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IMPOSING CYCLIC STRAIN ON OSTEOGENIC STEM CELLS:

THE EFFECTS OF STRAIN LEVELS AND REPITITION OF

CYCLIC STRAIN IN AN IMPLANT ENVIRONMENT

By

Daniel Henlee Smith

A Thesis
Submitted to the Faculty of
Mississippi State University
In Partial Fulfillment of the Requirements
For the Degree of Master of Science
In Biomedical Engineering
In the Department of Agricultural and Biological Engineering

Mississippi State, Mississippi

December 2004

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Bone and bone cells have been shown to respond to mechanical forces placed upon them. Particularly, strain plays an important role in osteogenic differentiation of marrow cells around artificial implants in bone. These strains, depending on their magnitude, duration, and repetition, can alter the proliferation and matrix synthesis of osteoblasts. To test how strain parameters influence osteoblast behavior, a four-point bending apparatus was used to impose cyclic strain on osteogenic stem cells isolated from rats and seeded on titanium plates. Cells were stimulated at 1 Hz for 15 minutes daily and compared to an unstrained control. Stimulation occurred at two magnitudes: 400 and 1000 micro-strain, and three levels of repetition: one, three, and five consecutive days. DNA, protein, alkaline phosphatase, and calcium levels were measured to determine the proliferation and matrix synthesis activity of the cells. No statistically significant effect was found for the tested parameters under these conditions.

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CHAPTER I

INTRODUCTION AND BACKGROUND

Background and Significance

Structure and Function of Bone Tissue

Bone tissue serves several functions within animals, the most notable of which is providing a strong structural support for locomotion and protection of vital organs. However, bone also serves as an important reservoir for calcium and other minerals. Several cell types are responsible for the formation and maintenance of bone. The first cell type is the osteoblast which is responsible for forming new bone. These osteoblasts may eventually encase themselves in bone matrix, at which point they are then termed osteocytes and serve primarily to maintain their surrounding matrix rather than forming new matrix. A third cell type is the osteoclast, a large multinucleated cell that is responsible for breaking down old or damaged bone for replacement by new bone formed by osteoblasts. All of these cells are constantly active in the process of maintaining, forming and degrading bone. The extent of their activity and the balance between building and breaking down bone is regulated by several factors including mechanical stress and hormonal response to blood calcium levels.

Bone tissue can be described on both the macroscopic and microscopic levels. Different structures exist at both the visible and the molecular level. Macroscopically, the outer

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layer of bone is made up of dense tissue known as compact bone, while the inner layer is made of a much more porous tissue called cancellous bone or spongy bone (Fig 1.1). In compact bone, osteocytes and layers of intercellular matrix are arranged in a concentric circular pattern called osteons around central canals. The osteocytes reside in small cavities within the bone called lacunae. These osteons are oriented parallel to the long axis of the bone in a fashion to resist the compressive forces placed on the bone (Fig 1.1).

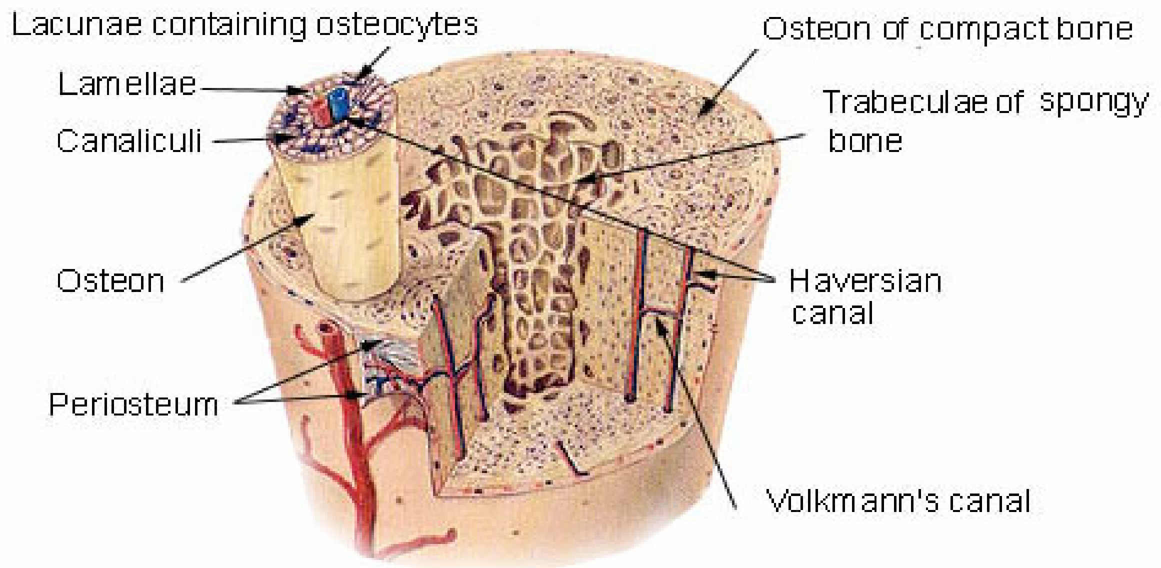

Figure 1.1: Gross Structure of Bone.
http://www.training.seer.cancer.gov/module_anatomy/unit3_2_bone_tissue.html

The Haversian canal, which runs through the center of the osteon, contains blood vessels and nerves. Haversian canals are linked to each other laterally by Volkmann's canals containing larger blood vessels and nerves. This interconnecting canal system containing blood vessels and nerves is known collectively as the Haversian system. The cells within the osteon communicate with each other and with the Haversian system via small openings in the bone known as canaliculi (Fig 1.1 and 1.2).

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Cancellous, or spongy, bone also contains osteocytes, but due to its porous nature does not contain an interconnecting canal system. Instead of the solid structure found in compact bone, cancellous bone contains a much more open structure. This web-like open structure resembles a sponge, as the name implies. The small individual pieces or struts that make up the web are composed of bone mineral, proteins and osteocytes and are known as trabeculae (Fig 1.1).

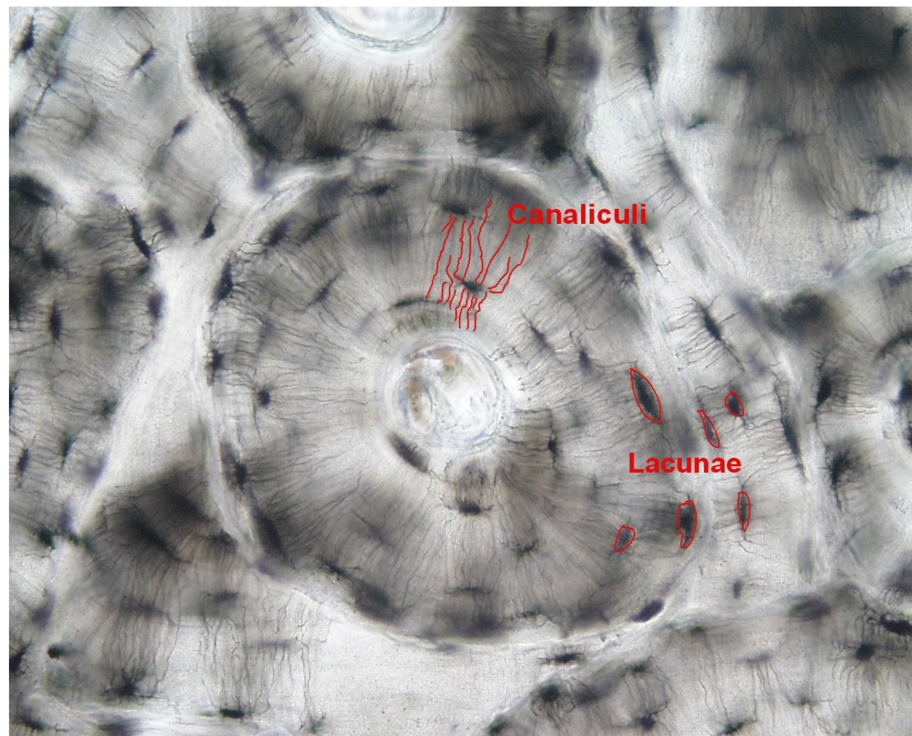

Fig 1.2 : Histological Section of an Osteon.
This demonstrates the central Haversian canal as well as small canaliculi communicating between lacunae.
http://www.lab.anhb.uwa.edu.au/mb140/

On the microscopic level, two types of bone tissue occur naturally in compact bone (Fig 1.3). The first is lamellar bone, which makes up almost all healthy adult bone. Lamellar bone is a mature bone type that consists of collagen fibers organized in a very regular parallel fashion. This parallel structure gives the bone a great deal of structural

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strength. Woven bone however is formed rapidly, generally after some damage has occurred to the bone tissue (e.g. a callus formed during fracture healing). This bone structure consists of collagen fibers oriented in all different directions and it is not as strong as the parallel structure of lamellar bone. Eventually, this woven bone will be changed and modified to form lamellar bone. This process is referred to as remodeling.

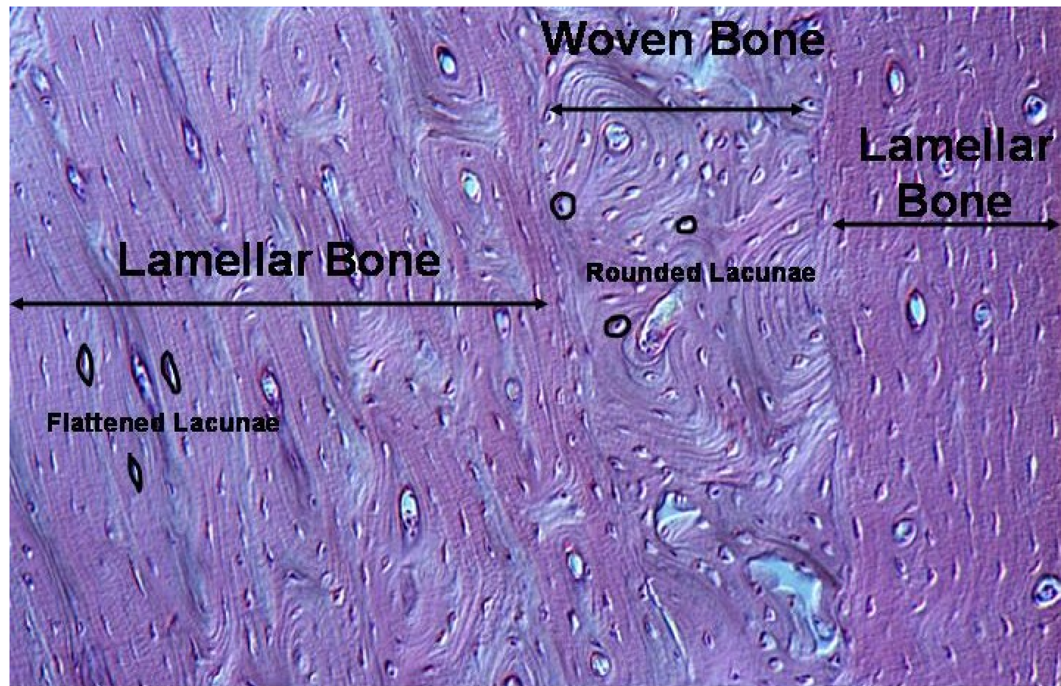

Figure 1.3: Histological Section of Woven Bone and Lamellar Bone.
http://www.lab.anhb.uwa.edu.au/mb140/

Differentiation of Bone Marrow Stem Cells

Bone marrow resides within the hollow compartment inside bone (principally long bone) and contains two major cell systems. They are the hematopoietic cells and the marrow stroma. The former is a cell line responsible for the production of blood cells and osteoclasts. The latter is a group of cells including fibroblasts, adipocytes and osteoblasts (near the inner surface of the bone) that serve to structurally and

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physiologically support the hematopoietic cells. Also contained in the stroma are cells with stem-cell-like qualities that can give rise to osteocytes, chondrocytes, adipocytes, and fibroblasts, among others (Krebsbach 1999). These individual cell lineages, including osteogenic, adipogenic and chondrogenic cells arise from common progenitor cells (Muraglia 2000, Houghton 1998). These progenitor cells have the ability for unlimited self renewal and have been termed mesenchymal stem cells (Bruder 1998). As these cells differentiate into more mature cell types, they begin to lose their pluripotent potential (Ter Brugge 2002, Muraglia 2000). Uniquely, these stromal stem cells are easily isolated from gross marrow since *in vitro* these cells will adhere to tissue culture plastic, and the remaining hematopoietic cells in the marrow will not (Krebsbach 1999).

Differentiation of these mesenchymal stem cells along the osteogenic lineage proceeds in several specific stages identified by morphological changes (Aubin 1998). A number of factors are important in the differentiation of mesenchymal stem cells. Hormonal influences, vascular supply, and mechanical environment all play a role in the guided differentiation of these cells. One proposed theory of tissue differentiation in fracture healing and embryonic development suggests that mesenchymal stem cells are highly influenced by both the hydrostatic forces and the strain of their environment. [(Fig 1.4); (Carter 1988, Blenman 1989)]. It should be noted that newly implanted devices in bone can be considered a unique form of fracture healing and is governed by the same principles of cell differentiation around the new implant.

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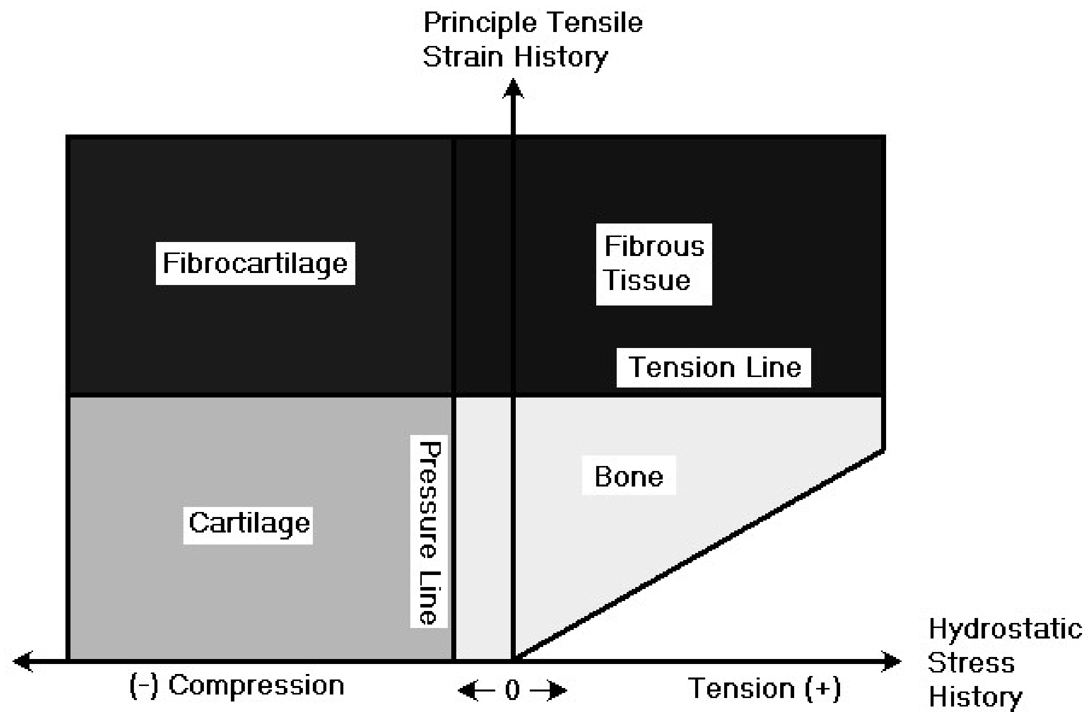

Figure 1.4: Tissue Differentiation Theory.
adapted from Carter D, *et al.* [1988]

The first step in this differentiation into osteoblasts is the formation of osteoprogenitor cells. These osteoprogenitor cells are somewhat rare, making up less than 1% of the stromal cells (Bellows 1990, Aubin 1999). *In vitro,* these cells show a great ability to proliferate, yet lack a fundamental ability for self-renewal (Bellows 1990, McCulloch 1991). That is, they are capable of forming great numbers of mature osteoprogenitor cells, but very limited in their ability to produce more immature osteoprogenitor cells. Unfortunately, to some extent, the same may be true *in vivo* and may be partly responsible for bone pathologies such as osteoporosis (Bellows 2003). The difference between the mature and immature cells lies in their ability to form osteoblasts. The mature cells are capable of spontaneously forming osteoblasts without the aid of inducers, while the immature cells need specific inducers to form osteoblasts (Long 1995,

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Turksen 1991). These inducers include some glucocorticoids such as dexamethasone, transforming growth factor β1(TGF-β), basic fibroblast growth factor (bFGF), bone morphogenic protein-2 (BMP-2), and 1,25-dihydroxy vitamin D_3 (1-25-OH D_3).

The next stage of differentiation following the osteoprogenitor is the pre-osteoblast. Though these cells are not fully characteristic of the final osteoblasts, they do produce small amounts of alkaline phosphatase, an indicator of active matrix mineralization (and therefore bone deposition). The final osteoblast is a post-proliferative cell that manufactures all the products used in the formation of bone matrix. These include collagen type I, osteocalcin, bone salioprotein, and osteopontin. They also possess the ability to fully mineralize the matrix since they produce characteristically large amounts of alkaline phosphatase or ALP (Aubin 1998 a and b). This enzyme facilitates the deposition of calcium phosphate crystals inside the collagen matrix of bone.

Mechanical Effects on Bone Tissue

For many decades it has been known that bone remodels in response to mechanical changes in its environment. The first to study the biomechanical environment of bone was the Swiss anatomist von Meyer in 1867(von Meyer 1867.) A German civil engineer, Culmann, collaborated with von Meyer and they first suggested that internal stress patterns regulated trabecular alignment (Roesler 1987.) Only a few years later Wolff popularized this idea in what he called his “Trajectorial Theory” of trabecular alignment (Roesler 1987, Wolff 1892.) It was Wolff’s writing that brought acceptance to this idea and sparked a longstanding interest in the study of bone adaptation

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and remodeling. Roux also recognized that connective tissue would adapt to its mechanical environment and suggested a self-regulating system (Roux 1895.)

Wolff predicted that bone remodeling would eventually be described quantitatively rather than qualitatively by establishing a set of mathematical rules of law for bone remodeling. To date, no firm set of mathematical laws has been discerned from research. However, basic principles of the biomechanical effects on bone are known. Many studies have evaluated the effects of varying mechanical environments from different gravitational forces to exercising only one side of the body (Hert 1969 & 1971, Churches 1979 & 1981, Saville 1969, Woo 1981, Watson 1973, Jones 1977, Vogel 1973, Whedon 1977, Smith 1977, Uhthoff 1978, Jaworski 1980). These studies all demonstrate one basic principle: increased levels of activity cause an increase in bone mass, while decreased levels of activity cause a decrease in bone mass. This hypothesis is based on the concept of bone maintaining a specific biomechanical environment to keep strain at an ideal level. That is, bone responds to increased strain by increasing matrix deposition in order to stiffen itself thereby lowering the mechanical strain given the same mechanical force. This idea is perhaps best explained in a widely sited diagram produced by Rubin (Fig 1.5). Support of this hypothesis has been provided in many *in vivo* studies that subject animals to a specific mechanical loading environment and observe the resulting increase or decrease in bone formation (Rubin and Lanyon 1987, Burr 1984, Churches 1979 & 1981, Hert 1971 a & 1971 b, Lanyon 1981, O'Conner 1982).

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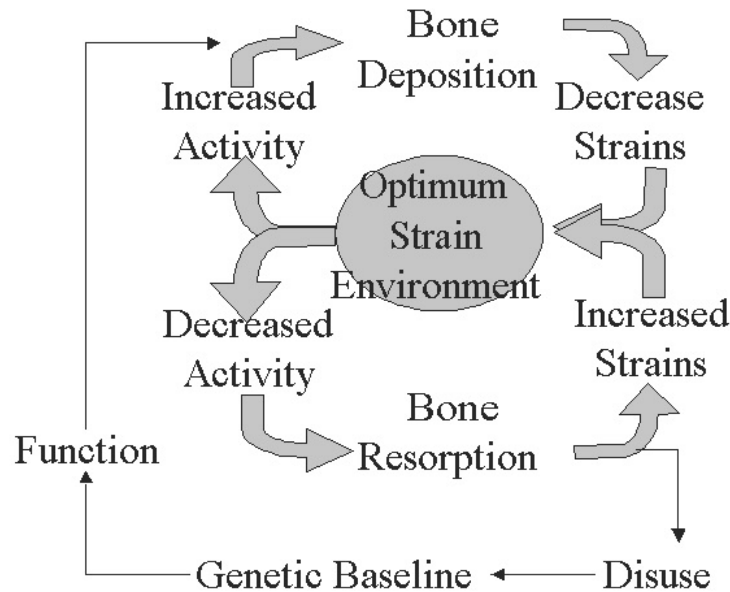

Figure 1.5: "Adaptation Hypothesis" of Bone Regulation.
[adapted from Rubin in *Calcified Tissue International*]

Several studies have been conducted to quantify the strain environment of bone. It has been shown that in humans, during strenuous activity, such as running, the average maximum strain encountered in a long bone is approximately 850 micro-strain and 400 micro-strain during a less strenuous activity like walking (Lanyon 1975). These levels of strain encountered in bone are similar for several species including rats, dogs, sheep, and turkey (Rabkin 2000, Fritton 2000). When the three species, dogs, sheep and turkeys, were monitored during the normal activity of an entire day, it was shown that large strains in excess of 1000 micro-strain rarely occurred while very small strains in the order of 10 micro-strain predominated and occurred several thousand times daily. These magnitudes were shown to be similar for all three species and for weight bearing and non-weight bearing bone (Fritton 2000).

The mechanical loading of bone fractures has also been studied. This is a unique instance in which load is applied primarily across woven bone, which is not as strong as

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the mature lamellar bone. However, it is this research that may prove most valuable in the clinical setting, as the majority of bone related treatments involve the healing of bone and not bone diseases. Studies have shown that both passive and active loading of an externally fixed fracture increase density and in some cases quality of callus formation around a fracture site (Aro 1990, Egger 1993). But these studies were only qualitative, simply comparing loaded vs. non-loaded fractures. When fracture movement is measured, it shows a dosage type response for callus formation around the fracture (Claes 1998, Wolf 1998.) That is, small and very large movements in the fracture gap will cause smaller callus formation than medium movements. This gives great insight into the effect mechanical forces have on new bone formation. However, the latter data only indicates a formation of more bone tissue and not higher quality tissue. The optimum treatment in this case is one that produces high quality bone very quickly, thus fully healing the fracture in the shortest time possible.

Artificial Implants in Bone Tissue

Implants in bone are widely used in orthopedic, dental and craniofacial applications. These implants are designed to be strong, corrosion resistant, and biocompatible with bone. To date a wide variety of materials have been chosen for these implants, including titanium and titanium alloys, stainless steel alloys, and various plastics. A great deal of research goes into the properties of each particular implant material both independently and through medical companies seeking to evaluate their products.

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In addition to the vast amount of research on mechanical properties of the implants themselves, much research has been done on the biological integration of the implants into bone tissue including mechanical loading (Bumgardner 2000, Chang 1996, Puleo 1999). For example, Barbier and Schepers found that axially loading oral implants produced "a more uniform, histologically quiescent remodeling response" than those implants loaded nonaxially (Barbier 1997). Others have used finite element analysis and recovered implants to develop a bone-remodeling model based on the localized strain at the tissue-implant surface (Simmons 2001). It is clear from the variety of experiments performed that loading affects the long term success of implants in bone. (Fischer 2004, Romanos 2004, Ko 2003). Thus it is important to understand how bone cells and bone precursors respond to strain in an implant environment.

In Vitro Mechanical Loading Studies

A great deal of research has been done regarding the response of bone cells to mechanical stimuli *in vitro*. Confusing the issue is the wide variety of stimulation techniques. Four-point bending apparatuses are very common (Winter 2002, Neidlinger-Wilke 1994, Bottlang 1997). They work by bending a substrate across two stationary support members with two moving outer members. Another common way to mechanically load cells *in vitro* is a longitudinal stretch system (Somjen 1980, Meikle 1979, Leung 1977, Murray and Rusthon 1990, Ives 1986, Winter 2003). Here, a substrate is stretched end to end to produce the desired strain on the attached cells.

Another method of applying strain is substrate distention, in which the substrate and cell monolayer is deformed by some force such as vacuum, fluid displacement, or

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displacement by stretching over some solid mass (Hasegawa 1985, Banes 1985, Gilbert 1990, Labat 2000). Generally, the strain produced by these devices is hard to characterize due to the non-uniformity of the stress applied to the substrate surface. These devices also produce strain in all directions, which for a given measured magnitude of strain produces a much greater mechanical stimulation than other mono-axial methods; cells are stretched equally in all directions instead of just one primary direction. This must be taken into account when reviewing the strain magnitudes used in these experiments. A unique subset of the substrate distention class is made of devices that produce in-plane substrate distention (Williams 1992, Schaffer 1994, Lee 1996, Ziegler 1997). These devices hold part of the substrate surface flat over a frictionless mass and either moves the mass or distends the surrounding surface to produce uniform strain in all directions across the flattened surface where cells are cultured.

Other devices include those that produce unconfined compression on the cells through direct hydrostatic compression (Bourret 1976, Elder 2000, Lippiello 1985, Burger 1992, Brighton 1996) and platen abutment (Torzilli 1997, Burton-Wurster 1993, Guilak 1994) in which a mass directly acts upon a matrix of cells to compress it. Fluid shear systems have also been used to mechanically stimulate cells (Davies 1995, Dewey 1984, Topper 1997, Levesque 1985, Frangos 1988, Chun 1997). For these fluid models, shear stresses must be estimated or theoretically calculated. The great variety of devices in use makes for difficulty in comparing experimental results.

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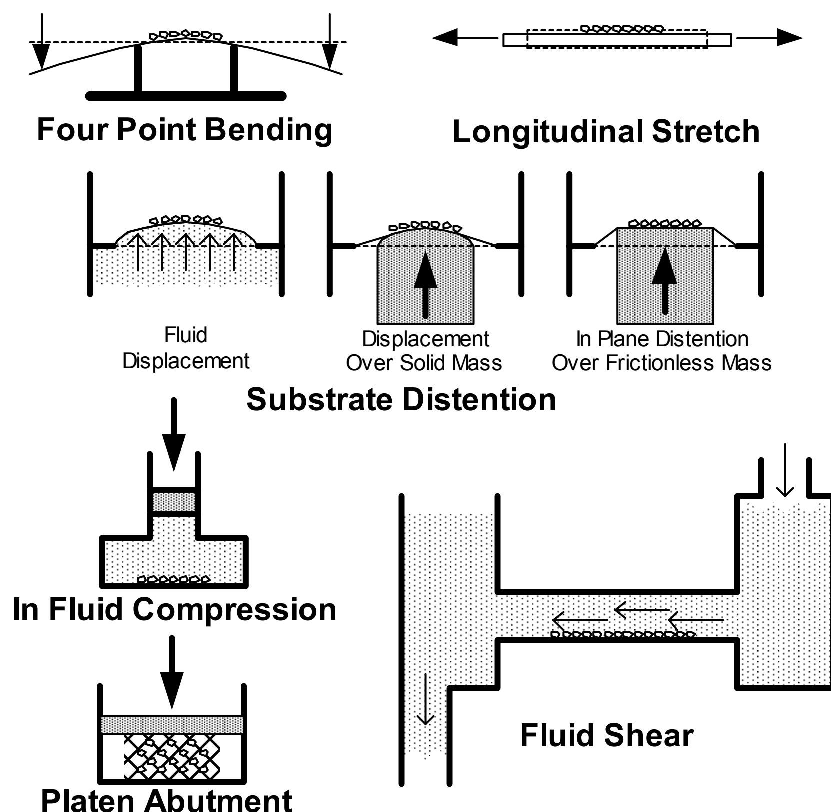

Figure 1.6: Various Methods of Mechanically Stimulating Cells.

To confuse matters even more, these devices use a variety of substrates such as titanium (Winter 2002), silicon (Bottlang 1997, Neidleinger-Wilke 1994; Walboomers 2004), ceramics (Labat 2000), and even plastics or glass (Jones 1991). Surface chemistry and surface roughness have been found to play a critical role in influencing the cells in their environment (Puleo and Nanci 1999, Boyan 1996). Because of this, studies using different substrates may not be directly compared. Therefore, it is imperative that researchers use in their *in vitro* experiments substrate materials and preparations comparable to those used in the intended application of the research. In the case of implant devices used in hard tissue, surfaces representative of implant alloys would be appropriate.

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Additionally, each of these devices has its own unique means of stress application (pneumatics, mechanical pulleys, electromagnets, etc.), its own unique method of strain measurement (direct application of strain gauges, optical measurements, theoretical calculations, etc.) and its own unique waveform pattern of strain application. Perhaps the most confusing factor in these experiments is the variety of cell types, isolation techniques, and culture techniques used to grow the different cell populations. Thus it is very difficult to compare "apples to apples" in these studies. However, some basic principles can be gleaned from these experiments.

The most obvious property of bone-forming cells found *in vivo* is their propensity to respond to mechanical stimulation in a trigger-like fashion. That is, they do not respond in a dose-like manner to the stimulation placed upon them, but instead, more of an all or nothing fashion. This cell type shows no response below a certain level of stimulation and above this threshold shows near peak response regardless of further stimulation. This threshold may be a certain strain level, strain duration, number of strain cycles, or a combination of these three. Jones et al. showed that periosteal cells doubled production of collagen after one cycle of a large strain (3000 micro-strain) and no greater response was seen following 30 cycles of strain (Jones 1991). Brighton et al. demonstrated that bone cells cyclically strained in a biaxial direction at 400 micro-strain increased proliferation and decreased bone matrix synthesis after 15 min of stimulation and had no effect after up to 48 hours of stimulation (Brighton 1991). In this same study it was shown that low magnitudes of strain (200 micro-strain) had no significant effect on the cells. Brighton et al. reported in a later study that although peak response occurred at 120 strain cycles, near-peak response occurred after as little as 20 cycles (Brighton 1992).

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Mellitou et al. demonstrated that proliferation increased after 36 strain cycles per day but not after only 4 (Mellitou 1992). Lanyon and Rubin as well as Recker et al. reported similar observations in *in vivo* animal models (Rubin 1984, Recker 1991).

Further along the lines of this trigger-like response, is the concept that these cells respond only within a certain window of stimulation. That is, below certain stimulation and above certain stimulation the cells have no response. Frost was one of the first to suggest such an adaptive window in osteoporosis research (Frost 1991). Neidlinger-Wilke et al. observed that for strain levels of 1%, human osteocytes *in vivo* showed a proliferative response, while no response was observed at higher strain levels of 2.4%, 5.3%, and 8.8%, suggesting that the cells some how ignore such strain. However, it has been shown by some members of the same group that such high levels of strain cause cells to align away from the axis of strain (Neidlinger-Wilke 2001). This may be because the high levels of strain cause breaking of mechanical bonds along the axis of strain or by some innate cellular response. Regardless, the final conclusion is the same: either by mechanical substrate-cell bonding limitation or some unknown cellular mechanisms, cells do not normally respond to excessively large strain.

Osteogenic cells also tend to exhibit a refractory response. After they are triggered by a certain level of stimulation, they will be unresponsive to further stimulation for some time. Brighton et al. observed that in mechanically deformed bone cells, inositol phosphate levels peaked after only 2 minutes of stimulation and then decreased back to control levels after 5 minutes despite further stimulation. Similar occurrences have been observed in mRNA levels in osteoclasts (Stanford 1994).

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These cells also seem to have a memory for previous stimulation. Some experiments have shown that alkaline phosphatase levels remain similarly high for cells that were deformed and then rested as for those that were rested then deformed (Conti 1993). Other experiments have shown that the proliferative response of cells is similar whether it is measured starting directly after stimulation or after some period of rest (Brunette 1984). Skerry et al. proposed that this memory came from some organization of macromolecules within the cells (Skerry 1987).

While several complex variables govern the response of osteogenic cells to mechanical stimuli, perhaps the most obvious and sometimes overlooked characteristic is the relationship between a cell population's ability to proliferate and its ability to produce matrix. These two normally hold and inverse relationship, in that a cell only has a limited energy source and can "choose" to either use its energy to make other cells, use it to produce a product, or use it to perform some balance of the two. This effect was observed in a pilot study performed by this investigator. In the pilot study, UMR cells (an osteogenic cell line) were cyclically strained at 1 Hz for 15 minutes a day over 5 days and sampled on each of the 5 days. Four strain levels were tested: 0, 200, 400, and 1000 micro-strain. The group strained at 1000 micro-strain had the highest level of proliferation and the lowest level of matrix synthesis based on measurements of their DNA content, total protein, and APL activity. The group strained at 400 micro-strain was just the opposite with the lowest level of proliferation and the highest level of matrix synthesis.

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Specific Aims

Wound healing around a medical implant is highly important to the success of the implant. In the case of implants in bone, this wound healing includes the formation of new bone around the implant. Bone tissue remodeling is influenced by the mechanical forces and the implantation of orthopedic and orthodontia implants change the mechanical environment of the surrounding bone. This mechanical environment determines the success of the implant placed in the bone tissue. It is therefore imperative to study how mechanical forces influence bone formation around implants i.e. osseointegration. In particular, it is of use to examine how different mechanical loading regimes affect the osseointegration of implants.

Many studies have been conducted regarding this topic. *In vivo*, it has been shown that cyclic loading of bone in animals will increase bone formation, with dynamic loading regimes leading to higher bone formation than static. Callus formation at the site of a fracture has also been shown to be affected by movement at the fracture. Here, intermediate levels of movement generated the largest calluses, while large and small levels of movement generated smaller calluses. Implant loading conditions have also been shown to affect the nature of implant osseointegration. *In vitro*, matrix synthesis by osteoblasts was shown to increase at levels of strain near the maximum encountered physiologically. Also, cell proliferation increased while matrix synthesis decreased under mechanical stimulation beyond the physiological level.

It is somewhat difficult to glean a precise image of the response osteoblasts have to a range of different mechanical stimuli. This is primarily because of the wide variation of research going on globally. Researchers use a variety of cell lines, substrates,

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mechanical loading regimes, and so on. These studies have begun to answer some questions regarding the behavior of bone around an implant, but they have yet to resolve issues associated with implant osseointegration, the relatively low strain levels that may exist at the bone-implant interface, or different repetitive loading regimes. It is believed that strain conditions in bone well beyond normal physiological levels (approximately 500 micro-strain), durations, and repetitions provoke the physiological response of cell proliferation, while levels at the upper end of and in the physiological level provoke the response of increased matrix synthesis. Therefore, the hypothesis of this study was:

> Repetitive mechanical loading will cause more cell proliferation and delay the onset of matrix synthesis than cells subjected to less mechanical loading. Also, the magnitude of strain will delay the onset of matrix synthesis, with high levels of strain causing a greater delay.

To test this hypothesis, osteoblast precursor cells isolated from rat bone marrow were subjected to a series of mechanical loading treatments. Specifically, the cells will be cyclically loaded for 15 minutes daily for one, three, and five days consecutively. Within these groups, one set of cells was loaded at a magnitude of 400 micro-strain and one set was loaded at a magnitude of 1000 micro-strain. Controls were cells not strained. The cells were monitored for 15 days, a time when calcium deposition can be seen. Periodically the cells were tested for alkaline phosphatase activity, DNA content (to measure cell number changes), total protein level, and calcium content.

It was hoped that this experiment would add to the existing knowledge base for osteogenic cells. Ultimately, research would provide insight into implant behavior *in vivo* as well. As well as in implant integration, this knowledge may be useful in the treatment of bone disorders, such as osteoporosis, or bone injuries. This may lead to

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changes in implant design as well as implementation of treatment programs or therapeutic activities.

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CHAPTER II

METHODS AND MATERIALS

Device for Imposing Cyclic Strain

The device used for straining cells was modified from a design first used by Winters et al. (Winters 2002). The modified four-point bending apparatus was modified to run off of computer controlled pneumatics, rather than a cam-shaft design. The basic design consists of two central points that support a substrate material that is flexed by two outer moving supports. (Fig 2.1) The base of this apparatus is constructed of aluminum for light weight and features two central supports. Mounted to this base is an adjustable stainless steel bracket that positions a pneumatic cylinder directly over the center of the two supports. Stainless steel was chosen for this application to resist any corrosion that may occur if the device was operated in the humid environment of a cell culture incubator. Attached to the piston of the pneutatic cylinder was a system of titanium rods that ultimately contacted the substrate material. These rods were secured with springs attached to the pneumatic cylinder bracket to offset the rods' own weight. They were also designed to be adjustable so that the point of contact may be changed. This device was originally intended to be used with thin titanium sheets as its substrate, but a variety of other flexible materials could possibly be used and some are currently under experimentation by other investigators.

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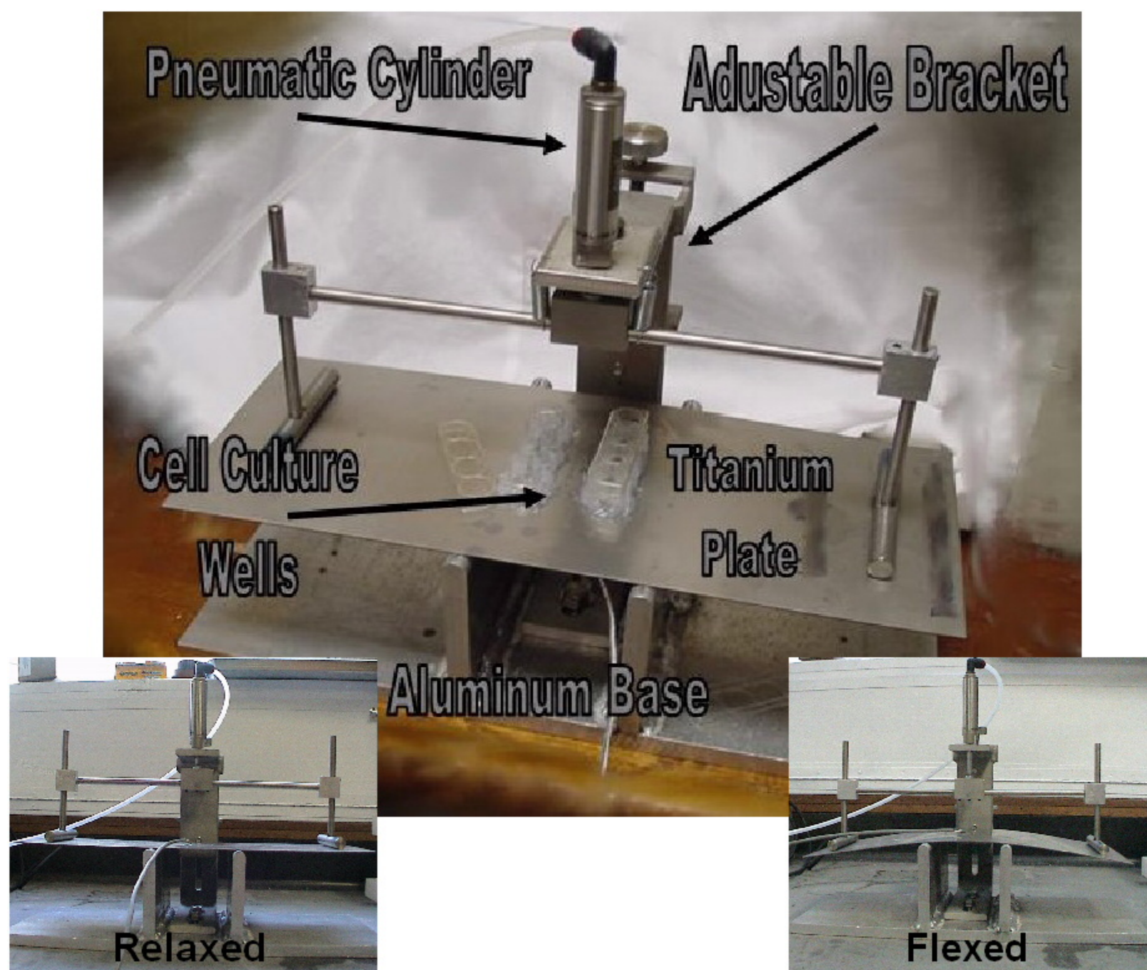

Figure 2.1: Strain Apparatus.

This device runs off of pneumatic pressure obtained from a pressurized air cylinder. This high pressure air is regulated down to approximately 150 p.s.i. and sent to a voltage to pressure transducer (Type 3110 Servo Pressure Regulator, Marsh Bellofram, Newell, WV). This transducer varies the air pressure sent to the Airpel pneumatic cylinder (Model E15D1.5-N, Airpot Corp., Norwalk, CT) and is controlled by a personal computer equipped with a multifunction I/O board (PCI-MIO E Series Model NI 6250, National Instruments, Austin, TX) and running National Instruments LabVIEW software (Version 5.0, National Instruments, Austin, TX). The particular LabVIEW application

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allows for the user selection of different voltage output waveforms along with varying amplitudes and offsets. Strain is measured via strain gauges affixed to the plates (Type CEA-06-250UN-350, Vishay Micro-Measurements Group, Inc., Raleigh, NC). These gauges are read by a strain indicator (Model 3800 Wide Range Strain Indicator, Vishay Micro-Measurements Group Inc., Raleigh, NC) that displays the actual strain and sends an analog signal to the computer to be read and saved in a LabVIEW data file. A schematic of the test set-up is shown in Figure 2.2.

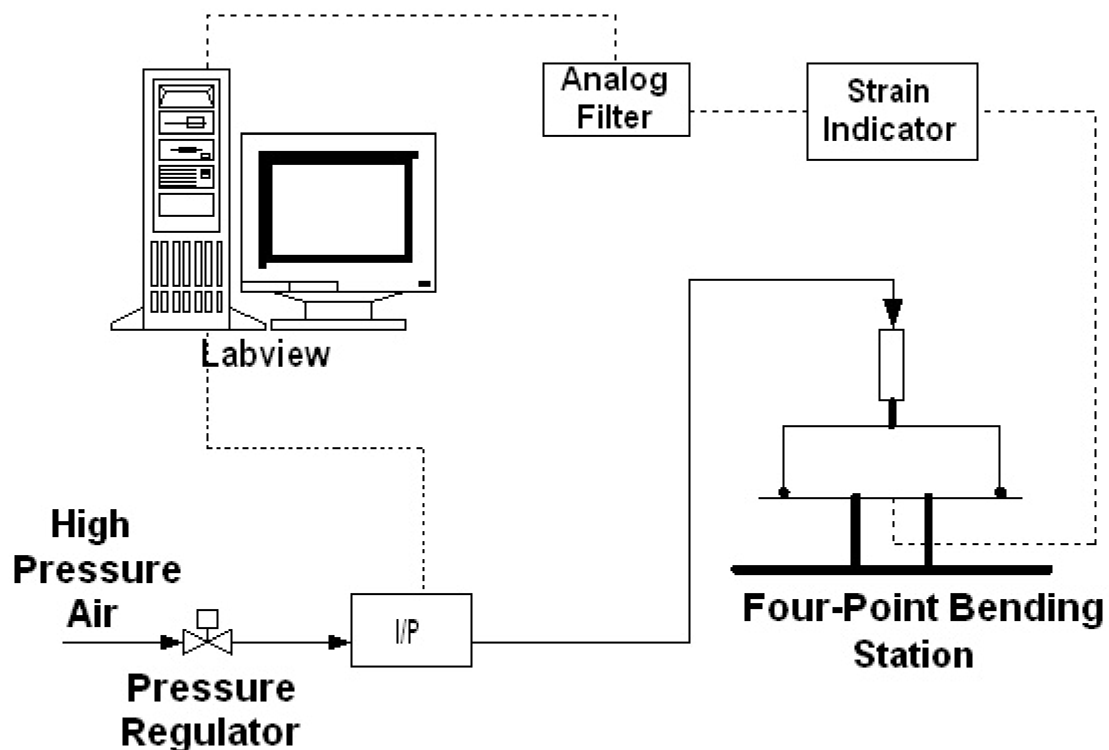

Figure 2.2: Strain Apparatus Control.

Each plate must be individually calibrated to the desire strain. Before the experiment begins, each plate is calibrated using a trial and error process to determine the appropriate voltage output from the computer that will produce the desired strain on that

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particular plate. After that is determined, the voltage is noted and is used throughout the experiment for that plate. This has been shown to have a high degree of repeatability, with strains not varying more than 1-2% between testing sessions for a given voltage output. Strain is verified throughout testing by collection of the output from the strain gage during each test for each plate.

This setup is capable of producing cyclic strain in accurate waveforms up to approximately 1Hz and infinitely low frequency. At frequencies higher than 1 Hz, the limitations of the machine begin to adversely affect the waveforms produced. The device can only flex and release a plate so fast and if the waveform frequency requires that it be faster, then an accurate waveform will not be reproduced. Figure 2.3 illustrates several of the possible strain waveforms that can be reproduced by this strain device. The sine wave was chosen for this experiment because it is the most researched waveform.

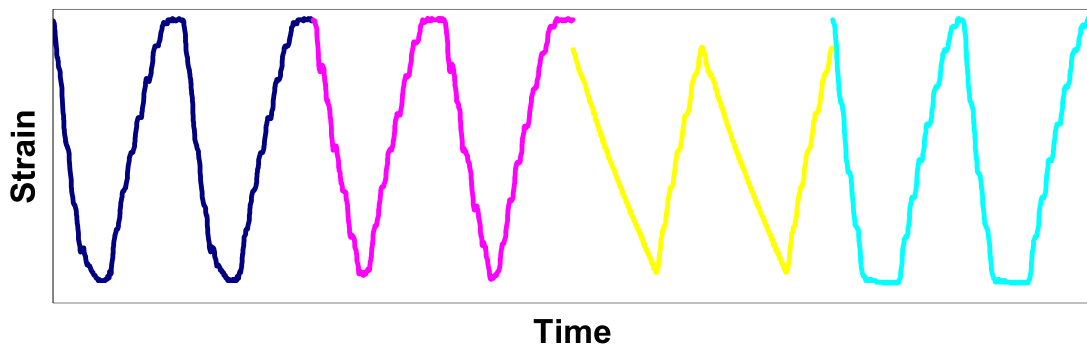

Figure 2.3: Strain Waveforms Produced at 1 Hz.
(Left to Right: Sine, Triangle, Sawtooth, and Square)

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Characterization of Strain

Pryor to beginning the experiment, several plates were prepared with strain gauges across both the top and the bottom of the plate in varying orientations. The plates were then strained under varying magnitudes to observe the characteristics of the strain across the area of the plates between the central supports. The first observation was that strain measurements on the bottom and the top of the plate at the same point are equal in value, but opposite in sign. The significance of this is that the strain encountered on top of the plate may be accurately measured on the bottom of the plate, allowing cells to be cultured on top free of strain gauge interference. The strain across the plates was also shown to be symmetric along the short axis and along the long axis of the plates.

Some researchers may assume that the plate represents an infinite slab in four-point bending and therefore has uniform strain across its entire surface. In reality, the strain is not uniform across the entire surface of the plate. At the very edges of the plate and near the central supports, there is considerable deviation of strain from the central measured value. Figure 2.4 shows the variation of strain across the plate. The highest deviation from center comes directly over the supports in the center of the plate. These values may be as much as 14% lower than the central strain, but just inside the supports the strain returns to more uniform levels. The very edges of the plate also cause some variations with up to 8% deviation from centrally measured strain. For the purposes of this experiment, the cell culture area was limited to areas where no more than 5% deviation from the centrally measured value occurred. The centrally measured value was then taken to represent the actual strain on the cell culture surface.

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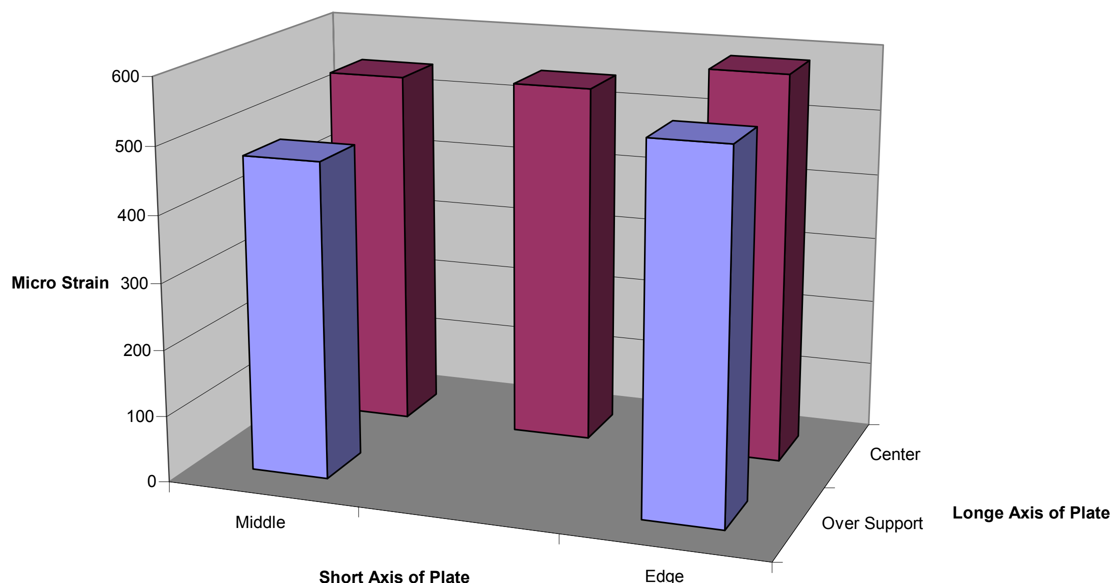

Figure 2.4: Strain Measurements across the Surface of a Test Plate.

Plate Preparation

A sheet of 0.125 inch thick titanium alloy, ASTM grade 4 (Titanium Industries, Parsippany, NJ) was cut into pieces approximately 6.2 inches wide and 16 inches long. This alloy was chosen as it is commonly used in dental, orthopedic, and craniofacial implants. The original plates were then rough sanded until all visible pits from the sheet-rolling process were gone. Before each experimental run, the plates were first rough sanded with 80 grit sandpaper to remove any surface contamination then progressively sanded with finer and finer sandpaper up to a 1000 grit wet polish. This procedure for surface preparation was chosen since it is commonly used in the implant industry. The

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plates were then cleaned with soapy water and rinsed with ethanol to remove any leftover contamination.

After the surface of the plates had been prepared, tissue culture wells were affixed to the surface of the plates. Nunc brand Sonicseal slides (Cat. No. 138121, Nalge Nunc International, Rochester, NY) were used for this application (Fig 2.5). This product is designed so that cells may be cultured on a clear plastic slide that is removable from its own tissue culture wells. For this study, Sonicseal slides were removed from the bottom of culture wells. Once the bottom portions of the wells were removed, the wells were roughly sanded and affixed to the plates, two sets of four wells per plate, with a small bead of clear RTV silicon rubber adhesive (732 Multi-Purpose Sealant, Dow Corning, Midland, MI). The control plate had one extra set of wells for use as the day one control for the study. Once the initial silicon was allowed to set up, more silicon was added around the outer edge of the wells to completely seal them.

Figure 2.5: Sonicseal Wells.
[As from the factory (left), removed from their base (left center), and affixed to a plate (right, 2)]

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Following at least a 24 hour curing time, the wells were washed with ethanol to remove any silicon contaminates from the wells. Further ethanol was allowed to sit overnight in the wells to fully cleanse them. The plates were again washed and placed in a sterile hood under a UV light overnight to sterilize them. The following morning cells were seeded onto the surface of the plates.

Cell Culture

Cell Isolation

Juvenile male Wistar rats between 80 g and 100 g were obtained from Charles River Laboratories (Wilmington, MA). Two rats were used for each experimental run. The rats were asphyxiated in carbon dioxide upon arrival. Shortly after asphyxiation, the fibula and tibia were removed by blunt dissection and cleaned of any remaining muscle. The bones were then rinsed in five sequential antibiotic/antimycotic baths in a sterile environment. Following this, the epiphyses of each bone were transected and the marrow was flushed into cell culture media using a large needle. Once in the cell culture media, the cell/media mixture was further mixed with an automatic pipetter to break up any clumps of cells. The cells were then placed in 5 large 75 ml cell culture flasks and further media was added. Two days later, the media in the flasks was removed and the cells were washed with PBS to remove non-adherent cells since the adherent cells are those which exhibit osteogenic potential (Kresbach 1999). These cells were then grown for seven to ten days until a sufficient amount was obtained. Medium used for the cells was Dulbecco's Modified Eagle Medium (Gibco BRL, Grand Island, NY) supplemented

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to contain 10% fetal bovine serum (F2442, Sigma, St. Lois, MO), 1% 1X antibiotic-antimycotics (A 9909, Sigma, St. Loius, MO), and 4 mM L-glutamine. Medium was changed every 2 or 3 days.

Cell Growth and Seeding

Cells were removed from flasks using 0.25% trypsin-EDTA (Cat No. T4049, Sigma, St. Loius, MO), resuspended in approximately 20 ml of medium and counted using a hemacytometer. The concentration of the cells was adjusted to seed the cells in each well on the titanium plates at 1 x 10^4 cells/cm^2. Each cell culture well has an area of approximately 1.5 cm^2, yielding 1.5 x 10^4 cells per well. The experimental plates were all seeded at the same time, with the wells seeded in a random order across all the plates. Upon subculture and seeding onto the experimental plates, the cells were maintained in Minimum Essential Medium – Alpha Medium with GlutaMAX glutamine substitute and ribonucleosides and deoxyribonucleosides (Gibco BRL, Grand Island, NY). This media was also supplemented to contain 10% fetal bovine serum and 1% 1X antibiotic-antimycotic. Further, 2 mg dexamethasone, 2.5 mg ascorbic acid, and 1.5 ml of 1 M β-glycerol phosphate were added to 500 ml of medium to enhance the cells' differentiation into osteoblasts and to facilitate deposition of calcium phosphate mineral. As with the original cells, the medium for the experimental cells was changed every 2 or 3 days for up to 15 days of culture.

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Experimental Treatment

Two replications of this experiment were perfomed. Six treatment groups and one control group were used in each experimental run. The groups were cyclically strained at 1 Hz for 15 minutes per day. This time of stimulation was chosen as a convenient amount that was almost certainly above they cells' threshold for stimulation (Jones 1991, Brighton 1991 and 1992, Millitou 1992). One set of two groups was subjected to strain on the first day, another set was subjected to daily strain for three consecutive days, and a final set was subjected to daily strain for 5 consecutive days. Within each of these sets, one group was strained at a peak magnitude of 400 micro-strain and the other at 1000 micro-strain. The control group was never mechanically stimulated. Cells were subjected to experimental strain regime beginning approximately 6 hours after seeding on the surface of the plates, giving the cells ample time to adhere to the titanium alloy surface. Treatments occurred at the same time on following days, giving almost 24 hours for cells to recover from any refractory period that might exist (Stanford 1994).

Due to limited incubator space, the treatments took place outside an incubator. To make sure that this did not influence the findings, all plates not receiving a treatment were taken out of the incubator for 15 minutes on each day that any group was treated. Thus every group was exposed to the same amount of time outside the incubator. Furthermore, the plates were positioned on supports similar to those on the strain device to ensure that the handling did not affect results.

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Sampling and Testing Procedures

A total of eight wells were available for sampling on each treatment group over the entire experiment. An additional four wells were placed on the control plate and sampled on day 1 prior to treatment to represent the entire population's starting point. Because the sampling technique is destructive, the eight wells were divided among each of the chosen sample days. On day 5, two of the eight wells were sampled, and three were sampled each on day 10 and 15. Unfortunately, during these experiments it was nearly impossible to keep all of the wells perfectly sealed to titanium, especially under heavy mechanical flexing. Therefore, a few of the wells (2 to 3 out of the 60 is normal) usually leaked during the experiment. Those wells were disregarded and sampling was adjusted so that all sample points would have at least two samples, with a preference for day 15 groups still having 3 samples.

In order to take samples, selected wells first had their media removed and their cells rinsed with sterile PBS. Next, 400 μl of cell lysing solution containing 0.5% Nonidet P-40 detergent, 50 mM Tris-Cl, 100 mM NaCl, and 5 mM $MgCl_2$ was placed in each well and allowed to lyse the cells for 2 hours. This lysed-cell solution was then mixed in the well to remove any adhering particles and placed in a sample tube and frozen at -20^0C. All samples were frozen and later thawed to perform various assays. Those wells being tested for mineralized calcium content were first lysed as normal, then 100 μl of 0.5 N HCl was added to the wells and allowed to dissolve any calcium matrix overnight. This fluid was then collected from the wells and frozen in sample tubes. A small aliquot of each sample was used for each of the assays. The lysed-cell solutions

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were used for DNA, protein, and ALP assays, while the HCl solution samples were used for calcium assay. All samples were tested in triplicate.

DNA Assay

DNA content was measured as an indicator of relative cell number and used as a basis to compare cell proliferation and to normalize enzyme activity. This was measured using a fluorescence assay kit (Cat. No. DNA-QF, Sigma, St. Louis, MO). This kit uses a fluorescent dye, bisBENZIMIDE, which binds to AT sequences in the minor groove of double stranded DNA. The dye is excited with light at 360 nm and the fluorescence is measured at 460 nm. This was performed on a SPECTRAmax PLUS flourometer (Molecular Devices Corporation, Sunnyvale, CA). A standard curve was prepared during testing to convert fluorescence values into ng of DNA.

Alkaline Phosphatase (ALP) Activity

ALP activity was measured using individually purchased reagents used in an assay kit that is no longer in production (Cat. No. 104-LS, Sigma, St. Louis, MO). This kit contains a substrate that reacts with ALP and changes color. Absorbance was read at 410 nm in a μQuant universal microplate spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT). A standard curve was prepared to convert measured absorbance into Sigma units (equivalent to one μmol of *p*-nitorphenol reacted per minute). These values were then normalized to DNA measurements to provide a per cell basis for comparison of ALP activity.

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Protein Assay

Total protein content was tested using an assay kit (Cat. No. 23227, Pierce, Rockford, IL). This kit is based on a bicinchoninic acid (BCA) reaction that reduces Cu^{2+} to Cu^{1+} by a protein, resulting in a purple-colored product. Absorbance was read at 562 nm in a μQuant universal microplate spectrophotometer. A standard curve was prepared to convert absorbance readings into μg/ml protein.

Calcium Assay

Concentrations of Ca were measured by the orthocresolphthalein complexone (OCPC) colorimetric method using OCPC fluid, ethanolamine/boric acid buffer, and hydoxyquinline. Samples were first diluted to get concentrations within acceptable ranges. Absorbance was read at 570 nm in a μQuant universal microplate spectrophotometer.

Statistical Analysis

Triplicate readings of assays were averaged and each sample point was represented by the averaged values for 2, 3 or 4 samples for that group on that day. Data from the two experimental runs was compiled and multi-factorial ANOVA was used to determine the statistical difference between various groups. Statistically different was defined to be significant at $p<0.05$.

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CHAPTER III

RESULTS

Figure 3.1 shows the observed DNA results for both experimental runs. DNA content was shown to increase over the course of the experiment, demonstrating that cells were in fact growing in all groups. Shown in the day 1 position is a separate set of control wells that were sampled prior to any experimental treatments. These are meant to provide a baseline reference for all groups. No significant interactions were found at a level where $p<0.05$. The only significant effect found was time ($p<0.05$). Day 5 and day 10 were found to be statistically different from day 15 ($p<0.05$). Table 3.1 shows the ANOVA table for the DNA test results.

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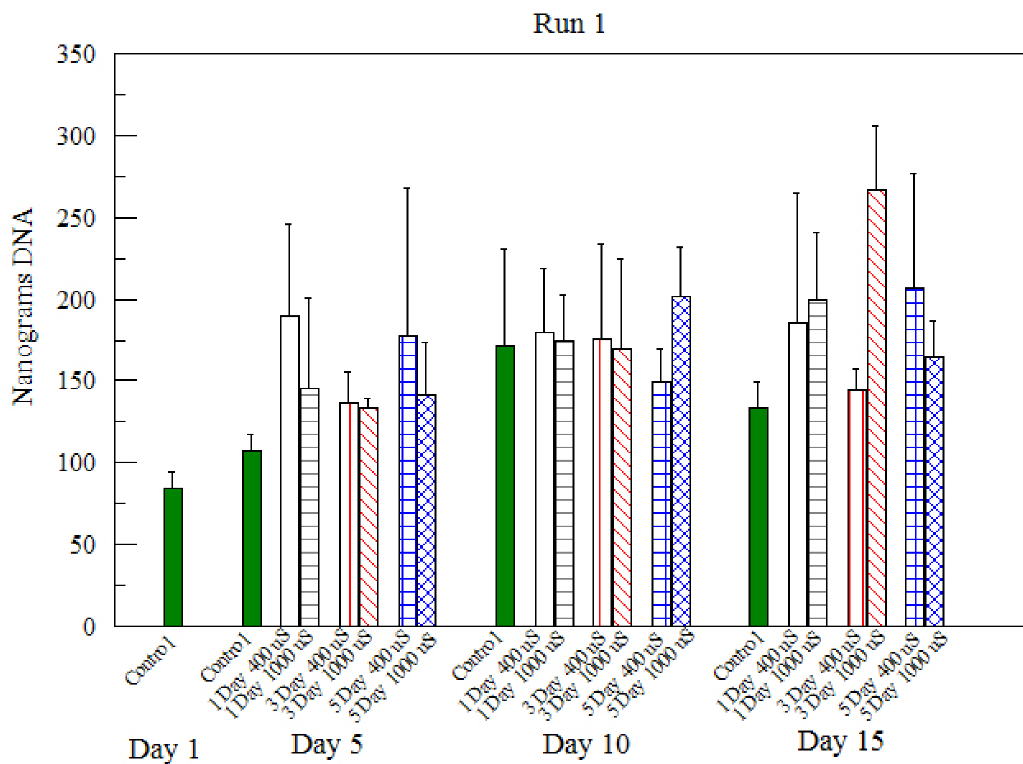

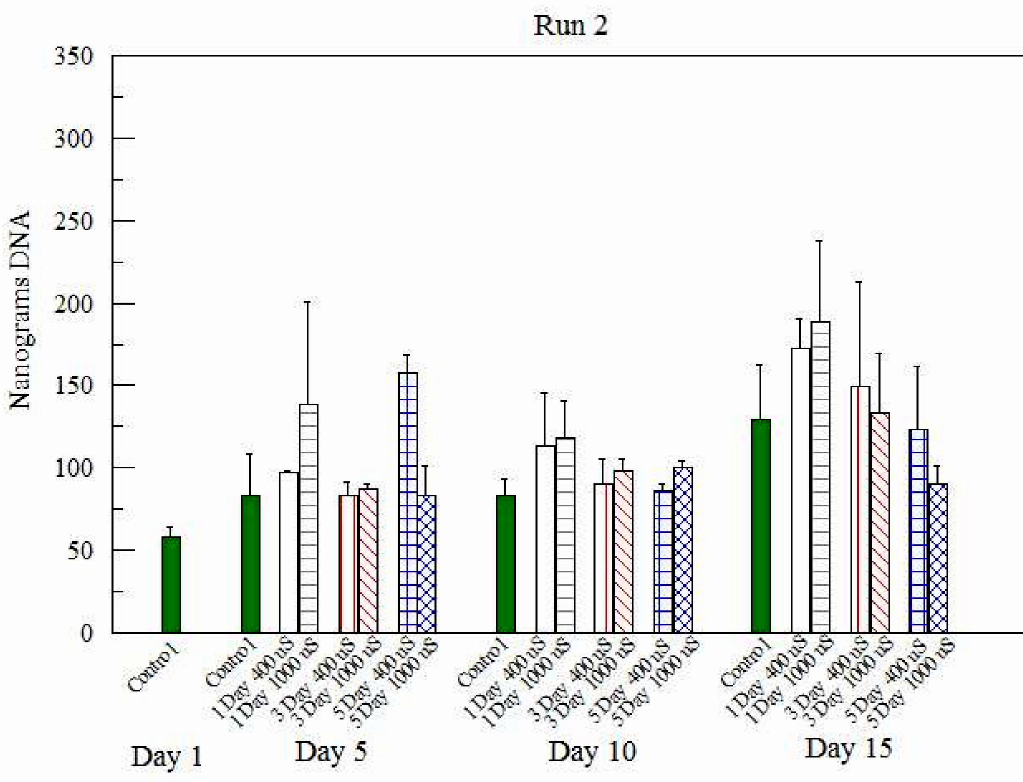

Figure 3.1: Total DNA Content.

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Table 3.1: ANOVA Test of DNA Results.

Effect	Num DF	Den DF	F Value	Pr > F
Day	2	12	6.48	0.0124
Strain Duration	2	5	2.71	0.1595
Strain Level	1	5	0.01	0.9206
Day x Duration	4	12	1.07	0.4146
Day x Level	2	12	1.05	0.3804
Duration x Level	2	5	1.74	0.2664
Day x Duration x Level	4	12	1.73	0.2088

The results for total protein assays are shown in Figure 3.2. As with DNA results, there were no significant interactions found and the only significant effect was time ($p<0.05$). Groups sampled 10 or more days apart were found to be statistically different ($p<0.05$). The statistical results were similar whether the protein was reported as total protein or total protein normalized to DNA content. The total protein also increased over time, showing that the cells were in fact making matrix.

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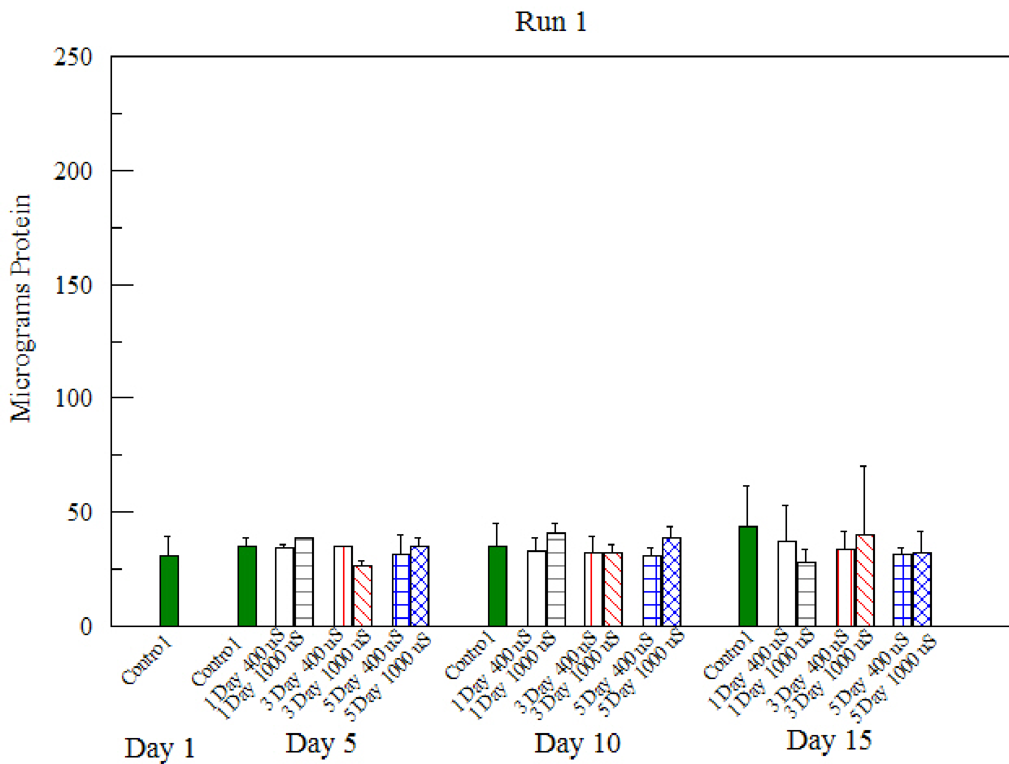

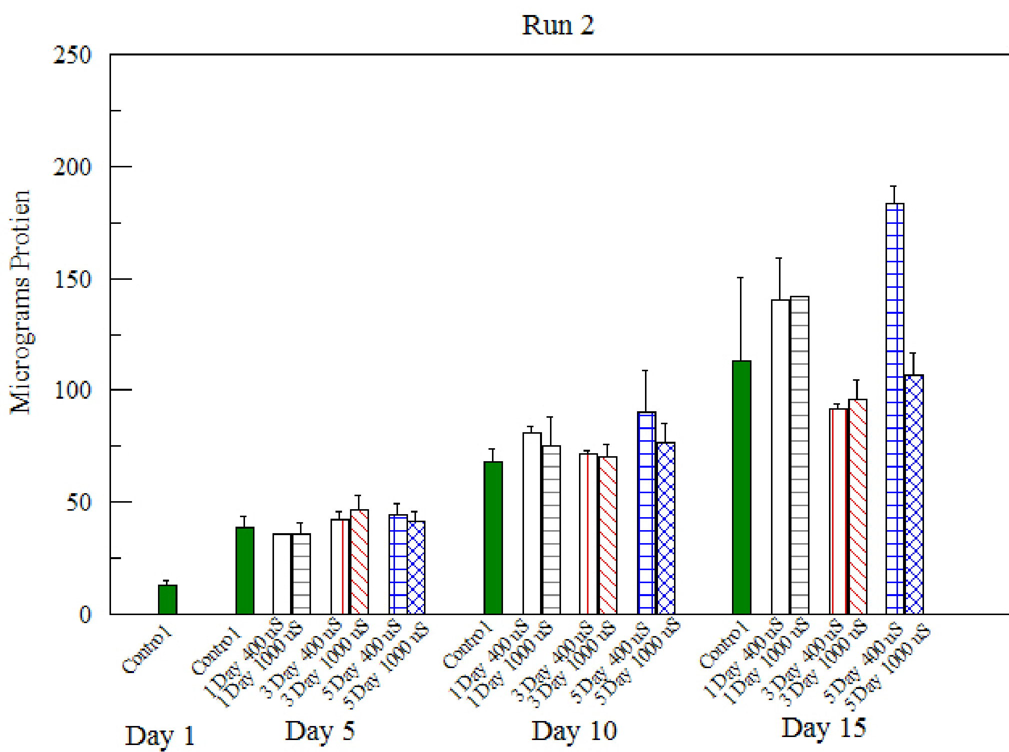

Figure 3.2 Total Protein Results.

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The alkaline phosphatase (ALP) assay results are shown in Figure 3.3. These results have been normalized to DNA content for accurate comparison. ALP is often normalized to total protein content; however, in cells producing a vast amount of matrix, the large amount of protein in the matrix can coincide with high ALP activity to produce a deceptively low normalized value. Therefore, DNA measurements were used to normalize bulk ALP activity to provide a more realistic per cell comparison of ALP activity. Values displayed on the graph are representative of mathematical correlations to a standard curve. Negative values occurred in samples whose adjusted absorbance was lower than that found for the blank standard solution. Though these values are reported as negative due to conversions using standard curves, they should be viewed as zero or below detection limits.

Between the two runs there is more than an order of magnitude difference between the measured ALP activities. However, for the purpose of this experiment, ALP activity may be used as a phenotypic marker for osteoblasts rather than a measure of matrix synthesis activity. The level of matrix synthesis can be estimated by the total protein content and final calcium results. There were no significant interactions between treatment variables ($p<0.05$). Time was only variable to have a significant effect.

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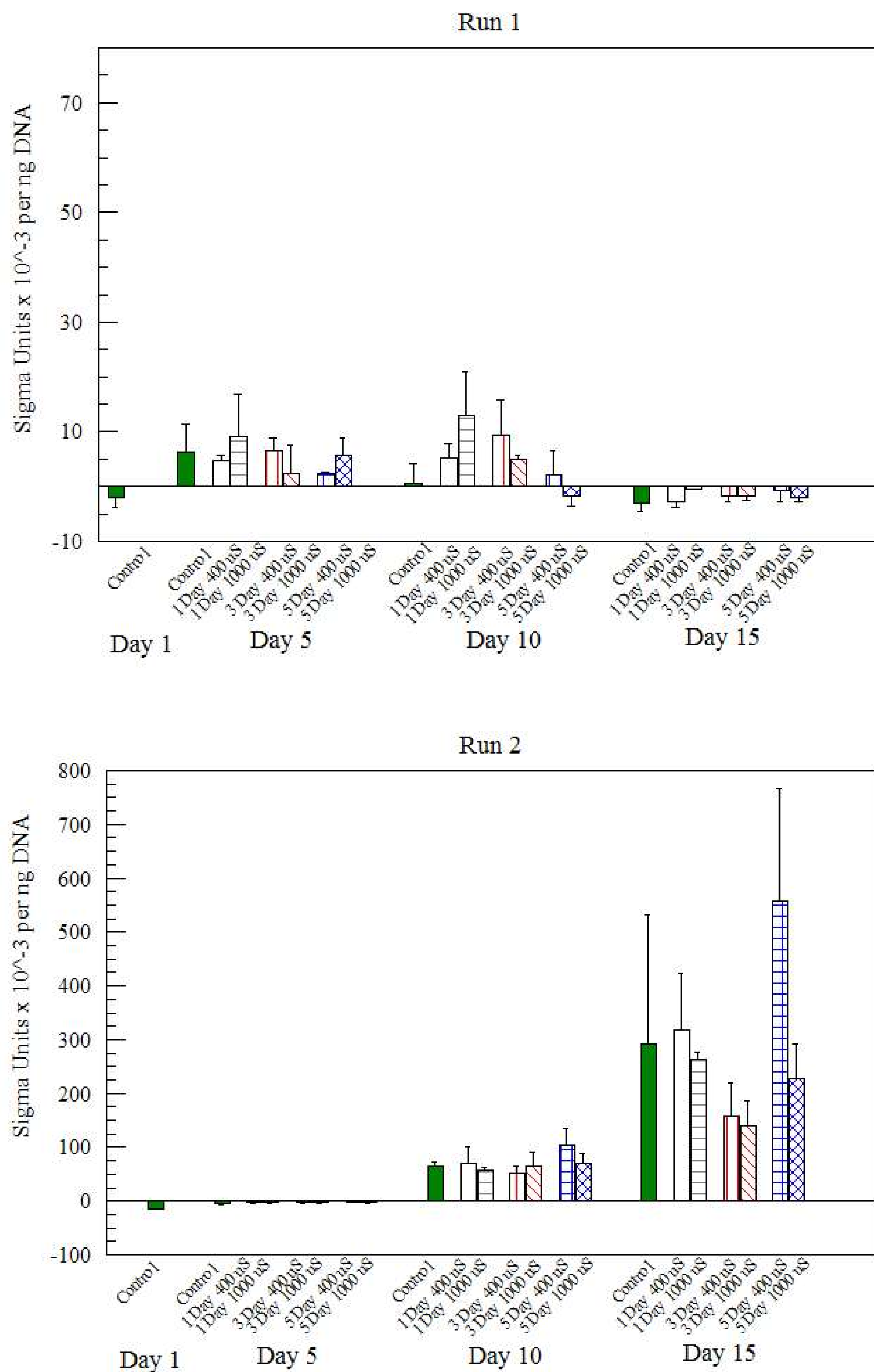

Figure 3.3: Alkaline Phosphatase activity normalized to DNA content.
(Note: the y-axis scales are different by 10 fold)

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The measurements for final calcium content are shown in table 3.2. No significant difference was found for any treatment combination, nor were any interactions found ($p<0.05$). However, significant calcium was present to indicate ossification of bone matrix had begun.

Table 3.2: Milligrams of Calcium ± Standard Deviation Measured on Day 15.

Strain Duration	Strain Level			
	Run 1		Run 2	
	400 μ-strain	1000 μ-strain	400 μ-strain	1000 μ-strain
1 Day	0.307 ± 0.012	0.245 ± 0.058	0.061 ± 0.014	0.040 ± 0.005
3 Days	0.311 ± 0.070	0.282 ± 0.010	0.108 ± 0.030	0.208 ± 0.051
5 Days	0.300 ± 0.061	0.275 ± 0.061	0.069 ± 0.011	0.194 ± 0.008
Control	0.303 ± 0.021		0.033 ± 0.013	

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CHAPTER IV

DISCUSSION

Bone tissue is responsive to its mechanical environment and this sensitivity to mechanical loading may be important in the osseointegration of implant devices. It is believed that strain conditions in bone well beyond normal physiological levels (approximately 500 micro-strain), durations, and repetitions provoke the physiological response of cell proliferation, while levels at the upper end of and in the physiological level provoke the response of increased matrix synthesis. Therefore, it was hypothesized that repetitive mechanical loading will cause more cell proliferation and delay the onset of matrix synthesis than cells subjected to less mechanical loading. It was also hypothesized that the magnitude of strain delays the onset of matrix synthesis, with high levels of strain having a greater effect. To test these hypotheses, rat mesenchymal stem cells were subjected to mechanical strains while growing on titanium metal and evaluated for proliferation and ability to deposit extracellular bone matrix material.

Unfortunately, this experiment did not result in any significant data related to the hypothesis of the experiment. The increases in DNA and protein over the course of the experiment indicated that the cells grew. However, there were neither significant differences between the treatment groups and the controls nor any significant differences among the treatment groups. Thus, the hypothesis was not supported. It is possible that

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the data simply indicate that these experimental treatments have no significant effects. It is also possible that adequate replications were not performed to observe specific trends.

Had this experiment yielded significant results, information would have been gained concerning how bone and osteoblasts respond beyond the level of normal physiological stimulation. Further, a greater characterization of the cells' memory for previous strain would have been gained by evaluating cells strained over different time periods. It is well documented that bone responds to strain according to Frost's hypothesis (Fig 1.5). That is, when higher than normal levels of strain are encountered, osteocytes and osteoblasts deposit matrix to stiffen the bone thereby lowering the resulting strain from the same encountered force. However, this seems to only be applicable in physiological conditions. Knowing how cells respond at stimulation higher than physiological levels may prove useful in creating new therapies for injuries or implant integration. Further it is known that strain environment affects tissue differentiation (Fig 1.4). It has also been suggested that the mechanical environment may not only guide differentiation towards a specific tissue type, but influence the growth rate of differentiating tissue (Henderson 2002). It was hoped that this experiment would provide evidence concerning this relationship between differentiation, proliferation, and strain levels at and beyond physiological conditions. As the hypothesis states, it was believed that strain levels beyond those encountered naturally would result in delayed differentiation and increased cell proliferation.

This experiment was expected to conform to cellular growth trends that are considered normal (Fig 4.1). These trends were observed by this researcher using both the UMR transformed cell line and mesenchymal cells obtained from live rats. This

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expected trend for mesenchymal cells includes DNA values that increase over time and plateau between 15 and 20 days as a result of cellular confluence. Normalized ALP values are expected to increase initially, then peak approximately sometime between day 8 and 12, and finally fall after the peak. Protein and calcium levels were expected to constantly rise over at least 20 days with calcification beginning several days after cell seeding. These trends are consistent with those obtained by other researchers in the field (Bellows 1990, Rickard 1994, Ter Brugge 2002). It was expected that the tested parameters would alter these trends by raising or lowering peaks and plateaus or by altering the time needed for peaks and plateaus to be reached. Unfortunately, not only did the experimental treatments not produce significantly different results, they in some cases did not produce results consistent with the expected trends. Specifically, the protein content for Run 1 did not increase consistently and the normalized ALP activity in Run 2 steadily increased rather than peaking near day 10.

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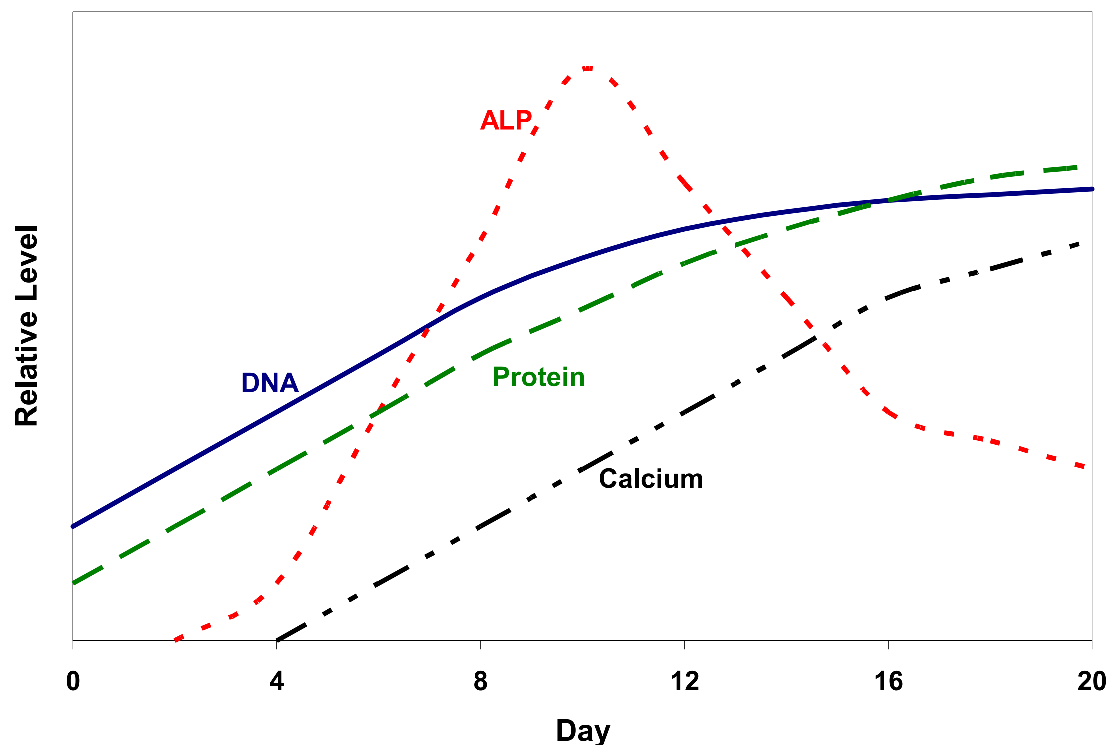

Figure 4.1: Expected Cellular Growth Trends.

Further, the design of this experiment intended that mesenchymal cells be used. This would be much more representative of cellular invasion and differentiation around a new implant in bone. For this reason, bone marrow stem cells were chosen for experimentation rather than the readily available but fully differentiated UMR cell line. Though the UMR cells would have added increased consistency between runs, they would not be entirely representative of normal conditions. This abnormality is partly because they are fully differentiated cells and partly because they are osteosarcoma cells and therefore have less restrictive growth rates as compared to normal cells.

To further ensure the use of the most pluripotent cells possible, dexamethasone was withheld from the isolated cells during growth. It has been shown that dexamethasone causes marrow stem cells to differentiate into osteoblasts. After even two

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passages of cell culture with dexamethasone, these cells begin to show decreased proliferate capacity and muted adherence to afore mentioned growth trends, both indicative of more differentiated cells (Ter Brugge 2002). It was hoped that by withholding dexamethasone from the culture of the original marrow isolation the most stem cell-like cells would be used in testing and thus be representative of actual wound healing conditions around a new implant. Dexamethasone was then added to culture media during the experiment in order to cause marrow stem cell differentiation into osteoblasts during the experimental testing. This however, may explain some unexpected results. Both the expectations for growth trends and the formation of the experimental hypothesis were based on research data concerning partially or fully differentiated osteoblasts. It is possible that these less differentiated cells do not respond to mechanical stimulation in a similar manner to their more differentiated lineages. Future studies would be useful to explore this possibility.

Sources of variance that might account for the failure to obtain significant effects abound in any experiment, but several are notable here. The two experimental trials were performed using different rats. Though the rats were from the same breed, obviously individual differences in genetics could affect the results of the experiment. Cell culture, in particular seeding cells onto the experimental plates, is a great source of variance. This can be seen in the measurements of the Day 1 total DNA content. Run 1 and Run 2 had standard deviations of 12.9% and 10.6% (respectively) of the average value of day 1 DNA content. The only way to overcome such variance is with more samples. Furthermore, the two experiments started with mildly different seeding density: a starting difference between 83.9 ng DNA for Run 1 and 58.3 ng DNA for Run 2.

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Normalizing other data to DNA content and normalizing DNA content to Day 1 DNA content can help account for these differences, though.

In essence this experiment proved to be too ambitious. One of the original goals was to provide a steady basis of comparison for cellular response over a broad range of conditions across several time points. Most studies in the literature consist of one treatment compared to a control measured at one or two time points, making the overall cellular response to different conditions difficult to ascertain as previously discussed. However, the beauty of these single treatment studies is in their simplicity and thus their propensity to produce significant results. The researcher is able to devote his or her efforts into testing a higher quantity of samples and generating a greater number of replicates. For example, had this experiment been limited to just two treatment groups rather than six, three times the replicates could have been tested in the same given amount of time with no additional effort.

The newly developed apparatus was proven, however, to produce consistent, reproducible, and repeatable strain conditions. Therefore, the limitation of this experiment lies in the ability to consistently isolate, culture, and sample cell populations. In the future this system may prove quite useful in evaluating the effect of different mechanical factors on the ability of cells to develop bone around an implant.

Future Work

This experiment is somewhat limited by the ability to test only 8 subgroups per treatment group per experimental run. This limitation is due to the cell culture wells that will fit on a single plate in an area that produces a uniform strain level. These subgroups

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must be further divided by assigning a certain portion of them to be destructively tested on each test day. Therefore, only two or three subgroups may be tested for each data point and in cases of well failure or contamination, this number may be cut to only one. Methods to increase the sample number with either larger plates or closer placement of culture wells should be explored.

Simply repeating the study several more times could lead to significant results. With two or three more runs, perhaps some trends would immerge. Unfortunately, because of material limitations in the lab and time limitations of the researcher, more repetitions could not be conducted for this experiment. Several runs were conducted but failed due to material problems or contamination.

Different logistics for sampling over time should also be evaluated for effectiveness. Rather than a researcher conducting multiple 15 or 20 day studies and sampling a portion of the wells each sample day, this research could be conducted as a series of single time point studies. That is, first a 5 day study would be conducted with all wells being sampled on day five, then a 10 day study, and so on. This would be another method of increasing the number of subgroups available leading to a more accurate depiction of the treatment group. This method would also lead to more immediately available results for publication of these individual, smaller scale studies.

As the multiple studies presented in Chapter 1 have shown, strain environment does influence the differentiation of osteocytes. It is highly valuable to understand how this occurs and how different levels and repetitions of strain application influence this process. It is therefore important to continue this research, using data taken from this experiment as a starting point.

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