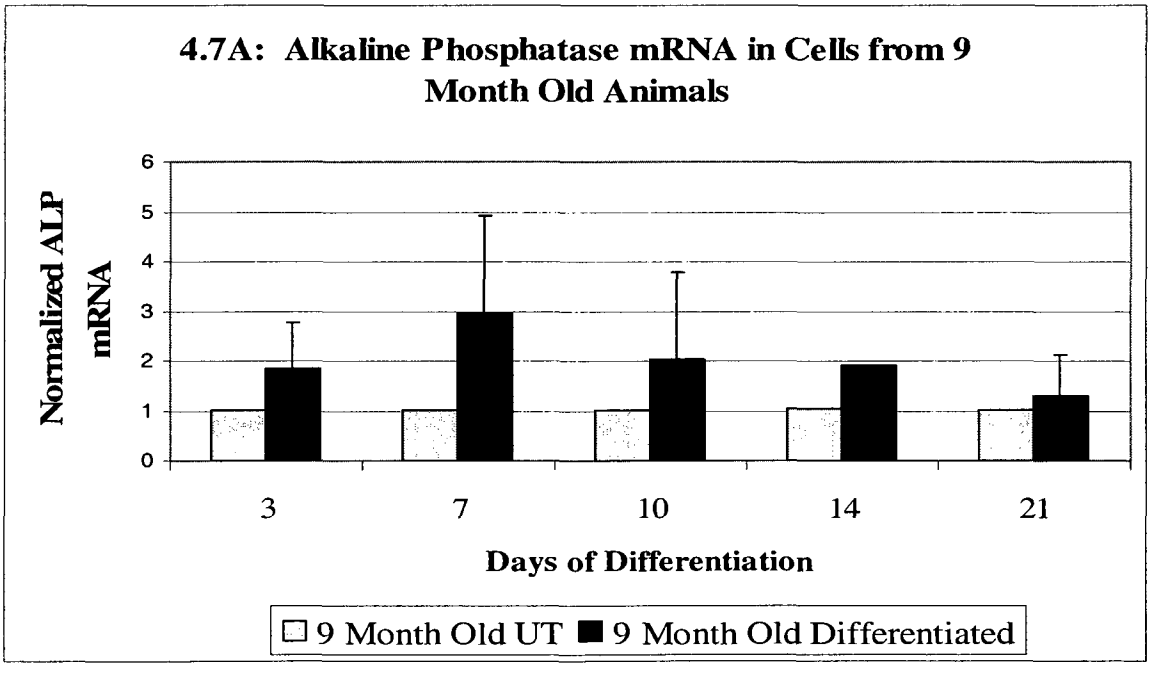
Figure 4.6B: Osteocalcin mRNA in Cells from 24 Month Old Animals



UT refers to cells treated with growth media and DM refers to cells treated with differentiation media. **(A)** Osteocalcin mRNA increases through day 14 and then decreases with differentiation of cells from 9 month old animals. **(B)** Osteocalcin mRNA increases through day 14 and then decreases with differentiation of cells from 24 month old animals.



(C) Osteocalcin mRNA increases through day 10 and then decreases with differentiation of MC3T3 E1 cells.



UT refers to cells treated with growth media and DM refers to cells treated with differentiation media. **(A)** ALP mRNA increases up to day 7 and then decreases with differentiation of cells from 9 month old animals.

Figure 4.7B: Alkaline Phosphatase mRNA in Cells from 24 Month Old Animals

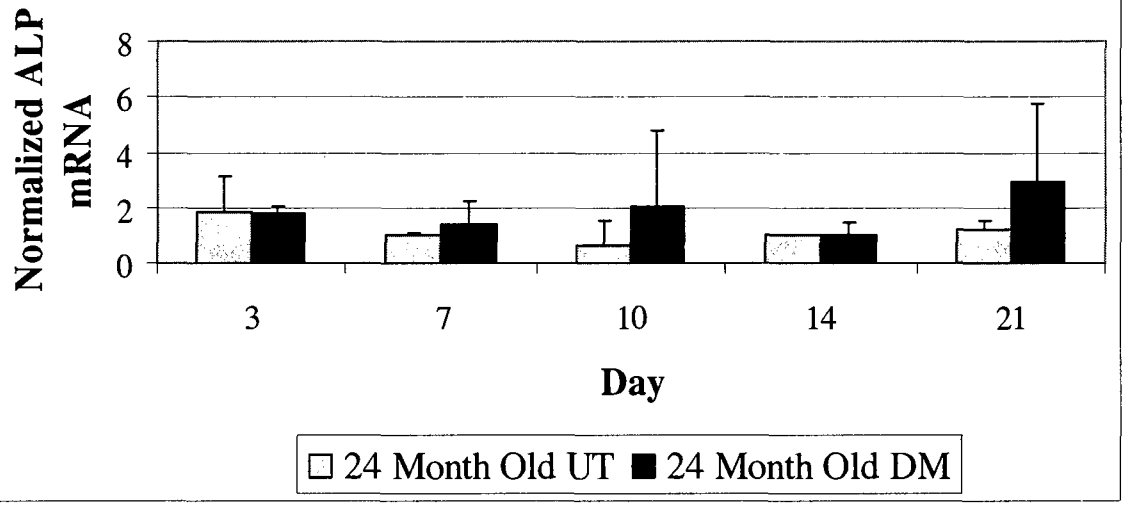
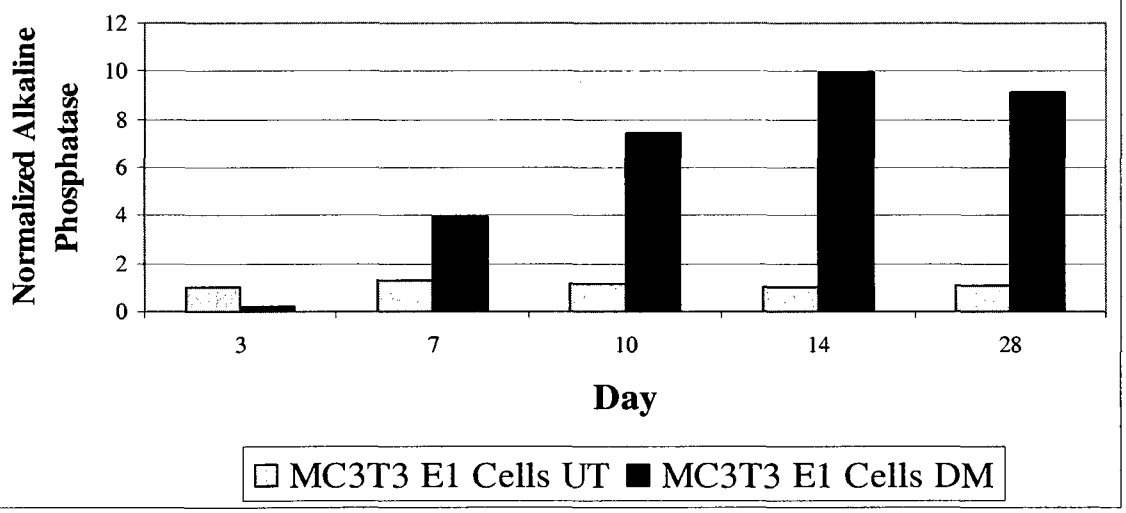
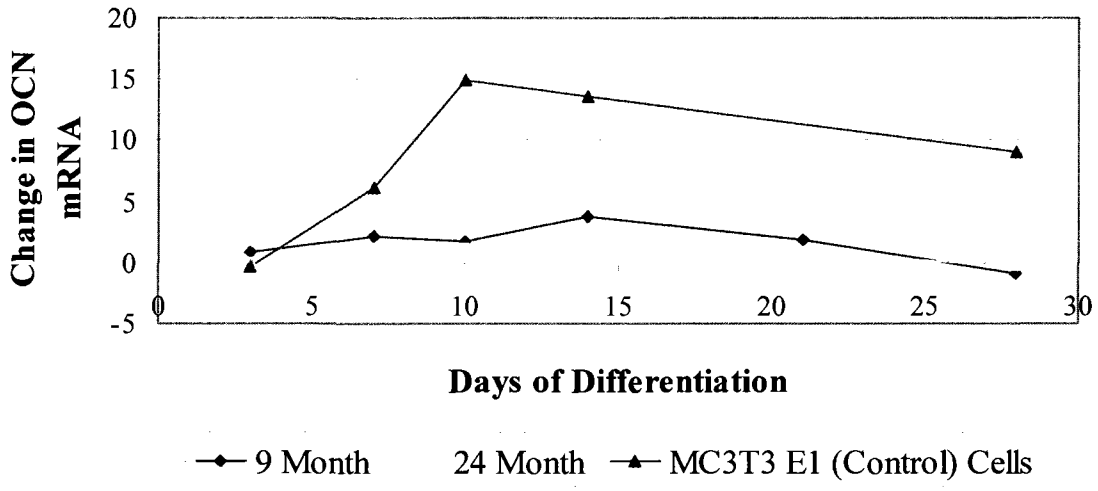


Figure 4.7C: MC3T3 E1 Cell Alkaline Phosphatase mRNA



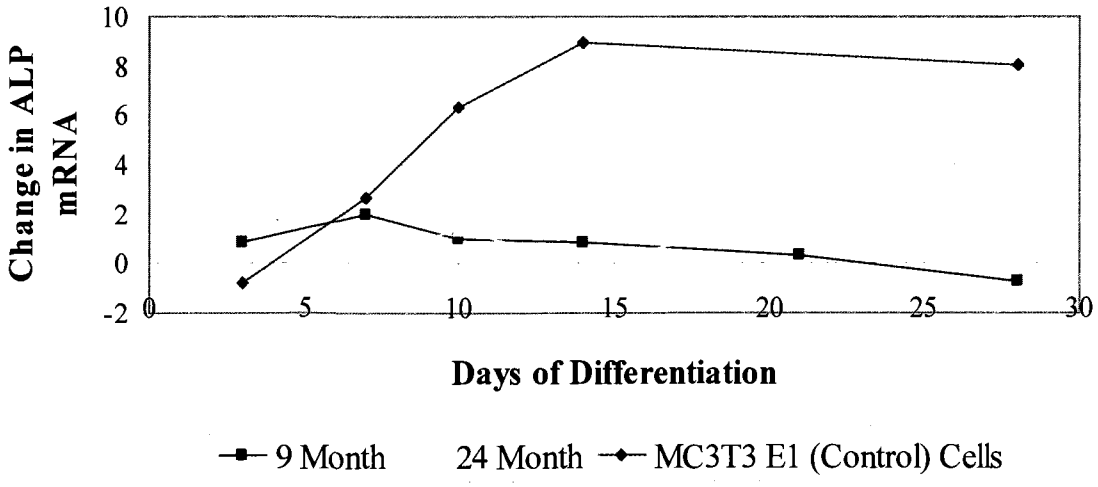
UT refers to cells treated with growth media and DM refers to cells treated with differentiation media. **(B)** ALP mRNA increases up to day 21 with differentiation of cells from 24 month old animals. **(C)** ALP mRNA increases up to day 14 and then decreases with differentiation of MC3T3 E1 cells.

Figure 4.8: Change in Osteocalcin mRNA with Differentiation



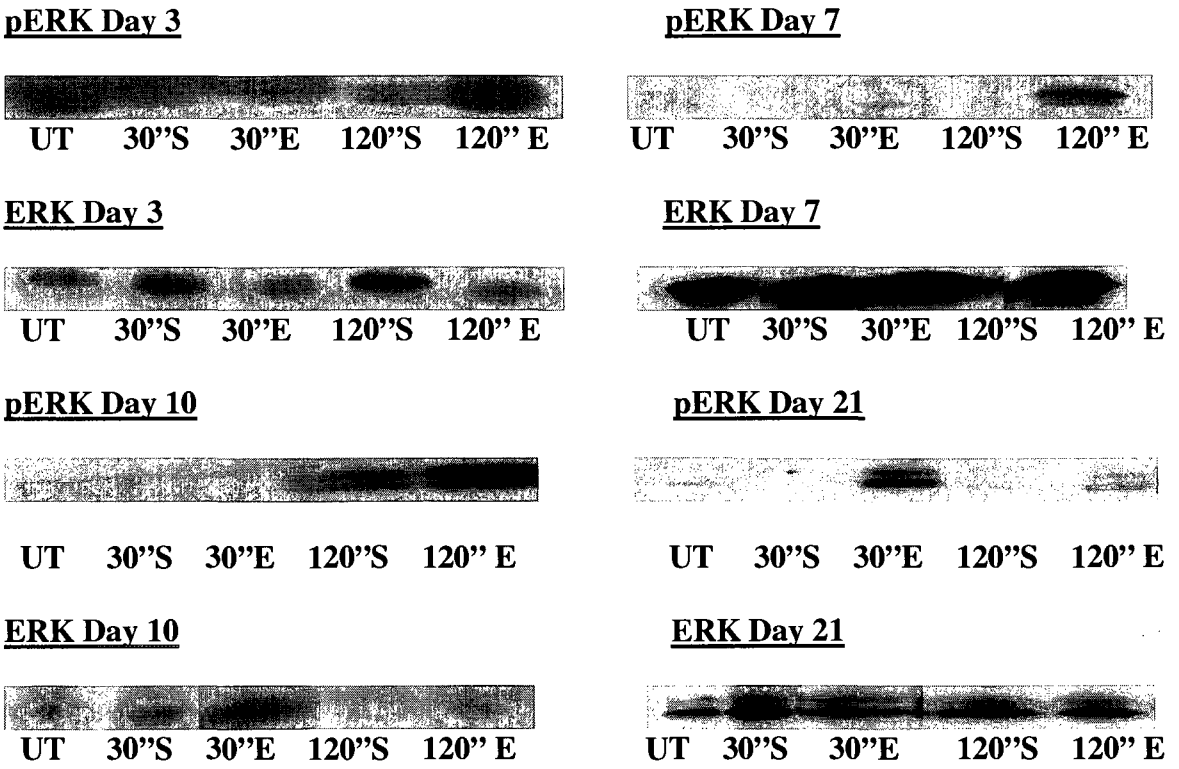
An increase in osteocalcin mRNA was observed in differentiated MC3T3 E1 cells and MSCs harvested from 9 month and 24 month old animals. This increase was highest in MC3T3 E1 cells and higher for 9 month old animals compared to 24 month old animals up through day 21.

Figure 4.9: Change in Alkaline Phosphatase mRNA with Differentiation



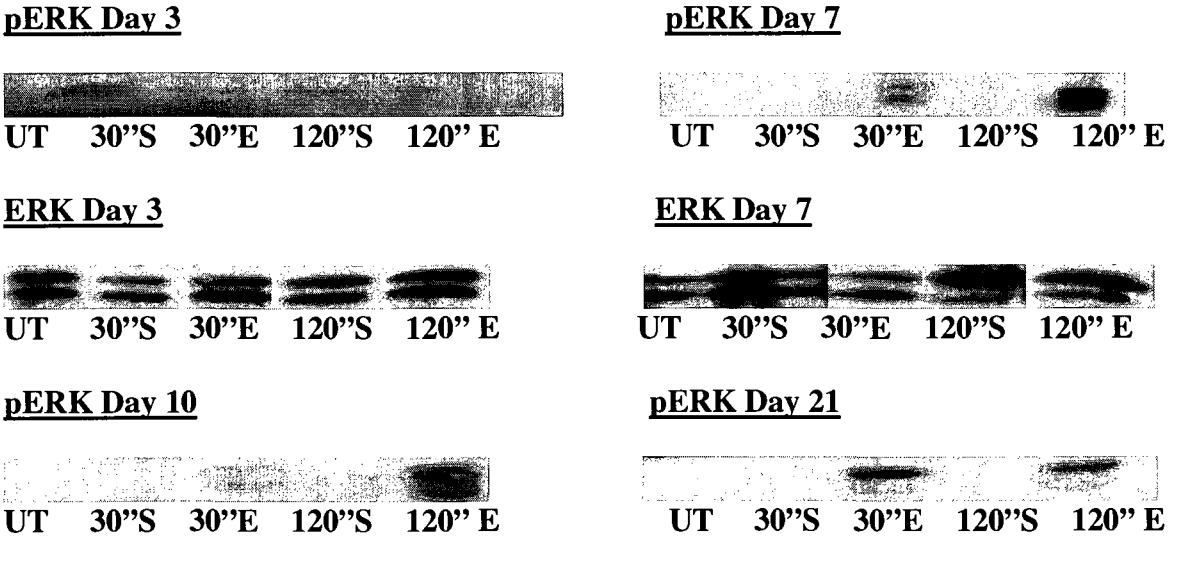
An increase in ALP mRNA was observed in differentiated MC3T3 E1 cells and MSCs harvested from 9 month and 24 month old animals. This increase was highest in MC3T3 E1 cells for most time points and higher for 9 month old animals compared to 24 month old animals after days 3, 7, and 14 of differentiation.

Figure 4.10A: 2 Month pERK and ERK Western Blots



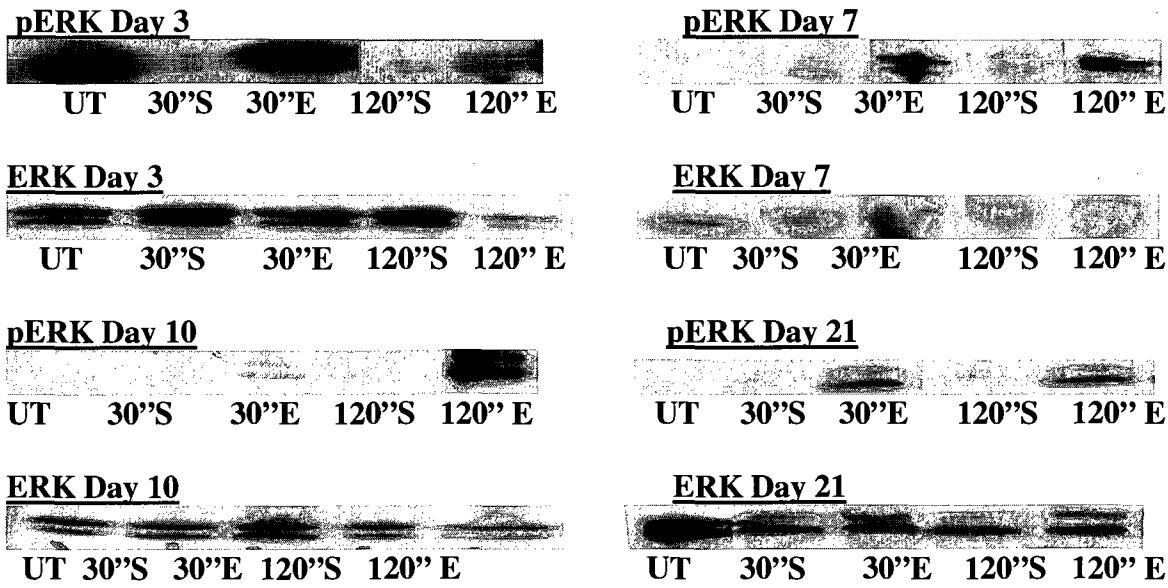
(A) ERK phosphorylated after mechanical stimulation of MSCs harvested from 2 month old animals and differentiated.

Figure 4.10B: 9 Month pERK and ERK Western Blots



(B) ERK phosphorylated after mechanical stimulation of MSCs harvested from 9 month old animals and differentiated.

Figure 4.10C: 24 Month pERK and ERK Western Blots



(C) ERK phosphorylated after mechanical stimulation of MSCs harvested from 24 month old animals and differentiated.

Figure 4.11A: 2 Month Old Animals Phosphorylated ERK to Total ERK Densitometry after 30 Minutes of Loading

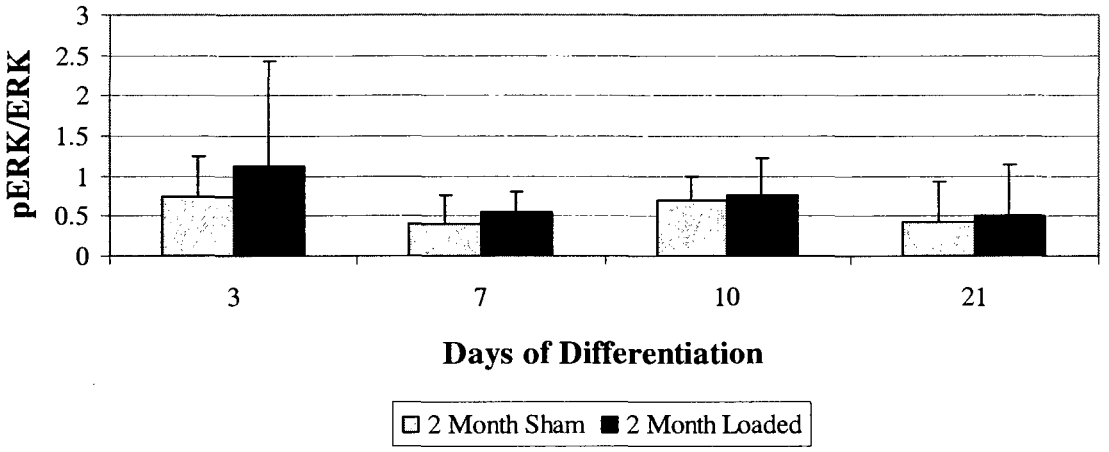
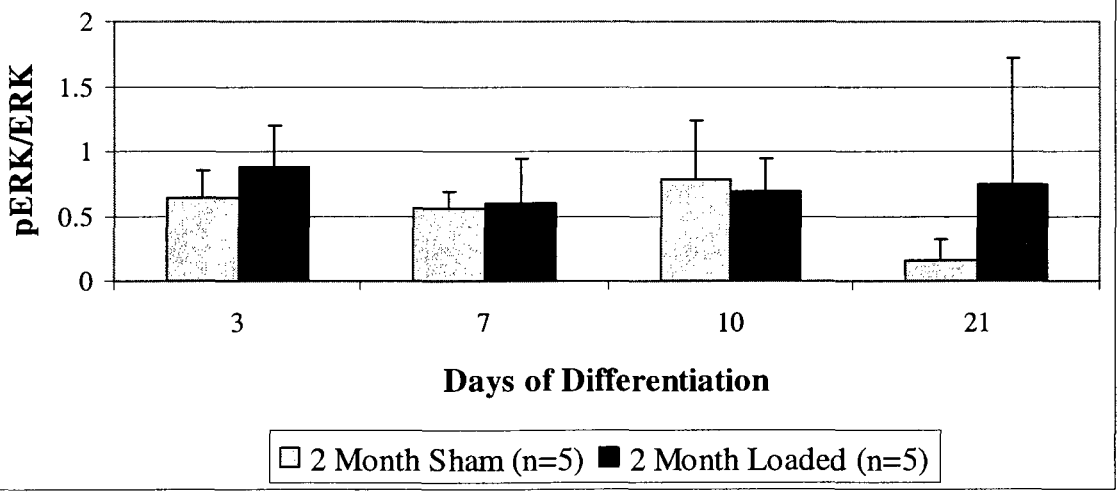


Figure 4.11B: 2 Month Old Animals Phosphorylated ERK to Total ERK Densitometry after 120 Minutes of Loading



(A) There was an increase in pERK in cells from 2 month animals loaded for 30 minutes after all differentiation time points **(B)** There was an increase in pERK in cells from 2 month animals loaded for 120 minutes after all differentiation time points

Figure 4.11C: 9 Month Old Animals Phosphorylated ERK to Total ERK Densitometry after 30 Minutes of Loading

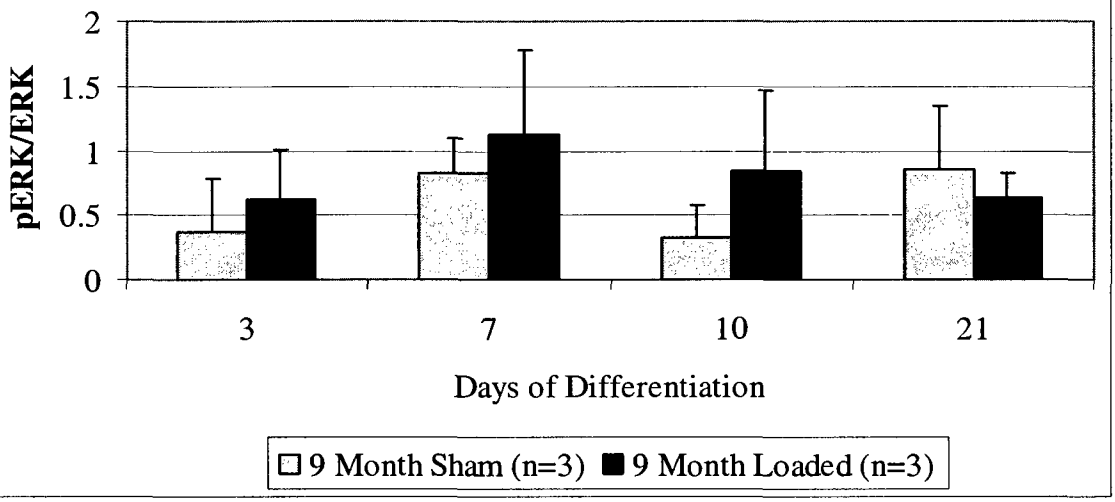
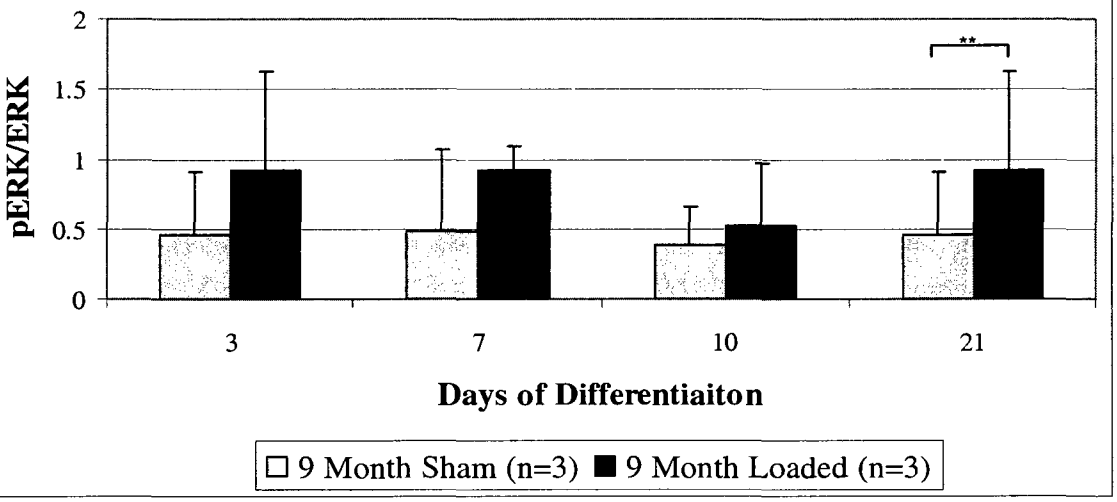


Figure 4.11D: 9 Month Old Animals Phosphorylated ERK to Total ERK Densitometry after 120 Minutes of Loading



(C) There was an increase in pERK in cells from 9 month animals loaded for 30 minutes after 3, 7, and 10 days of differentiation. **(D)** There was an increase in pERK in cells from 9 month animals loaded for 120 minutes after all differentiation time points.

Figure 4.11E: 24 Month Old Animals Phosphorylated ERK to Total ERK Densitometry after 30 Minutes of Loading

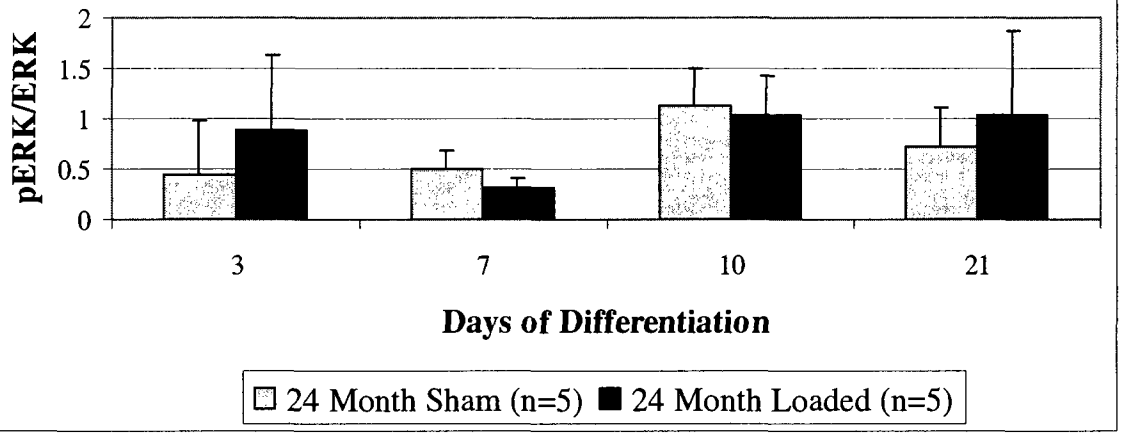
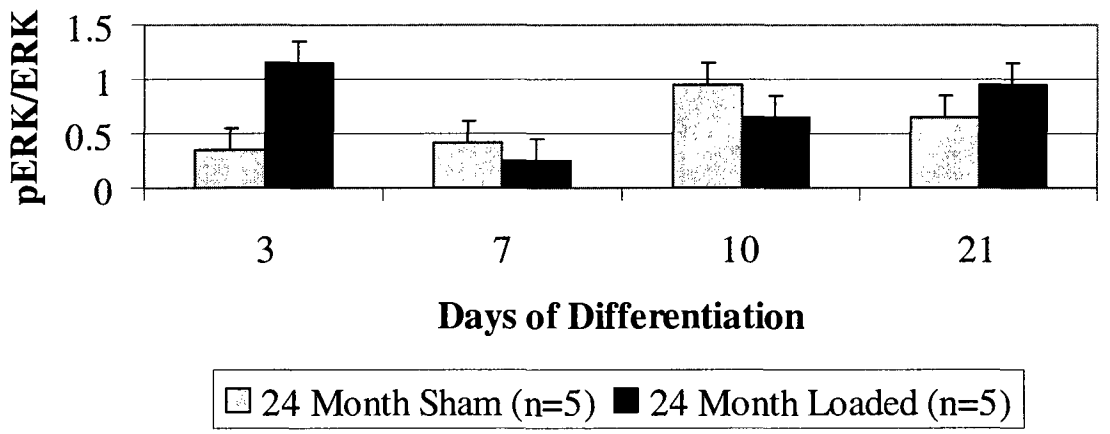


Figure 4.11F: 24 Month Old Animals Phosphorylated ERK to Total ERK Densitometry after 120 Minutes of Loading



(E) There was an increase in pERK in cells from 24 month animals loaded for 30 minutes after 3 and 10 days of differentiation. **(F)** There was an increase in pERK in cells from 24 month animals loaded for 120 minutes after 3 and 21 days of differentiation.

Figure 4.11G: Average Increase in MSC pERK Normalized to Total ERK per Animal after 30 Minutes of Loading

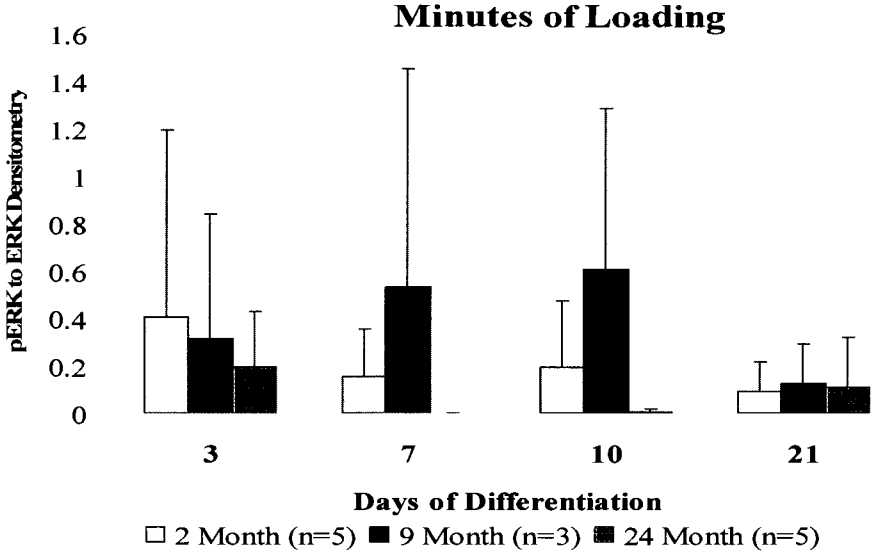
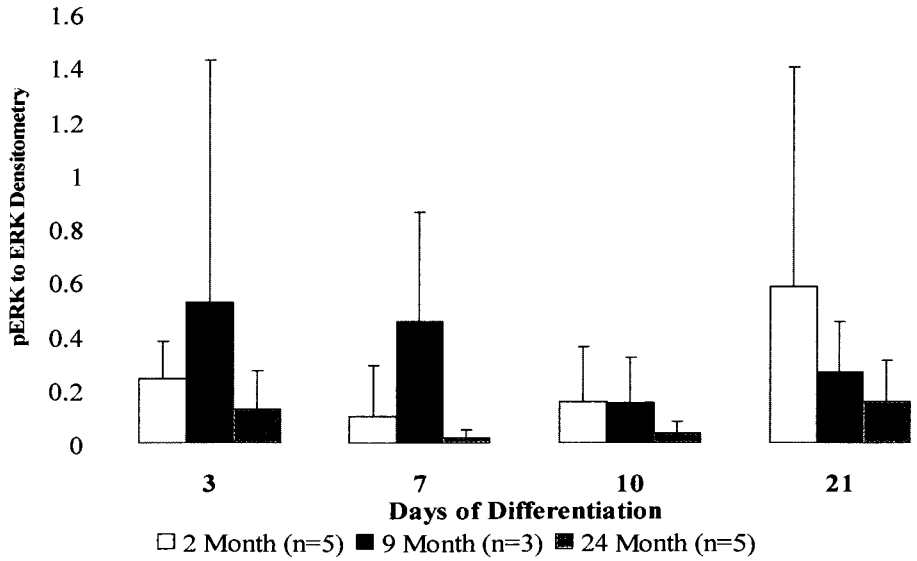


Figure 4.11H: Average Increase in MSC pERK Normalized to Total ERK per Animal after 120 Minutes of Loading



(G) The average increase in pERK per animal after 30 minute mechanical load was highest in cells harvested from 9 month old animals differentiated for 3, 7, and 10 days. **(H)** The average increase in pERK per animal after 120 minute mechanical load was highest in cells harvested from 9 month old animals differentiated for 3, 7, and 10 days

Figure 4.12A: 2 Month Old Animals Nitric Oxide Concentration after 30 Minutes of Loading

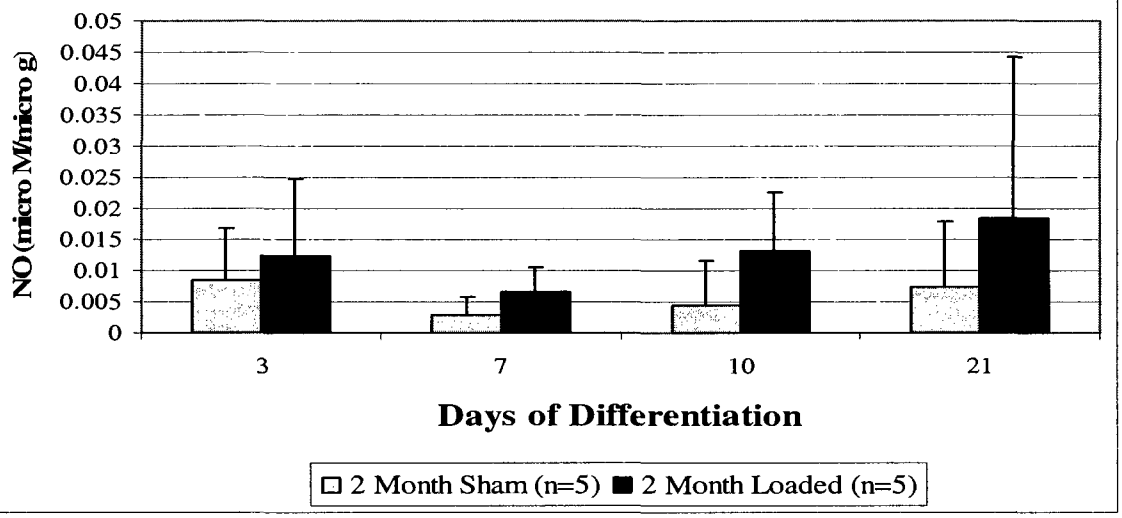
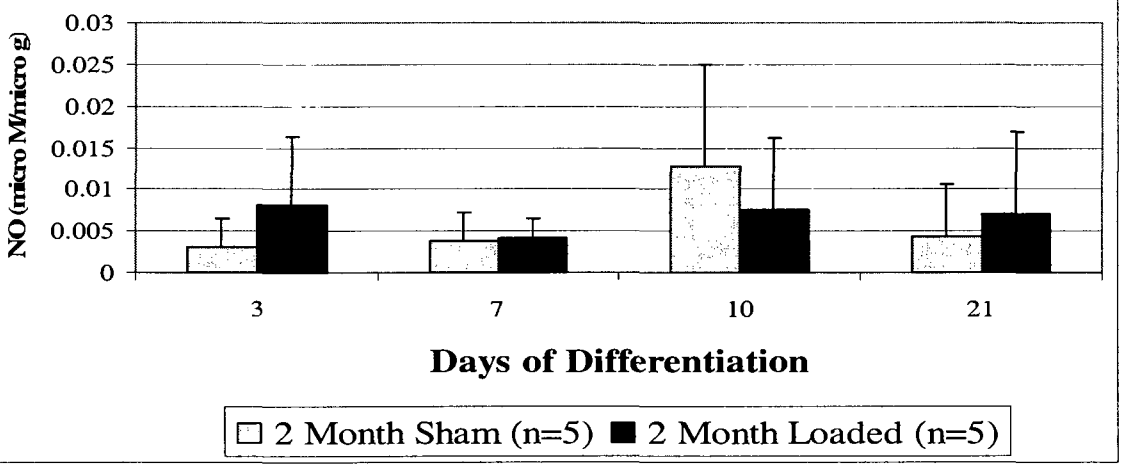


Figure 4.12B: 2 Month Old Animals Nitric Oxide Concentration after 120 Minutes of Mechanical Loading



(A) There is an increase in NO concentration in cells from 2 month old animals loaded for 30 minutes after all differentiation time points. **(B)** There is an increase in NO concentration in cells from 2 month old animals loaded for 120 minutes after all differentiation time points.

Figure 4.12C: 9 Month Old Animals Nitric Oxide Concentration after 30 Minutes of Mechanical Loading

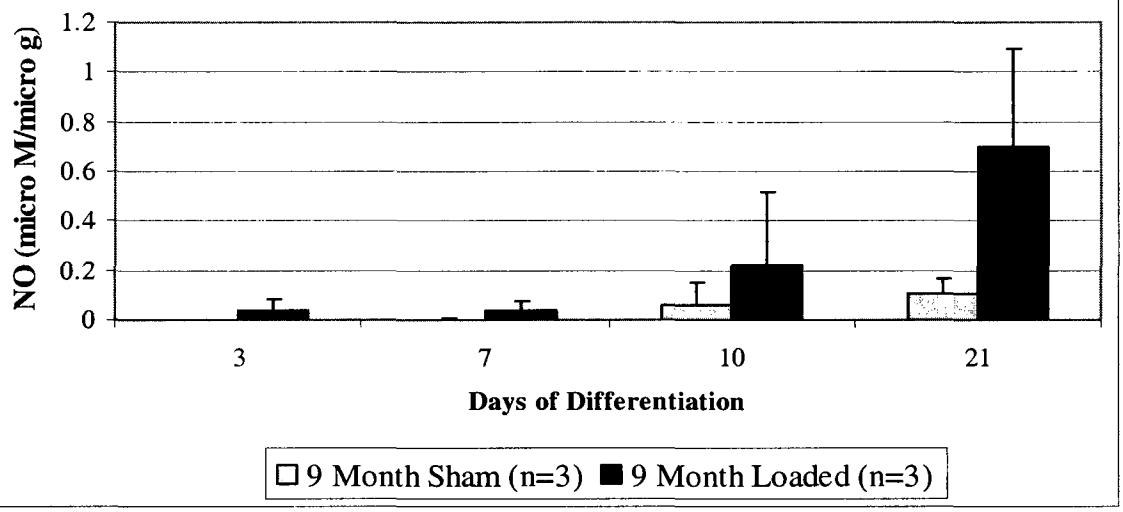
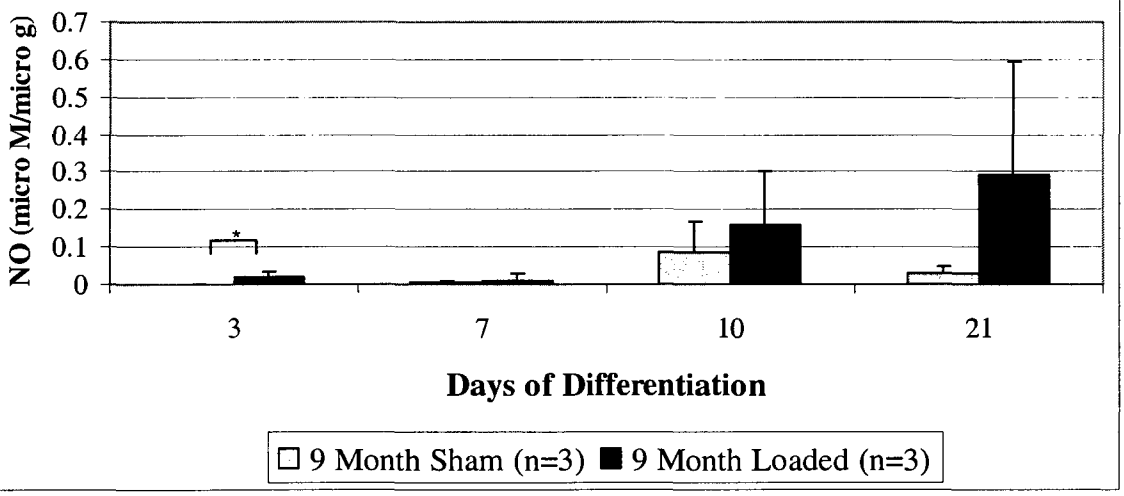
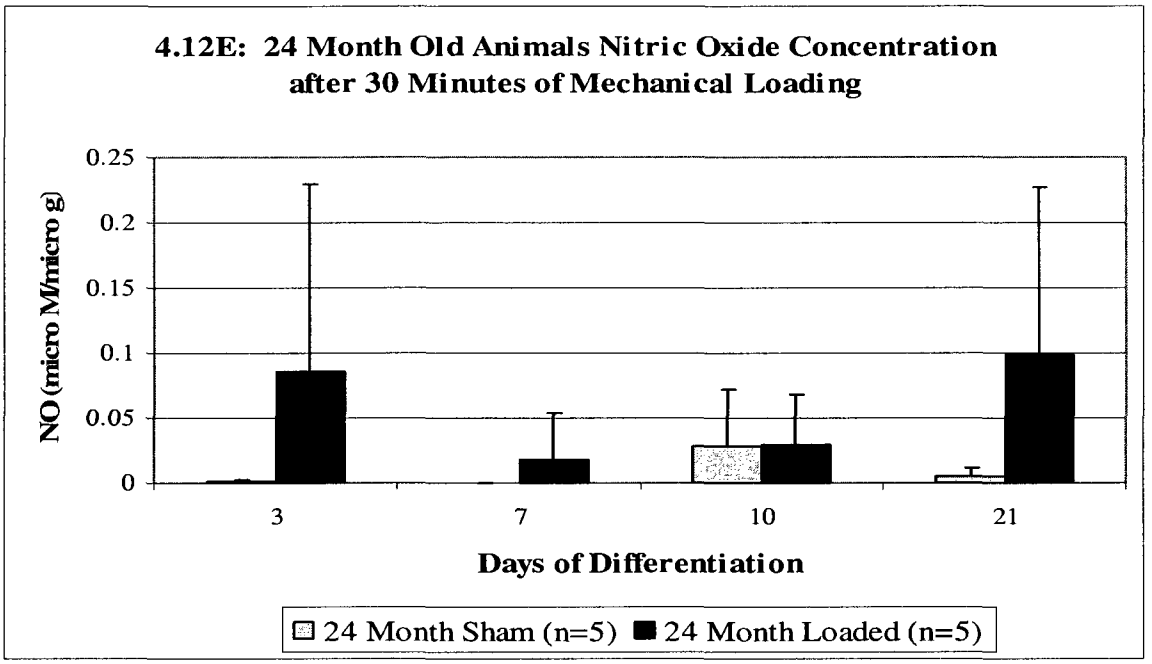
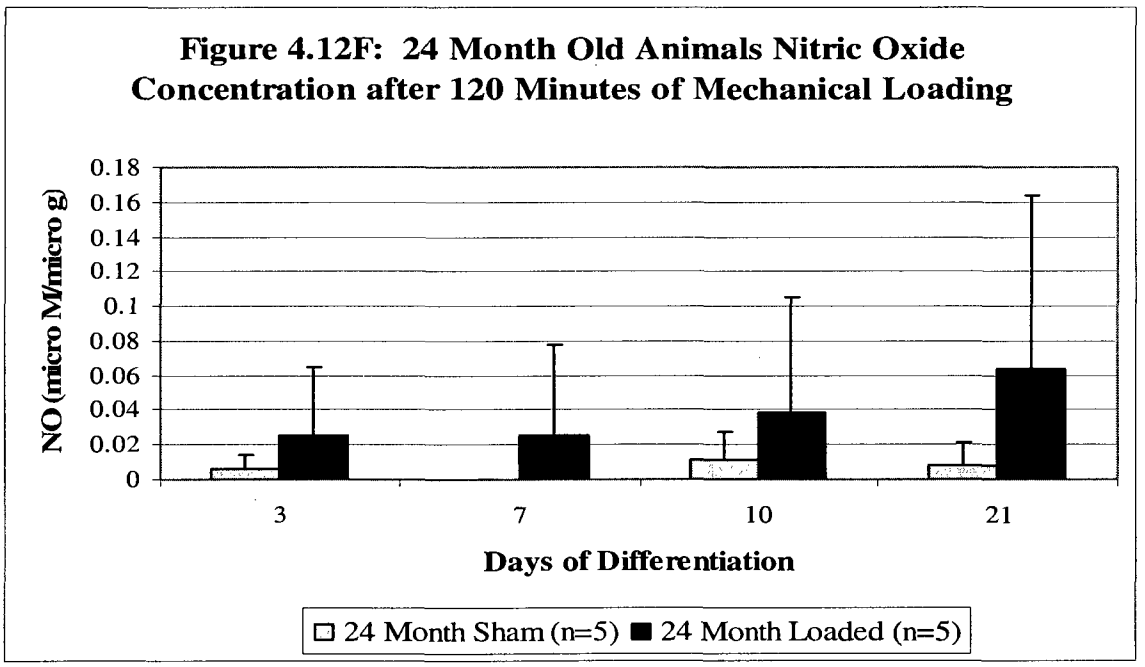


Figure 4.12D: 9 Month Old Animals Nitric Oxide Concentration after 120 Minutes of Mechanical Loading



(C) There is an increase in NO concentration in cells from 9 month old animals loaded for 30 minutes after all differentiation time points. **(D)** There is an increase in NO concentration in cells from 9 month old animals loaded for 120 minutes after all differentiation time points.





(E) There is an increase in NO concentration in cells from 24 month old animals loaded for 30 minutes after all differentiation time points. **(F)** There is an increase in NO concentration in cells from 24 month old animals loaded for 120 minutes after all differentiation time points.

Figure 4.12G: Average Increase in NO per Animal after 30 Minutes of Loading

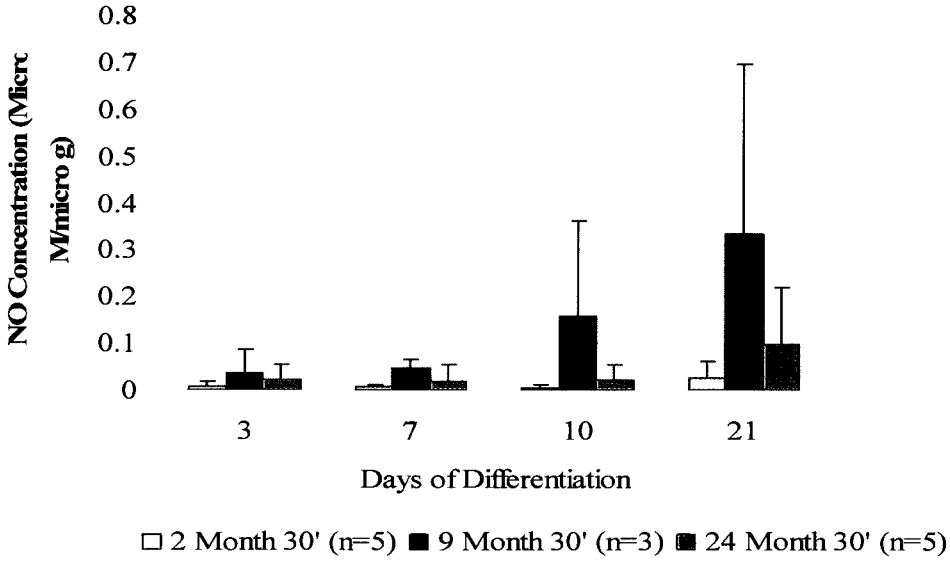
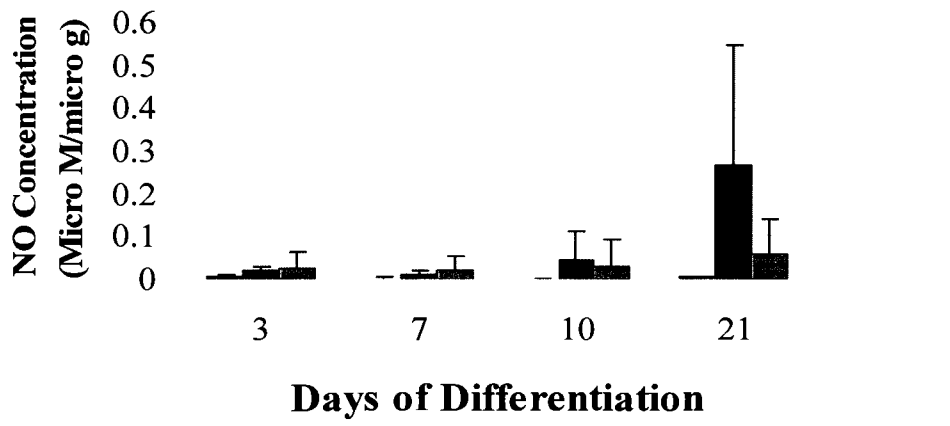


Figure 4.12H: Average Increase in NO per Animal after 120 Minutes of Loading



(G) The average increase in NO concentration per animal after a 30 minute mechanical load was highest in cells harvested from 9 month old animals after all differentiation time points. **(H)** The average increase in NO concentration per animal after a 120 minute mechanical load was highest in cells harvested from 9 month old animals differentiated for 10 and 21 days.

Figure 4.13A: Prostaglandin E_2 Concentration in 2 Month Old Animals after 30 Minutes of Mechanical Stimulation

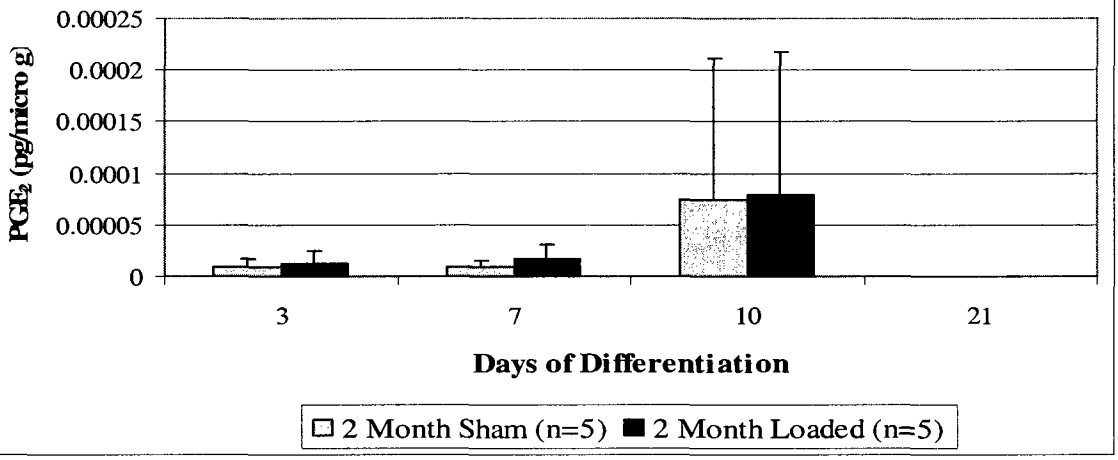
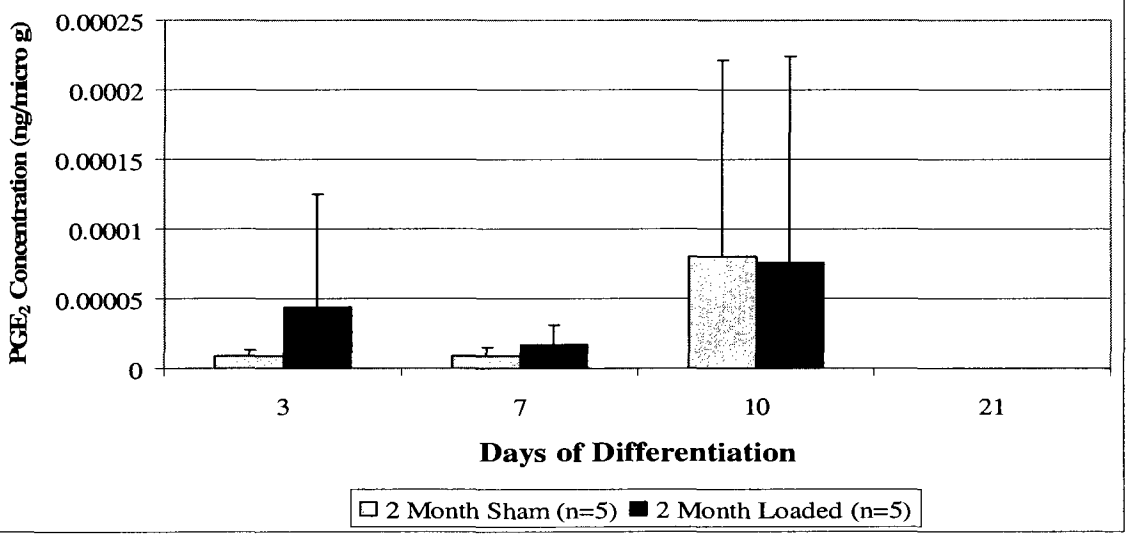


Figure 4.13B: Prostaglandin E_2 Concentration in 2 Month Old Animals after 120 Minutes of Mechanical Stimulation



(A) There was an increase in PGE_2 concentration after 30 minutes of loading in cells from 2 month old animals after 3, 7, and 10 days of differentiation. **(B)** There was an increase in PGE_2 concentration after 120 minutes of loading in cells from 2 month old animals after 3 and 7 days of differentiation.

Figure 4.13C: Prostaglandin E_2 Concentration in 9 Month Old Animals after 30 Minutes of Mechanical Stimulation

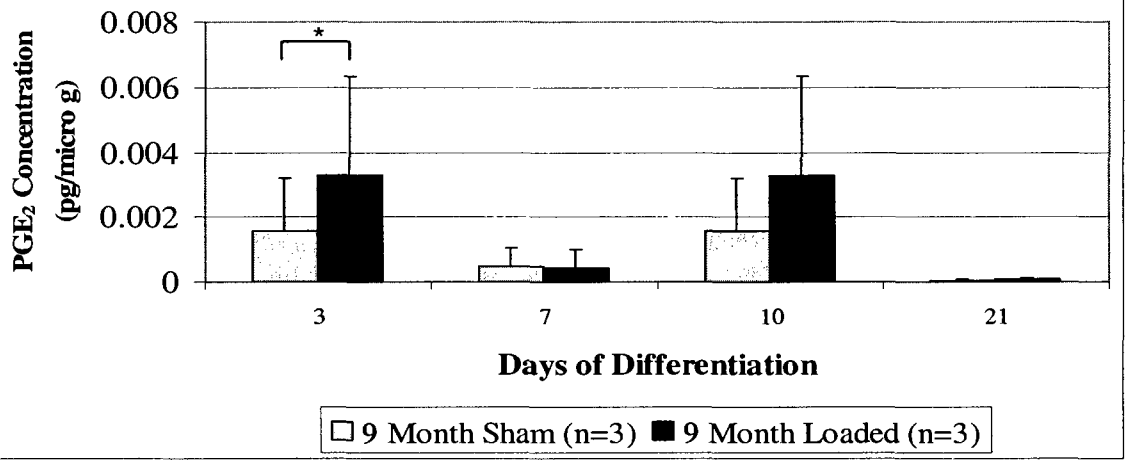
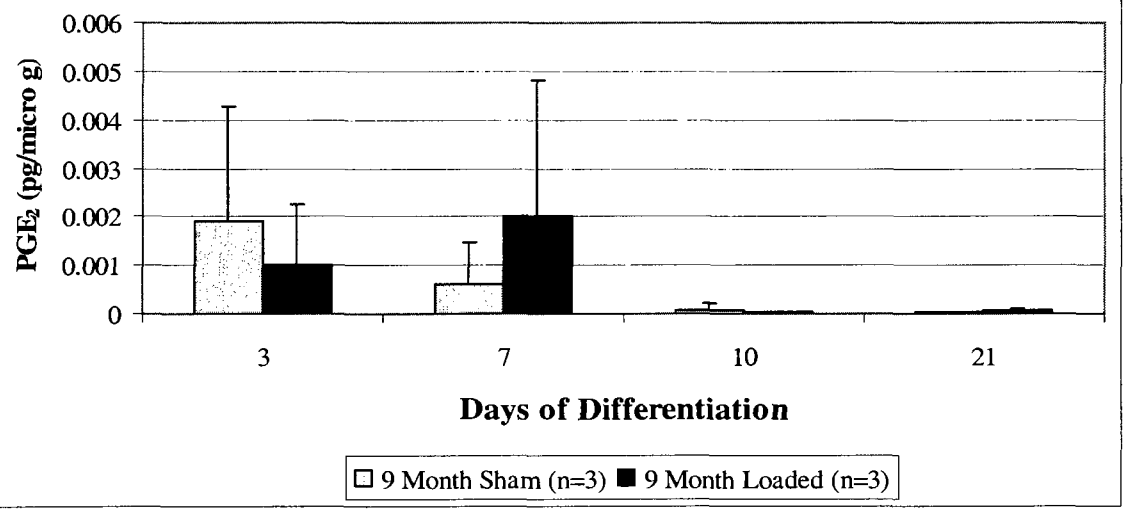


Figure 4.13D: Prostaglandin E_2 Concentration in 9 Month Old Animals after 120 Minutes of Mechanical Stimulation



(C) There was an increase in PGE_2 concentration after 30 minutes of loading in cells from 9 month old animals after 3, 10, and 21 days of differentiation. **(D)** There was an increase in PGE_2 concentration after 120 minutes of loading in cells from 9 month old animals after 7 and 21 days of differentiation.

Figure 4.13E: Prostaglandin E_2 Concentration in 24 Month Old Animals after 30 Minutes of Mechanical Stimulation

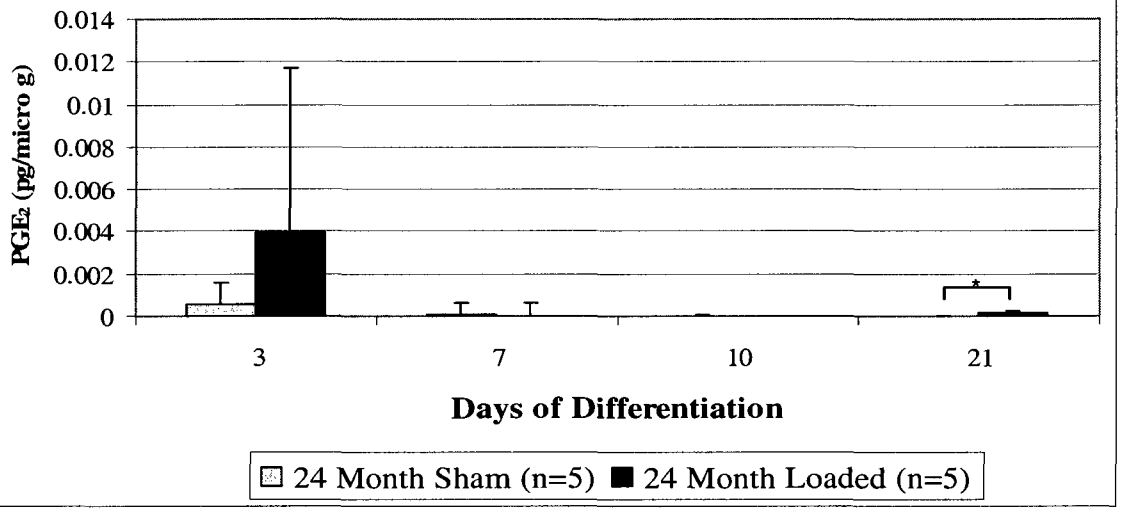
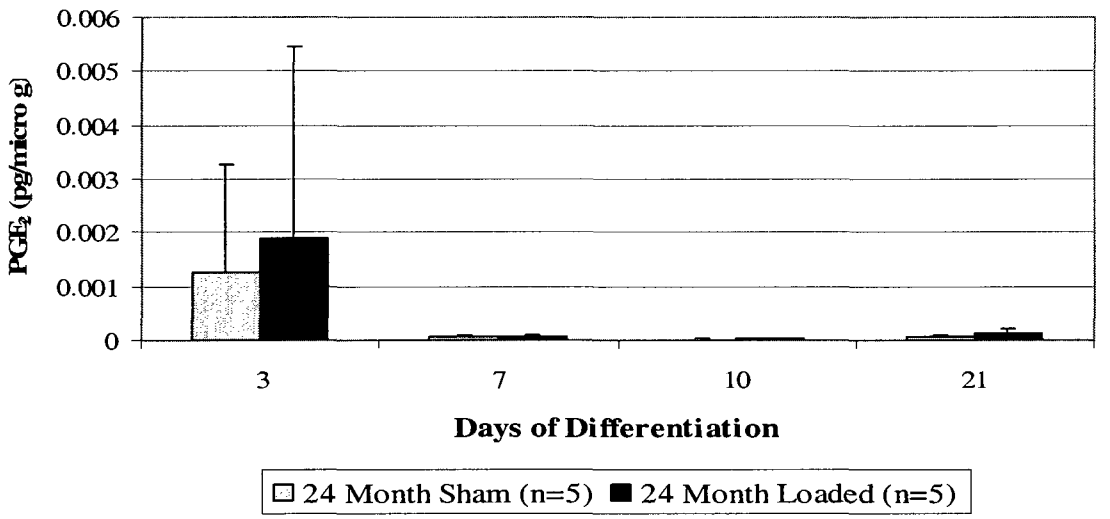


Figure 4.13F: Prostaglandin E_2 Concentration in 24 Month Old Animals after 120 Minutes of Mechanical Stimulation



(E) There was an increase in PGE_2 concentration after 30 minutes of loading in cells from 24 month old animals after 3 and 21 days of differentiation. **(F)** There was an increase in PGE_2 concentration after 120 minutes of loading in cells from 24 month old animals after 3, 10, and 21 days of differentiation.

Figure 4.13G: Average Increase in PGE_2 per Animal after 30 Minutes of Mechanical Stimulation

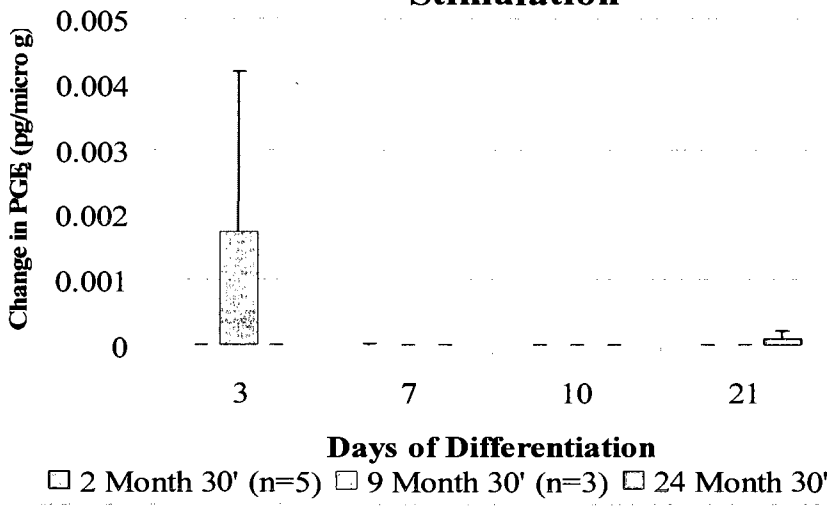
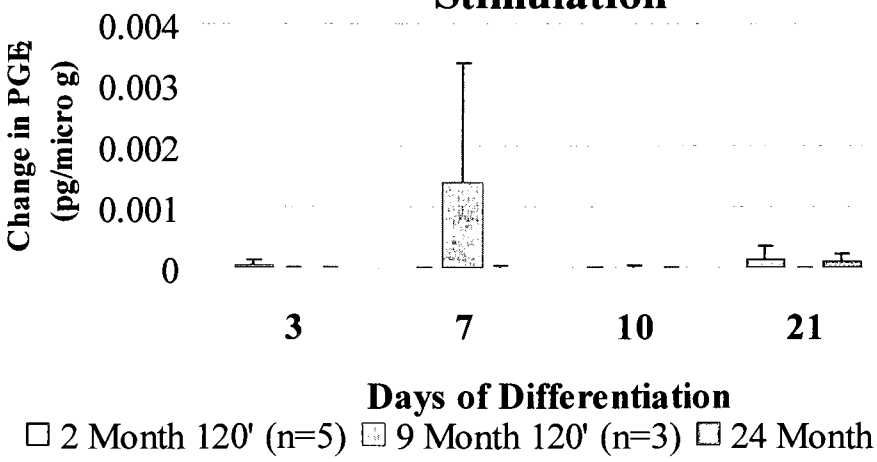


Figure 4.13H: Average Increase in PGE_2 per Animal after 120 Minutes of Mechanical Stimulation



(G) The average increase in PGE_2 concentration per animal after a 30 minute mechanical load was highest in cells harvested from 9 month old animals differentiated for 3 days. **(H)** The average increase in PGE_2 concentration per animal after a 120 minute mechanical load was highest in cells harvested from 9 month old animals differentiated for 7 days.

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CHAPTER V

CONCLUSION AND FUTURE WORK

You don't have to put up a fight. You don't have to always be right. Let me take some of the punches for your tonight.

~Sometimes you Can't Make it on your Own

5.1 Conclusion

Currently there is inconsistent data on the effect age has on the ability of bone to respond to mechanical stimulation. There is also limited knowledge of what occurs in an aging skeleton during conditions of regeneration and particularly to the osteocyte which is thought to be the primary mechanosensor. In older populations a difference in the fracture repair process has been identified and micro-cracks are increasingly prevalent, thus a more complete understanding of how age affects regenerative bone tissue and its response to mechanical load is necessary. In these studies the effect of age on bone under conditions of regeneration and during the maintenance of mature bone tissue was examined.

In chapter two the effect of age on regenerative bone tissue and its response to mechanical loading was investigated. Specimens produced in older animals had a higher degree of mineralization and mineral to matrix ratio than specimens produced in younger animals. This difference in TMD likely caused a reduction in local cell deformation in specimens from old animals and thus a reduced net increase in nitric oxide and prostaglandin E_2 which was observed subsequent mechanical loading.

In chapter three the response of regenerative bone tissue to mechanical loading was compared to the response of mature bone tissue to mechanical loading. Regenerative tissue may be more responsive to mechanical load than mature bone through the secretion of nitric oxide and osteopontin in both age groups, however these changes are observed in regenerative specimens from young animals as early as 3 months of regeneration. A dramatic response to mechanical loading at three months may initiate remodeling which maintains bone tissue age and its resistance to brittle failure. The increase in nitric oxide and osteopontin expression observed in regenerative specimens produced in 3 months in old animals was lower than that observed in young animals and could delay remodeling. This could be reflected in the increase in tissue mineral density observed in regenerative specimens from old animals after a 4 month implantation time period. This increased TMD could make regenerative specimens from old animals more susceptible to brittle failure. Interestingly the net change in PGE_2 was higher for mature bone specimens from both age groups when compared to PGE_2 secretion of regenerative specimens. PGE_2 may be an important signaling molecule during mechanical transduction in mature bone.

In chapter four the effect of age and differentiation on the response to mechanical stimulation was assessed in progenitor cells from animals of various ages. Mineralization appeared to be delayed in cells from old donors, however similar to that of cells from young donors at later time points which are consistent with TMD measures in regenerative bone tissue. Cells from 9 month old animals appeared to be more responsive to mechanical load through NO, PGE_2, and ERK signaling. At early time points there may be a higher proportion of mature bone cells in the cell populations from young animals, which have a greater capacity to respond to mechanical stimulation. At

later time points when mineralization is similar with age there may be a difference in sensitivity to mechanical load with age which was not investigated in these studies.

In regard to tissue engineering constructs it may be possible to use cells from old donors, however perhaps beneficial if the cells have been differentiated for some amount of time prior. Some pre stressing of cells from old donors might also aid in their integration during tissue engineering applications. Cells from old donors were able to respond to mechanical stimulation with changes in NO, PGE_2, and pERK and thus could promote bone formation if mechanically stimulated and implanted *in vivo*. Data from this study highlights the complex effect age has on bone and its response to mechanical stimulation. Key differences in mechanical response were highlighted which have the potential for further investigation to develop therapeutics for bone loss in aging populations.

The data from this study suggests that differences in mechanical response with age could be the result of differences in mineralization which alters the local mechanical environment of the cell. If the embedded cells in the matrix are locally sensing a different magnitude of strain as an animal ages and experiences mechanical stimulation it is likely that the resultant measures of a mechanical response are altered. It could be important clinically to investigate methods of increasing the local strain that is placed on cells in elderly population despite this increase in TMD which could result in an increased up-regulation of mechanical response markers. Perhaps during fracture healing the application of a basal mechanical stimulation could increase local strains beyond what daily activity places on the cells and be sufficient to induce a mechanical response which

could initiate remodeling and maintenance of a mean tissue age which is less susceptible to brittle fracture.

In this study specific molecular pathways were examined which could also be important to consider during clinical treatment. During the fracture healing process additive stimulators or the enhancement of MAPK and NO could in combination with secreted and NO and phosphorylated MAPK which occurs as a result of daily loading be sufficient to initiate an anabolic response to load in bone in aged populations that is similar to what is observed in young populations.

Future Work

Future work should investigate the local mechanical environment of the cells loaded in these studies. It is important to understand the stimulus cells are sensing prior to their mechanical response. If there are differences in local strain with age, investigating whether the exposure of cells to the same local mechanical environment results in a difference in mechanical response would be important. If the mechanical response is matched with age when the local strain placed on the cells is similar future therapeutics could focus on matching these local strains during mechanical loading. Manipulation of the remodeling cycle could be investigated as a means to produce bone in young and old animals with a similar tissue mineral density.

It is also extremely important to understand the cellularity of regenerative specimens which could have a large impact on the local mechanical environment. If there are fewer cells present in regenerative specimens from old animals perhaps the ability of these cells to perform as a unit is impaired. Perhaps the targeted addition of

molecules that would enhance local proliferation during the repair process would increase the number of cells in aged animals that are able to communicate as a unit.

Aging is defined as the progressive accumulation of changes with time that are associated with or responsible for the increasing susceptibility to disease and death. The sum of deleterious free radical reactions which occur continuously throughout cells and tissue are a major component of the aging process. Stolzing and colleagues found that mesenchymal progenitor cells from their oldest age group accumulated raised levels of oxidized proteins and lipids and show decreased oxidative enzyme activity colony forming unit numbers, and increased levels of apoptosis and reduced potential for proliferation (Stolzing, A. et al., 2006). Aging and death of single cells due to the aging process are under genetic control which is subject to modification by the environment which also includes the effect of aging cells on each other.

DNA encodement could be altered with aging as well the accuracy of protein synthesis, crosslinkage of macromolecules, and free radical reaction damage (Harman, D. et al., 1991). DNA damage accumulates with age and cellular DNA damage responses may contribute to manifestations of aging (Lomnard, D. et al., 2005). During aging telomere length, which contributes to its stabilization and is a key to avoiding replicative senescence, shortens (Magalhaes, J. P. et al., 2004). It would be interesting to examine if these changes take place in bone cells with aging.

The effect of age on morphology and function has been well documented for other cell types. It has been established that age induced mtDNA deletion mutations expand within individual muscle fibers, eliciting fiber dysfunction and breakage (Herbst, A. et al., 2007). In addition, reduced intrinsic excitability was observed in hippocampal

pyramidal neurons from normal aging subjects which demonstrated an enlarged postburt afterhyperpolarization and increased spike frequency adaptation (Disterhoft, J. et al., 2007). Campell and colleagues found an aging related increase in long lasting calcium dependent and calcium mediated potentials which could be attributed to greater channel activity (Campell, L. et al., 1996). Similarly an aging related increase in the slow afterhyperpolarization, calcium spikes and currents, and L-type voltage gated calcium channel activity was observed in studies by Thibault, Murchinson, and colleagues (Aging Cell). Schwann cell (SC) population doubling time was reduced by a factor of almost three compared to those of young SC (Funk, D. et al., 2007). Data has shown that peripheral neurons can compensate for an age related decline in the function of at least one of the neuronal calcium buffering systems by increasing the function of other calcium buffering systems (Buchholz, J. et al., 2007). During aging neurons undergo morphological changes such as reduction in the complexity of dendrite aborization and dendritic length (Dickstein, D. et al., 2007). While McCreadie et al. did not find differences in the size (volume) or shape (anisotropy) of the lacunae between women with and without osteoporotic fracture, which would affect local strain and rates of molecular transport through the tissue, it would be interesting to examine osteocyte the structure of the osteocyte as a function of age through use extensive three-dimensional reconstructing software such as IMARIS, process length measuring software NEURON TRACER, and cell surface area per cell volume analyzing software SURPASS (McCreadie, B. et al., 2004; Sugawara, Y. et al., 2005).

While this study does offer insight into the changes that can occur in bone with advancing age and its ability to respond to mechanical forces under regenerative and

mature conditions it does have its limitations. Organ culture and *in vitro* models lack the complex forces and interaction between all cell types which bone tissue is exposed to under normal conditions. For example, muscle forces are absent and studies have shown that energy providing enzymes and antioxidant enzymes levels and activities are increased in young animals post exercise, however in old animals a reduction in activity of these enzymes was found at the completion of training (Bar-Shai, M. et al., 2008). Muscle forces are a major component of skeletal adaptation various studies have shown that muscle strength has effects on bone mass or bone mineral density that are independent of age, weight, height, or years of estrogen use (Burr, D. et al., 1997, Villa, M. L. et al., 1995, Bauer. D. C. et al., 1993, and Frost, H. M. et al., 1987).

Furthermore circulatory hormones and growth factors are absent in the models used in this study. Aging is hormonally regulated and with aging it is well established that estrogen and androgen levels fluctuate (van Heemst, D. et al., 2005). Insulin-like growth factor-I (IGF-I) insulin like growth factor binding protein-3, dehydroepiandrosterone sulfate, testosterone, estrodial, and free androgen index all decrease with advancing age (Fatayerji, D. et al., 1999). Estrogen concentration has been shown to increase PGE_2 production by primary human bone cells and *in vivo* data suggests a synergistic effect of weight bearing exercise and hormone replacement therapy on whole body bone mineral density in elderly women and *in vitro* data support an additive effect of mechanical load and estrogen on osteoblast proliferation (Rubin, C. J Bone and Joint Surgery Am and Bakker, A. Osteoporosis International). In addition, ERα knockout mice have an impaired adaptive bone formation response to mechanical

loading (Lee, K., et al. 2003). Locally and systemically delivered rhIGF-I can produce a significant increase in new bone formed in aged animals (Fowlkes, J. L. et al., 2006).

In addition the studies in this thesis focus primarily on a mechanical response through ERK signaling and nitric oxide and PGE_2 production. There are other pathways implicated in bone tissue mechanotransduction and other factors affected by this process which play a role in bone adaptation. For example the canonical Wnt-β Catenin signaling pathway is key regulator in bone development and bone homeostasis. Function loss in Wnt co-receptor Low density lipoprotein Receptor related protein 5 (LRP5) results in osteoporosis while function gain in LRP5 results in high bone mass. *In vitro* studies show that laminar fluid shear stress can induce translocation of β-Catenin to the nucleus and activate a TCF-reported gene (Norvell, S. et al. 2004). *In vitro* osteoblasts from Lrp5-/- mice were defective for mechanotransduction and failed to synthesize bone matrix after mechanical loading (Sawakami, K. et al., 2006).Antagonists of the Wnt- β Catenin signaling pathway signaling pathway such as Sclerostin and dickkopf-1 (*Dkk1*) also play a major role in bone homeostasis. *In vivo* mechanical loading was found to reduce sclerostin expression and increase the rate of bone formation and elevated levels of alkaline phosphtatase and osteocalcin are associated with SOST mutation carriers (Robling, A. et al., 2006, Dijke, P. et al. 2008, Kikuchi, A. et al., 2007)

Focal adhesion kinase (FAK) is also a critical component of bone cell mechanotransduction. When activated, FAK autophosphorylates tyrosine 397 which enables interaction of with a number of src-family proteins and other molecules with SH2 domains. Tyrosine phosphorylation mainly in FAK occurs in osteoblasts upon mechanical stimulation and FAK contributes to MAPK activation. FAK inactivation

specifically blocked the ability of bone marrow cells to sense a mechanical stimulus and reproducibly and rapidly repressed osteoblasts from depositing a mineralized matrix (Leucht, P. et al., 2007). The application of fluid shear to MC3T3 E1 osteoblasts can induce the development of stress fibers and the formation of focal adhesions containing β1-integrin and α-actinin and the disruption of microfilaments can inhibit shear induced increases in COX-2 (Pavalko, F. M. et al., 1998).

Connexins are also involved in bone cell mechanotransduction and mechanical stimulation results in an increase in the expression of connexins *in vitro* and *in vivo*. In addition to regulating cell to cell communication, connexins can also form regulatory channels between the cell and the extracellular environment, and knockdown of connexin inhibits the release of PGE_2. In addition, increased serine phosphorylation of connexin 43, the primary connexin in bone, correlates with a flow induced in gap junction intracellular communication (Alford, A. et al., 2003). Mechanical stimulation also results in the mobilization of intracellular calcium which is an important early second messenger. Studies suggest that when it is blocked during mechanical loading mechanically regulated gene expression alterations do not occur (Rubin, J Gene). Studies also suggest it is a potent transcriptional inducer of COX-2 expression and PGE2 production in osteoblasts via the ERK pathway (Choudhary, S. et al., 2003).

The surface proteoglycan layer (glycocalix) is the primary sensor of mechanical signals and can transmit force to the plasma membrane of submembrane cortex. Lipid rafts and caveolae may serve as cell surface mechanotransduction sites within the plasma membrane. Recent data suggests that mice which lack caveolae have increased trabecular and cortical bone caused by the gene deletion and these structural changes are

accompanied by increased mechanical properties. Furthermore, caveolin deficiency leads to increased osteoblast differentiation (Rubin, J. et al. 2007). Transduction may occur here or at intracellular junctions (adherens junctions) and cell matrix contacts and in these adhesion complexes recruitment and reorganization of both the integrin and cadherin proteins is induced. Expression of cadherins which are proteins of the adherens junctions which interlink the cytoskeleton between nearby cells is increased by mechanical strain. Future work could investigate the effect of age on these mechanical sensors in regenerative and mature bone tissue. Perhaps with advancing age the ability of the cell to sense mechanical stimulation and transduce this signal at various junctions is altered.

In this thesis the primary focus was the effect of normal aging on regenerative and mature bone tissue. Future work could incorporate the same models used in this thesis to investigate the effect of age and disease on regenerative and mature bone tissue, which might have a more clinical application. For example the animal model could be modified to examine the mechanical responsiveness of regenerative bone tissue from knock out animals. In addition, menopause plays a critical role in rapid bone loss in women, and these animal models used in conjunction with oviariectomy studies could provide useful data on the way regenerative bone tissue responds to mechanical stimulation during states of hormonal alterations.

Mechanical response was applied to bone tissue in these studies for a maximum of 120 minutes. Studies show that nitric oxide production can continuously increase during 12 hours of mechanical loading (Johnson, D. et al., 1996). Fluid shear stress can also affect osteoclast proliferation and differentiation. The examination of signaling

molecules associated with the osteoclast is not part of this study and should be included in future work.

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APPENDIX A: VALIDATION OF THE IMPLANT-EXPLANT MODEL FOR AGING STUDY

I'm not afraid of anything in this world. There's nothing you can throw at me that I haven't already heard

-Stuck in a Moment

A.1. Introduction

Previously Hoffler demonstrated that an implant-explant model could successfully produce micro specimens when implanted in 6 month old male Sprague-Dawley rats. In order to determine if this same model could be used to study regenerative bone specimens produced in aged animals a pilot study was conducted in which 8 and 21 month old male animals were used.

A.2 Materials and Methods

Five 21 month old animals and five eight month old animals received bilateral implants in the femora as outlined in chapter two. After an implantation period of twelve weeks regenerative specimens were surgically removed from the chambers as described in chapter two. Control regions of the femora proximal to the defect served as controls.

A.2.1 Micro CT

Five incomplete micro specimens from each age group were scanned by a cone beam μCT system (GE Healthcare PCI, London, ON), and reconstructed at a voxel size of 18 microns. A region of interest was created with a cortical tool and morphological

parameters were determined using a commercially available voxel analysis software program (MicroView v. 2.18).

A.2.3. Histology

After micro ct analysis specimens were stored in 10% NBF for 48 hours and then fixed in 70% EtOH. Specimens were incubated overnight in 2% procion red, mounted in Prolong Gold Anti Fade with DAPI, which stains DNA (Invitrogen, OR), and imaged with an Olympus FV-500 Confocal Microscope. Procion red stained the lacunar canalicular network. Five random sections were examined at 10μm increments throughout the entire depth of the sample (approximately 254 μm).

A.2.4. Mechanical Loading

Regenerative specimens were either sham (n=5 old and n=4 young) or cyclically loaded for one hour in three point bending (n=5 young and n=5 old) in an incubator set to 37□C and 5% CO_2. Specimens were loaded to a maximum displacement of 14μm which produced ±17.63μ□. A calibrated load cell monitored a 19.5-30g load placed on specimens during three point bending.

A.2.5. Quantification of Nitric Oxide and PGE_2

Media was harvested from specimens after 15, 30, 45, and 60 minutes of either loading or sham treatment. Protein was harvested from specimens following treatment and prepared for western blot. Nitric oxide and Prostaglandin E_2 concentration was calculated for media specimens with colorimetric assay kits (Cayman Chemical, Ann Arbor, MI). Data was normalized to protein data which was determined with a BCA assay.

A.3 Results

Regenerative specimens were successfully produced in both 8 month and 21 month old animals during a 12 week implantation period. Complete regenerative specimens were found in 45% of the chambers placed in 8 month old animals and 50% of those placed in 21 month old animals. Representative images of the partially formed microspecimens is shown in **(Fig. A.1)** There was no significant difference in the average thickness of microspecimens produced in young and old animals **(Fig. A.2)**. Regenerative bone tissue from older animals had a higher average degree of mineralization or tissue mineral density (TMD) than younger animals, and TMD average values for young and old animals were less than respective average TMD measurements for mature bone controls **(Fig. A.3)**. Alpha blends also show that more voxels were mapped to greater HU values in regenerative specimens from old animals when compared to those from young animals **(Fig. A.4)**. This trend is supported by histogram data in **Figure A.5**.

Average mineral to matrix ratio was higher in regenerative specimens produced in old animals compared to those produced in young **(Fig. A.6)**. However, procion red stain suggests that there were a greater number of cells present in regenerative specimens from young animals compared to old animals **(Fig. A.7)** and this data is supported by the significant increase in number of DAPI positive cells observed in young regenerative specimens compared to old regenerative specimens **(Fig. A.8)**. Control bone, however, suggests no apparent difference in cellularity **(Fig. A.9)**. Normalized Nitric Oxide increased with load for both age groups during all time points it was assessed during loading. The increase was greater for young animals compared to old after 15 and 60

minutes of loading, while the reverse trend was observed after 30 and 45 minutes of loading **(Fig. A.10)**.

Discussion

In this study the effect of age on regenerative bone and its ability to respond to mechanical load was examined. Micro CT, histology, and nitric oxide data suggests that age does affect the morphology of regenerative bone and its response to mechanical load. This study supports the growing amount of research investigating the role of age on bone repair, transduction of external mechanical cues, cell density, and degree of mineralization; and highlights nitric oxide, an important modulator of bone forming and resorbing cells, as a component involved in the difference observed between young and old regenerative specimens' response to mechanical stimulus.

It is well established that with increased age there is a decrease in the osteogenic potential of progenitor cells to form new bone, and this study found that the number of osteocytes in regenerative bone from old specimens was less than that found in young specimens. However, the degree of mineralization observed in regenerative tissue from old animals compared to young was significantly higher, and no difference was found in the degree of mineralization of mature bone. Perhaps there is a compensatory mechanism that enables the bone in old animals to obtain such a degree of mineralization despite the decrease in osteogenic potential observed in marrow stromal cells. Furthermore, the specimens in this study were primarily woven bone which differs in organization from trabecular and cortical bone specimens which are most often used to obtain aging data. In addition, the higher degree of mineralization observed in regenerative specimens from

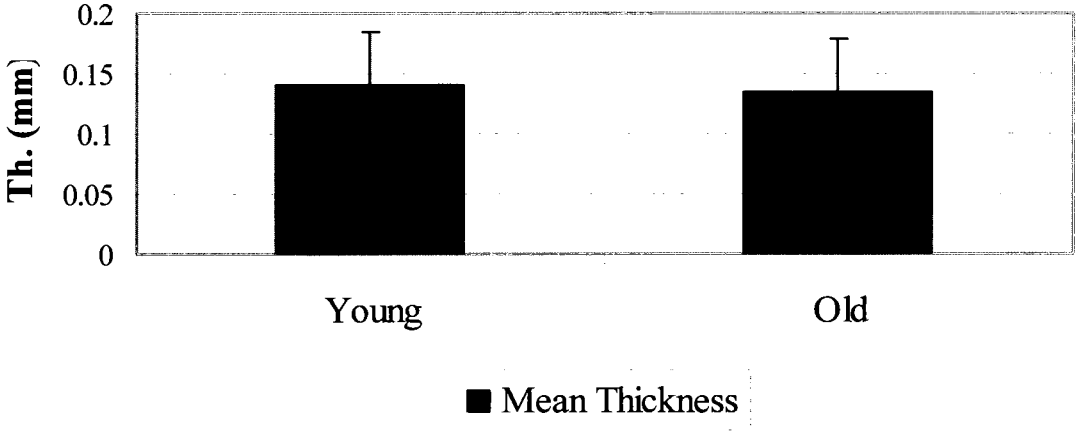
old animals may not be advantageous as it could make them more brittle and susceptible to the accumulation and propagation of micro-cracks.

Nevertheless, findings from this study suggests that decreased osteocyte cell density, increased degree of mineralization and time related differences in the increase of soluble nitric oxide during loading in regenerative bone samples are affected by age, and could highlight therapeutic routes to curtail age affects on decreased bone mineral density and structural integrity observed in elderly populations.

Figure A.1 Regenerative Tissue Isosurfaces

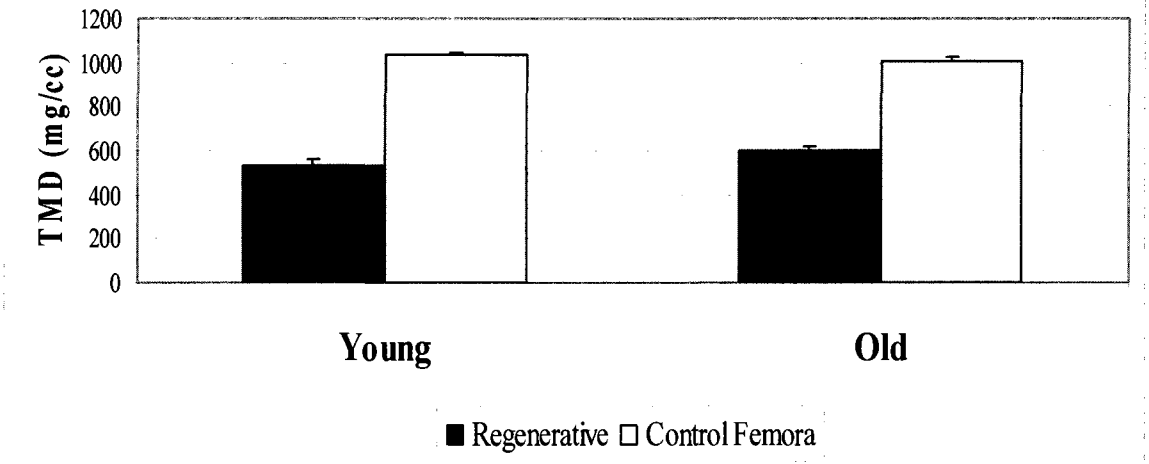
Representative isosurfaces of partially formed microspecimens that were used for CT and histology analysis.

Figure A.2: Regenerative Bone Specimen Mean Thickness (mm)



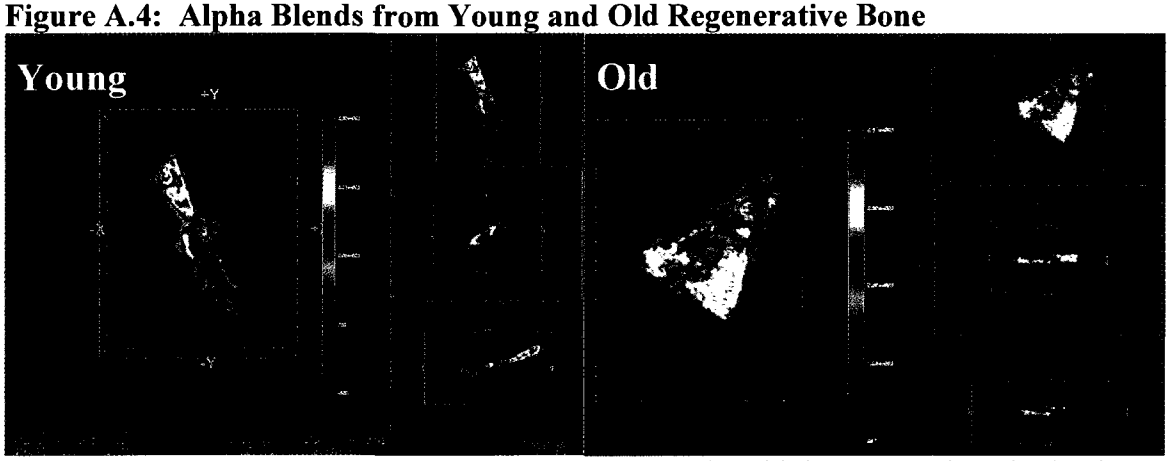
There was no statistically significant difference in the average thickness of regenerative microspecimens from young and old animals.

Figure A.3: Regenerative and Mature Bone Tissue Mineral Density (TMD)



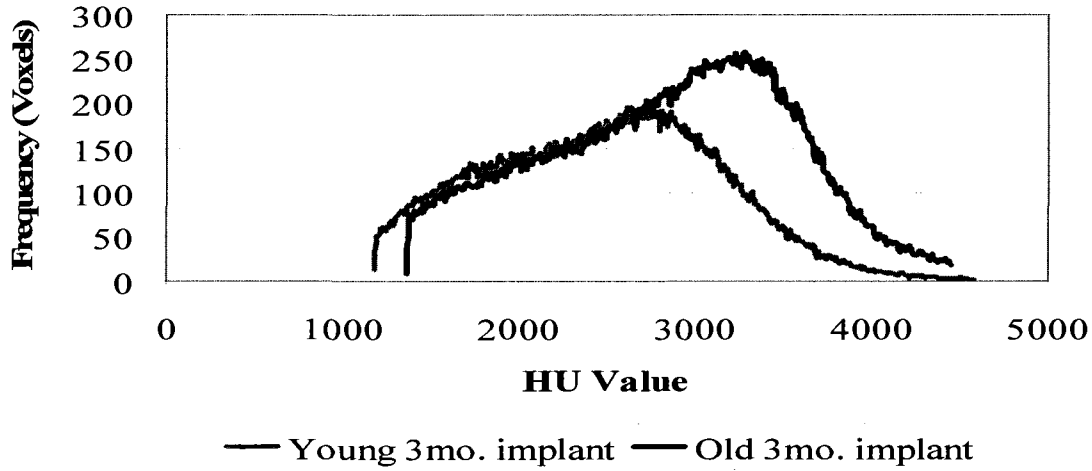
There was a significant difference in the average degree of mineralization between young and old regenerative bone tissue and similar values for mature bone from both age groups.

Figure A.4: Alpha Blends from Young and Old Regenerative Bone



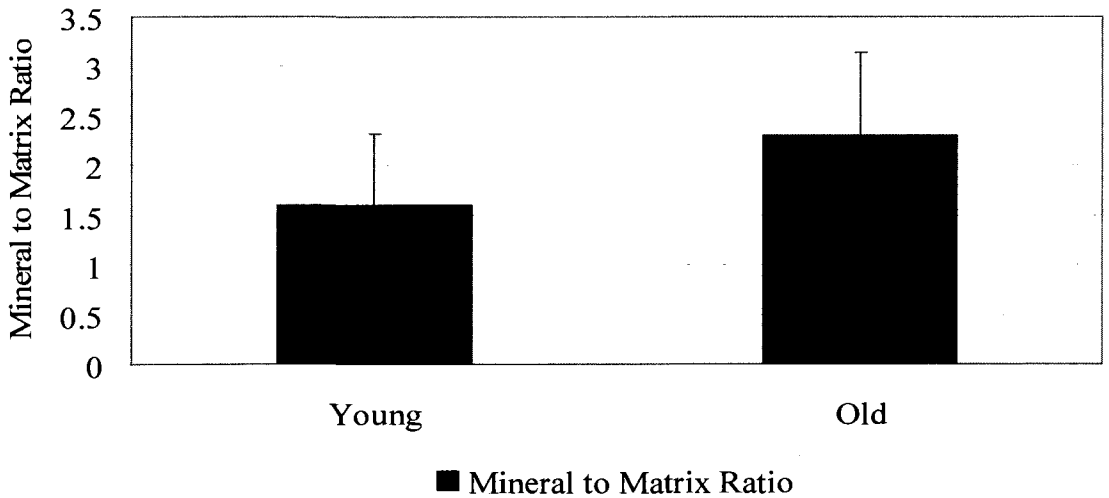
There were a greater number of bone voxels mapped to higher HU values indicating a greater radio density for bone voxels from regenerative tissue in old animals compared to young

Figure A.5: Histograms from Young and Old Regenerative Bone



There was a shift in the degree of mineralization between regenerative microspecimens from young and old animals. There is a greater number of bone voxels mapped to higher HU values indicating a greater radio density for bone voxels from regenerative tissue in old animals compared to young

Figure A.6: Mineral to Matrix Ratio from Young and Old Regenerative Bone



There was a higher average mineral to matrix ratio in regenerative microspecimens from old animals when compared to young.

Figure A.7: Procion Red Stain of Osteocytes in Regenerative Bone Specimens from Young and Old Animals

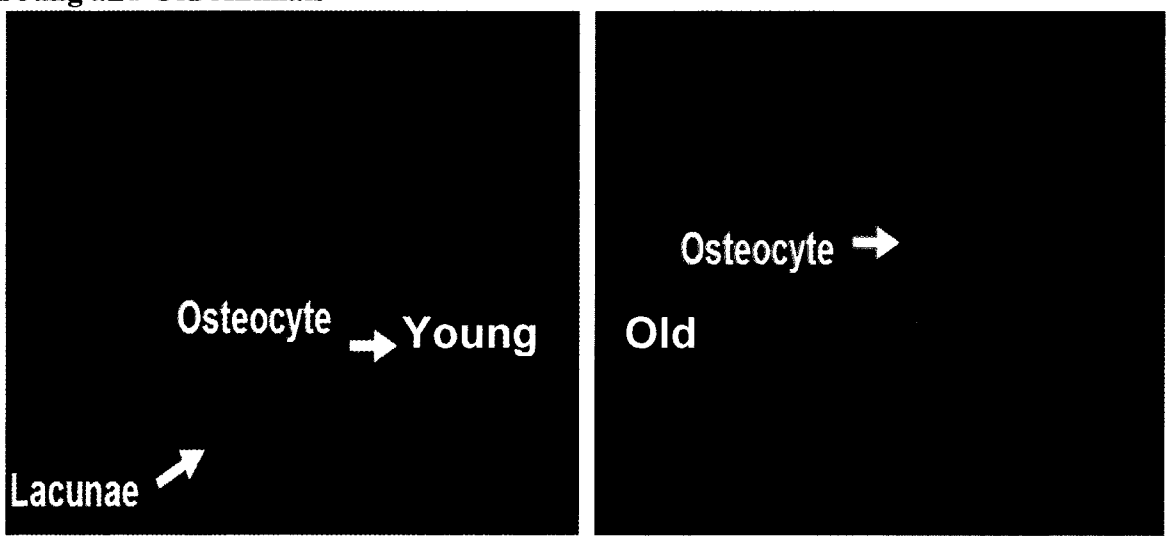
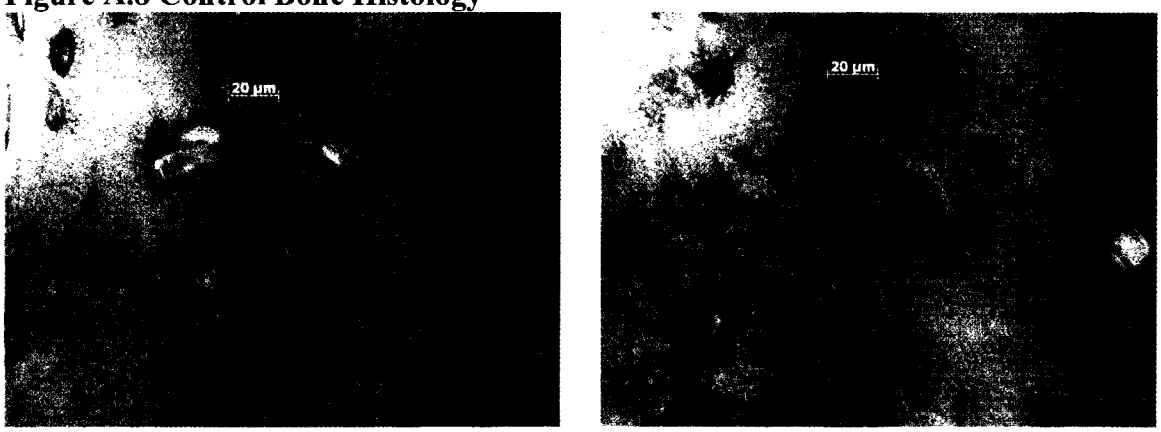


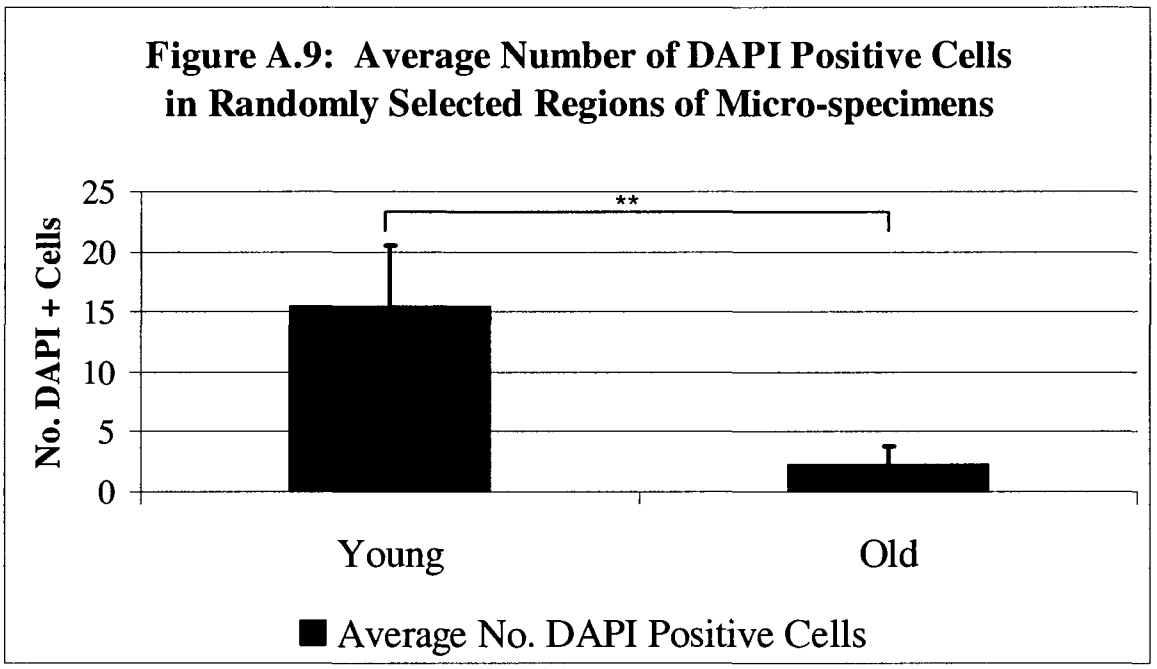
Fig. A.7 shows the greater number of procion red stained osteocytes in regenerative bone tissue from young animals compared to old.

Figure A.8 Control Bone Histology

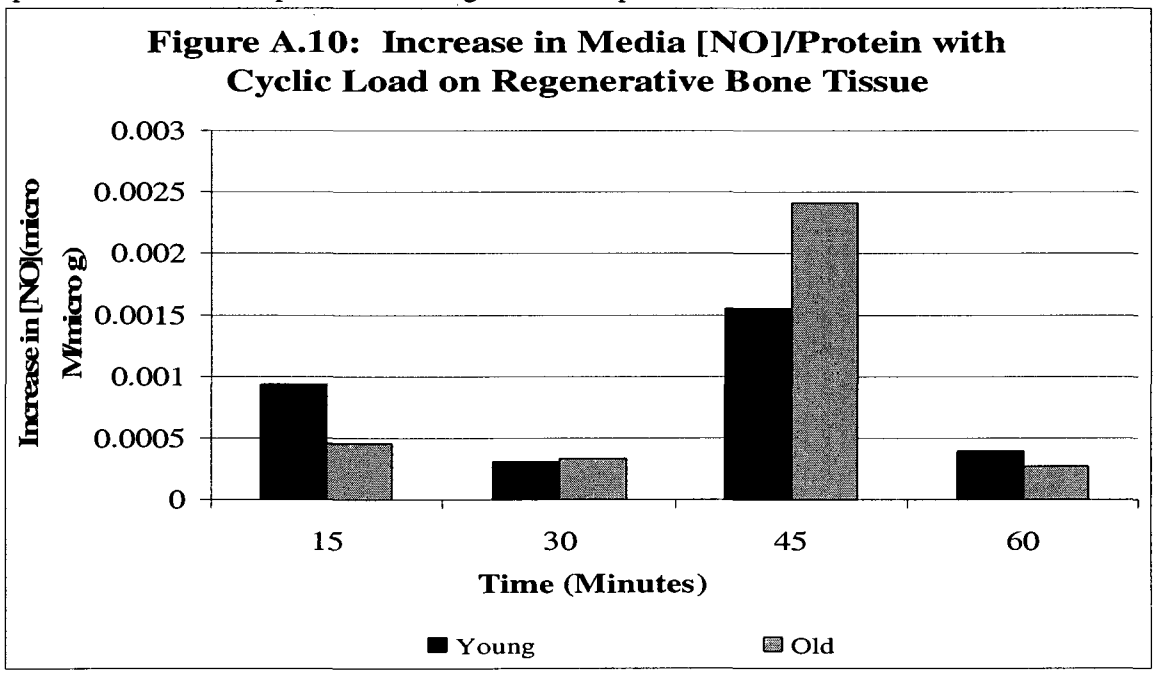


Young **Old** **40X**

There was no apparent difference in cellularity between mature specimens from young and old animals.



There was an increase in number of DAPI positive cells observed in young regenerative specimens when compared to old regenerative specimens.



There was an increase in normalized nitric oxide concentration for young regenerative specimens and old regenerative specimens over time.

APPENDIX B

VALIDATION OF OSCILLATORY FLUID SHEAR SYSTEM

One life but we're not the same we get to carry each other carry each other one one

~One

B.1. Introduction

Prior to oscillatory fluid shear experiments conducted in chapter four the system was validated with MC3T3 E1 cells cultured over a twenty one day time period. The load response in MC3T3 E1 cells was observed in addition to primary cells harvested from the long bones of rats.

B.2 Materials and Methods

B.2.1. Isolation of Primary Cells

Femora and tibiae were dissected from 1 month (n=3), 6 month (n=2) and 21 month (n=2) old male Sprague-Dawley rats. After marrow was flushed from both ends of dissected femora and tibia, bones were diced into smaller pieces and rinsed in PBS. Specimens were incubated at 37°C and 5% CO_2 for two hours in filter sterilized collagenase A diluted (Sigma-Aldrich 5-10ml/gm bone) in PBS (1mg/ml). After 2 hours bone fragments were washed three times with PBS. The final PBS wash was replaced with defined media (α-MEM+5% Calf Serum+5% FBS+1% Pen Strep). MC3T3 E1 cells were cultured separately in 10 cm culture dishes in defined media (α-MEM+1%Pen Strep+5%FBS).

B.2.2. Characterization of Cells

The mineralization capacity of primary cells harvested from the long bones of rats was assed with alizarin red, sirius red, and alkaline phosphatase stains. Cells were plated into 6-well tissue culture plates (BD Falcon) at a seeding density of 25,000 cells per 9.6cm^2 circular well. Media was replenished every three days was consisted of (α-MEM+5% Calf Serum+5% FBS+1% Pen Strep). Cells were rinsed with 1X PBS, fixed for 1 hour in 70% ethanol, and stained overnight with Alizarin Red (1:100:10 Alizarin red, ddH_2O, and 0.1% Ammonium Hydroxide) after 3, 7, 10, 21, 21, and 28 days of culture. Cells were imaged with a Zeiss microscope at 20x magnification. A separate population of cells cultured under the same conditions were rinsed with 1X PBS, fixed for 1 hour in 70% ethanol and stained for Sirius Red (100mg/ml in saturated aqueous picric acid) and imaged with a Zeiss microscope at 20x magnification after 3, 7, 10, 14, 21, and 28 days of differentiation. A separate population of cells cultured under the same conditions were rinsed with 1X PBS, fixed for 30 seconds in citrate-acetone formaldehyde and stained for Alkaline Phosphatase according to manufacturer's instructions **(Sigma Diagnostic St. Louis, MO)**.

B.2.3 Oscillatory Fluid Shear Stress

Cells were plated and either sham or experimentally loaded at 2Pa 0.5Hz for 30, 60, and 90 minutes as described previously in chapter four. Subsequent treatment media was collected, snap frozen in liquid nitrogen, and stored at -80□C until further processing. Cell protein was harvested with a lysis buffer comprised of 95% RIPA buffer, 1% sodium orthovanadate, 1% phenylmethanesulphonylfluoride or

phenylmethylsulphonyl fluoride (PMSF), and 3% protease inhibitor cocktail. Cell protein was snap frozen in liquid nitrogen and stored at -80□C until further processing.

B.2.4 Quantification of Nitric Oxide and Prostaglandin E_2

Media was assayed in duplicate according to manufacturers' instructions with colorimetric assay kits for nitric oxide and prostaglandin E_2 (Cayman Chemical, Ann Arbor, MI) as described previously in chapter 4.

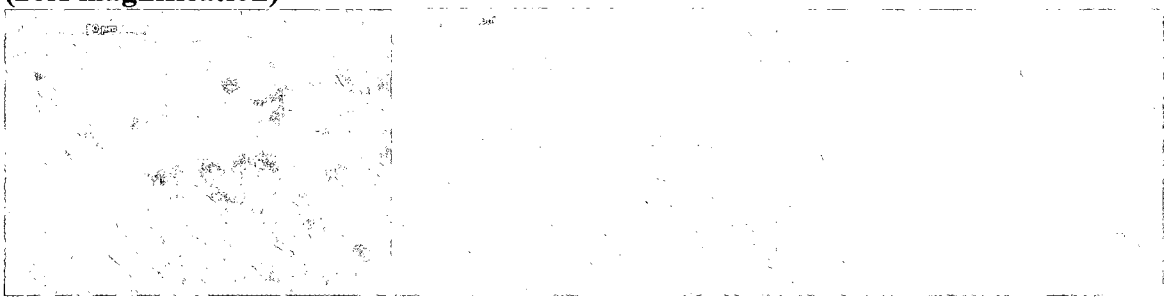
B.2.5 Western Blot

Ten μg of protein were loaded into the lanes of a 10% Tris-HCL gel. The gel was run for one hour at 150V and was transferred onto a PVDF membrane at 80V for 40 minutes as described previously. The membrane was blocked for one hour at room temperature on a shaker with a 5% Blotto solution (5% Carnation dry milk in 0.1% PBS-Tween) and incubated overnight on shaker at 4□C with primary antibody for phosphorylated extracellular regulated kinase (pERK) at a dilution 1:1000 (abcam, Cambridge, MA) and primary antibody for ERK (abcam, Cambridge, MA). The blot was then rinsed three times for 10 minutes each with 0.1% PBS-Tween. The membrane was then incubated for two hours at room temperature on a shaker with a secondary antibody at a dilution (1:25000) (Thermo Scientific, Waltham, MA). The blot was rinsed three times for 10 minutes each with 0.1% PBS-Tween. The membrane was then developed with Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA). Densitometry was calculated with Image J as described previously. Blots were then stripped for 8 minutes on a shaker at room temperature with 0.01N HCL and rinsed three times for 10 minutes each at room temperature on a shaker in 0.1% PBS-Tween.

B.3 Results

MC3T3 E1 cultured cells also stained positive for alizarin red and type I collagen **(Figure B.1)**. Both primary cells and MC3T3 E1 cells exhibited a load response through changes in nitric oxide, PGE_2, and pERK expression. There was an increase in nitric oxide expression in loaded MC3T3 E1 cells after all time points and days of culture **(Figure B.2)**. A dramatic decrease in the up-regulation of nitric oxide was observed in mechanically stimulated cells harvested from 6 month and 21 month old animals compared to 1 month old **(Figure B.3)**. Similarly a significant decrease in the up-regulation of prostaglandin E_2 occurred in mechanically stimulated cells harvested from 6 and 21 month old animals when compared to cells from 1 month old animals **(Figure B.4).** Representative western blots for ERK **(Figure B.5)** and pERK **(Figure B.6)** are shown. The densitometry of pERK to total ERK was highest for cells from 1 month old animals after all loading time points **(Figure B.7)**. Runx2 protein expression was highest in cells harvested from 1 month old animals **(Figure B.8)**.

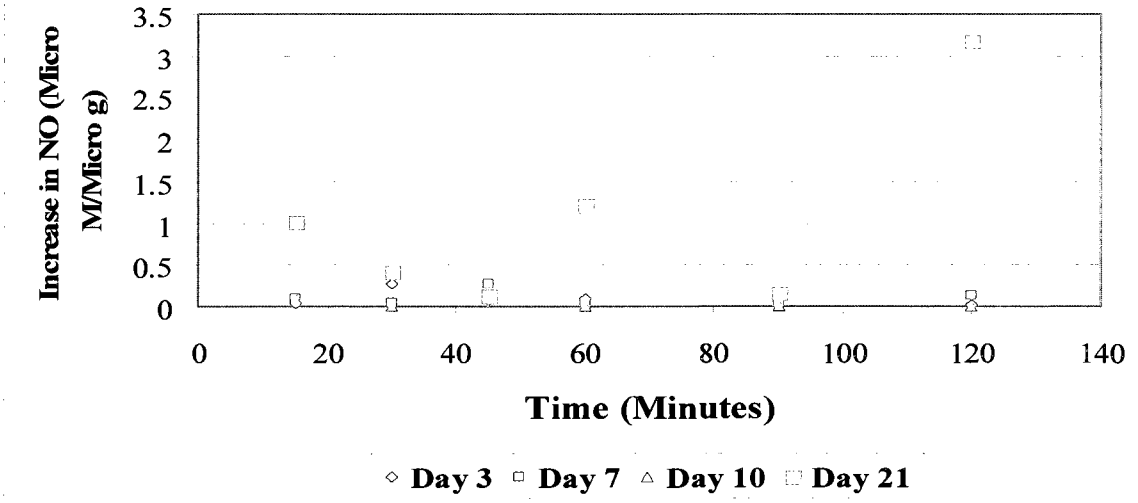
Figure B.1 Alizarin Red, Sirius Red, and Alkaline Phosphatase MC3T3-E1 Cell (20X magnification)



Alizarin Red **Sirius Red** **Alkaline Phosphatase**

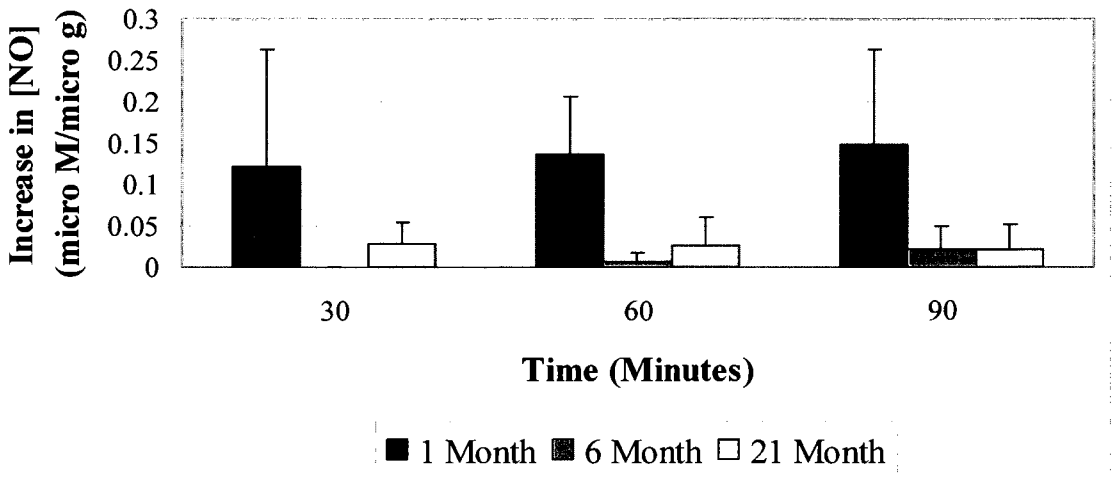
MC3T3 E1 cells stained positive for AP, ALP, and SR.

Figure B.2: MC3T3 E1 Cells Increase in Nitric Oxide after 2Pa Oscillatory Fluid Shear Stress



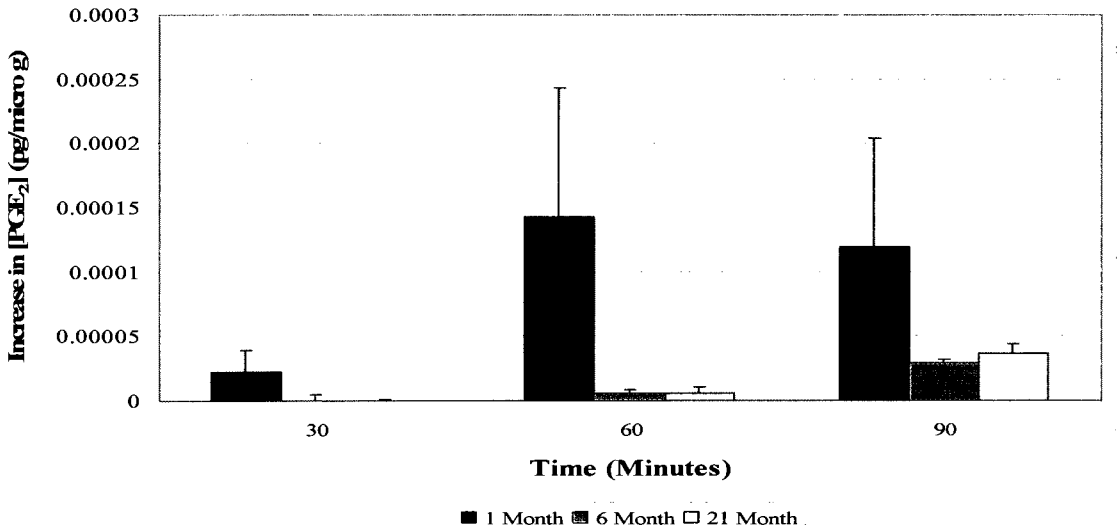
MC3T3 E1 cells increased nitric oxide production after exposure oscillatory fluid shear stress for all time periods of loading and all time periods of differentiation.

Figure B.3: Increase in Nitric Oxide after 2Pa Oscillatory Fluid Shear Stress



Cells increased NO production after exposure to oscillatory fluid shear stress for all time periods of loading. This increase was highest in cells harvested from 1 month old animals.

Figure B.4: Primary Cell Increase in Media [PGE_2] after 2Pa Oscillatory Fluid Shear Stress



Cells increased PGE_2 production after exposure to oscillatory fluid shear stress for all time periods of loading. This increase was highest in cells harvested from 1 month old animals.

Figure B.5 ERK Western Blots

UT 30"S 30"E 60"S 60"E UT 30"S 30"E 60"S 60"E UT 30"S 30"E 60"S 60"E

1 month 6 month 21 month

UT 90"S 90"E UT 90"S 90"E UT 90"E 90"S

1month 6 month 21month

UT represents untouched specimens, S represents sham treated specimens, and E represents experimentally loaded specimens. ERK expression was observed in cells from 1 month, 6 month, and 21 month old animals.

Figure B.6 pERK Western Blots

UT 30"S 30"E 60"S 60"E UT 30"S 30"E 60"S 60"E UT 30"S 30"E 60"S 60"E

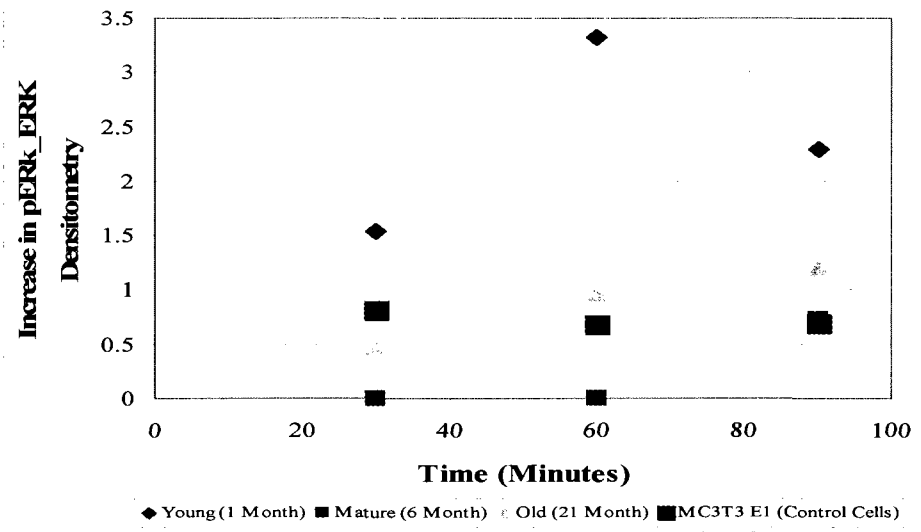
1 month 6 month 21 month

UT 90"S 90"E UT 90"S 90"E UT 90"E 90"S

1month 6 month 21month

UT represents untouched specimens, S represents sham treated specimens, and E represents experimentally loaded specimens. Phosphorylated ERK was observed in cells harvested from 1 month, 6 month, and 21 month old animals subjected to oscillatory fluid shear stress

Figure B.7 Increase in pERK Densitometry after 2Pa Oscillatory Fluid Flow



pERK densitometry increased in cells from 1month, 6 month, and 21 month old animals after exposure to oscillatory fluid shear stress. This increase was highest in cells from 1 month old animals.

Figure B.8 Primary Cell Runx2 Western Blot

1moUT 6moUT 21moUT

UT represents untouched specimens. Runx2 expression was highest in cells harvested from 1 month old animals.