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Mechanobiological Response of Stem Cells Derived from the Periodontal Ligament and Dental Pulp to Compressive Loading

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UNIVERSITY OF MIAMI

MECHANOBIOLOGICAL RESPONSE OF STEM CELLS DERIVED FROM THE PERIODONTAL LIGAMENT AND DENTAL PULP TO COMPRESSIVE LOADING

By

Anthony Nicholas Khoury

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Among the debilitating disorders associated with the musculoskeletal system, osteoarthritis and osteogenesis are the most prevalent disorders. These diseases are commonplace among people throughout the world and related healthcare options do little to improve on the condition. While the causes are yet to be fully understood, novel approaches involving stem cell treatments are currently being evaluated. The understanding of metabolic, formation, and degradation processes is simply a stepping-stone leading to the formulation of an innovative treatment.

The goal of this study was to assess the chondrogenic and osteogenic differentiation potential of stem cells derived from two different cell lines in the teeth. While there is a plethora of available stem cell lines found in the human body, cells from the Periodontal Ligament (PDL) and Dental Pulp were utilized to perform these experiments. Due to the many characteristics shared between teeth and bone it was hypothesized that compressive forces, both dynamic and static, on both cell lines would have a positive differentiation impact. Cells were loaded into 2% Agarose hydrogels and tested at a frequency of 1Hz and 0.1Hz for dynamic compression as well as static compression. A custom designed bioreactor was used to provide the sinusoidal dynamic compression in addition to the static compression. All experiments were performed under 15% strain for two hours a day, for three consecutive days.

Samples were quantified using RT-PCR in association with ten genes varying for chondrogenesis or osteogenesis.

Results from this study suggested that mechanical loading had a positive impact on the differentiation of cells derived from the PDL and Dental Pulp. Dynamic loading conditions resulted in an increase of up regulation for osteogenic and chondrogenic genes, while static loading had displayed no positive effects.

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complete my experiments. Additionally I would like to thank Gaby for her assistance while I was conducting the final experiments of this project. Everyone in the lab was extremely kind and generous throughout my research efforts and I graciously appreciate all of the support.

This thesis has been one of the most satisfying yet challenging projects I have encountered. However, during this research I have learned countless techniques and problem solving skills that I will utilize for the rest of my life. I am blessed to have met such wonderful people along the way.

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CHAPTER 1: INTRODUCTION

1.1 Musculoskeletal System

The musculoskeletal system is one of the most complex organ systems in the human body. This particular system encompasses the bones and tissues including cartilage, tendons and ligaments; which work synergistically to provide movement, form, structure and stability. In addition to providing movement, protection of internal organs is a vital function. While the musculoskeletal system is a very robust and resilient organ assembly it does not have the ability to retain itself. Due to the intense strains encountered throughout the course of one's life, deterioration or loss of function begins to settle in resulting in painful living conditions. Diseases such as osteoporosis and osteoarthritis are currently at the top of the list for researchers due to the high prevalence in today's society.

1.1.1 Bone Biology

The human skeletal system is made up of bones that provide several necessary functions required for daily life. A key function of bone is to provide a rigid framework in which organs are protected and the body is supported. This is accomplished through the high tensile strength of a combination of cortical and cancellous bone. As a highly organized tissue, bone is an organic matrix comprised of a hydroxyapatite mineral phase and calcium phosphate crystals [30]. Throughout one's lifecycle bones are constantly adapting and transforming in order to maintain the reliability of the skeletal system. Cortical and cancellous bones are integrated osseous tissues that make up the tissue on

the macroscopic level. The outer layer is referred to as the cortical portion while the inner section is known as cancellous. Eighty percent of bones within the human skeletal system are cortical bones due to its high density [30]. As previously mentioned, bone contains several minerals and composites. The concentration of these composites are generally 70% hydroxyapatite, 22% proteins such as type I collagen, and 8% water. These properties dictate the strength and quality of cortical bone in a human [2]. Within cortical bone resides cancellous bone. It is a loosely organized mesh composed of thin plates known as trabeculae [23]. The spaces between trabeculae are filled with red marrow and it is through this marrow in which osteocytes obtain nutrients. The concentrations of cortical and cancellous bone vary from bone to bone depending on the size of load required of the area.

Within the structures of bone are several key cells that play a role in structural and remodeling efforts. The extracellular matrix of bone is comprised of glycosaminoglycans and its fibrous protein is collagen. The collagen is an important factor of the extracellular matrix in that it provides the bone's tensile strength and form [33]. Osteoblasts are cells responsible for the formation of bone, also known as osteogenesis.

Extensive research into the bone remodeling process has revealed key remodeling markers which signal formation. These markers are resultant of enzymes related to osteoblast activity, or the construction of bone [18]. One specific marker is Type I Collagen. This is secreted as Type I procollagen by osteoblasts prior to the alignment as fibrils. Another marker is Alkaline Phosphatase (ALP). This is an osteoblastic enzyme with an unclear function; however, it is assumed to be significant in

the mineralization of bone matrix. While there are two isoforms of ALP, bone and liver forms, it is a strong marker for bone formation in the absence of liver disease. Additionally osteocalcin (OCN) is a widely known marker for bone. It is the most plentiful matrix protein in bone that is noncollagenous, and is expressed by highly differentiated osteoblasts [9]. However, it has been shown that portions of OCN are resultant of bone matrix degradation. This may propose that OCN can be a marker for bone resorption [14].

1.1.2 Cartilage Biology

An additional aspect of the musculoskeletal system is cartilage. Cartilage is found throughout the body and is regarded as a crucial connective tissue that supports functions such as shock absorption, and the end coating of bones. In addition, cartilage can be found in the ear and nose where it provides shape. There are three types of cartilage found in the body: elastic, fibrocartilaginous, and hyaline. The latter is the form most predominately found in the body, as it is commonly associated with the skeletal system. Unlike most systems in the human body, cartilage is avascular. Because blood does not pass through it, it relies on diffusion to receive nutrients needed for survival and repair. Cartilage that becomes injured beyond the subchondral bone has a limited ability to repair itself [10]. Overtime it becomes more difficult for cartilage to repair itself leading to debilitating diseases such as Osteoarthritis.

Classified under the Hyaline cartilage group is Articular cartilage. It is organized into four distinct groups based on the collagen organization and amount of proteoglycans present. While Type II Collagen is most commonly found in healthy tissue, collagens III, VI, IX, X, XI, XII, and XIV supply some amounts to the final matrix [39].

These collagen types are especially important to articular cartilage because they form the dense fibril networks upon which cartilage is based. Type II Collagen is the most abundant form contributing approximately 90-95% of total collagen. The fibrils are very resistant, having a half-life of several years under normal physiologic conditions due to collagen metabolism being slow. However it is when the tissue is in a diseased state that the turnover can greatly increase and surpass the ability of chondrocytes to provide an auxiliary layer. This state leads to further degradation of the matrix [38].

Chondrocytes are cells specific to only cartilage. They are the only viable cells to reside within healthy articular cartilage. There are two main functions of chondrocytes, which are dependent upon the cell's location. The primary function is to synthesize and sustain an extracellular matrix. This is especially important to articular cartilage applications where there is joint articulation. The second function of chondrocytes is for growth, specifically in association with epiphyseal plates [1].

1.2 Neural Crest

The neural crest is derived from the dorsal region of the neural tube at the ectoderm. It is a collection of cells that proliferate into a variety of tissues and organs in the body during development [11]. Among these cells are four types: cardiac, vagal, cranial, and trunk neural crest. Eventually these go on to form several tissues including the heart, nerves, and skin [17]. In 1992 Stemple, et al. proved that murine neural crest cells do in fact have the potential to form smooth muscle, neurons, and glia in addition to having the ability to renew themselves [35]. More recently Dupin et al. discovered an additional cell population within the cephalic neural crest that can give rise to the

previously mentioned cells along with chondrocytes, melanocytes, and osteoblasts [6]. The PDL is among the several types of tissues from which neural crest cells become.

1.3 Periodontal Ligament

The human PDL is a specialized collection of fibers attaching the tooth to the alveolar bone. It lies between the cementum and the inner wall of the alveolar bone socket [34]. The function of the PDL is to provide support while keeping the tooth within the confines of the jawbone. Not only does it provide structural stability for the teeth, but also has a role in homeostasis, tooth nutrition, and damaged tissue repair. It is a highly vascularized tissue allowing the surrounding areas as well as its own cell populations to receive adequate nutrient supply. There is an additional extracellular zone of matrix proteins and glycosaminoglycans. These collective properties allow the teeth to adjust their position while maintaining a strong hold to the bone. The ligament is home to a vast number of cells needed for the re-modeling of not only the bone, but also the cementum and PDL itself [15]. The most abundant type of cells found in the PDL are fibroblasts, which are primarily producers of collagen and assist in the maintenance of the ligament. These fibroblasts are crucial to the remodeling efforts of the PDL in that they are continually repairing collagen fibers therefore safeguarding the attachment sites to the alveolar bone [40]. Alongside fibroblasts are osteoblasts and osteoclasts. Whereas the osteoblasts form bone, osteoclasts resorb bone; more specifically in this application of alveolar bone matrix. In the vascular system reside hematopoietic stem cells, which go on to differentiate into pre-osteoclasts when RANKL and M-CSF are present. These pre-osteoclasts eventually travel to the bone where they become osteoclasts [29, 31, 37, 40].

Dating as far back as 1974, scientists have known of the special cellular properties of the PDL [19, 25, 26]. Over the years research has shown the promise of mesenchymal stem cell (MSC) properties of the PDL. However the proportion of viable stem cells within the PDL is still being determined. Nagatomo et al. conducted a study in which the ratio of available stem cells is present in the PDL. Using fluorescence activated cell sorter (FACS) they tested for MSC markers CD105, and CD166. In addition STRO-1 was examined due to it being an osteogenic precursor. Nagatomo et al. concluded that PDL cells do in fact express these characteristics [20]. It is through the MSCs that osteoblasts differentiate when osteogenic growth factors such as transforming growth factor β (TGF-β) are present [7, 40].

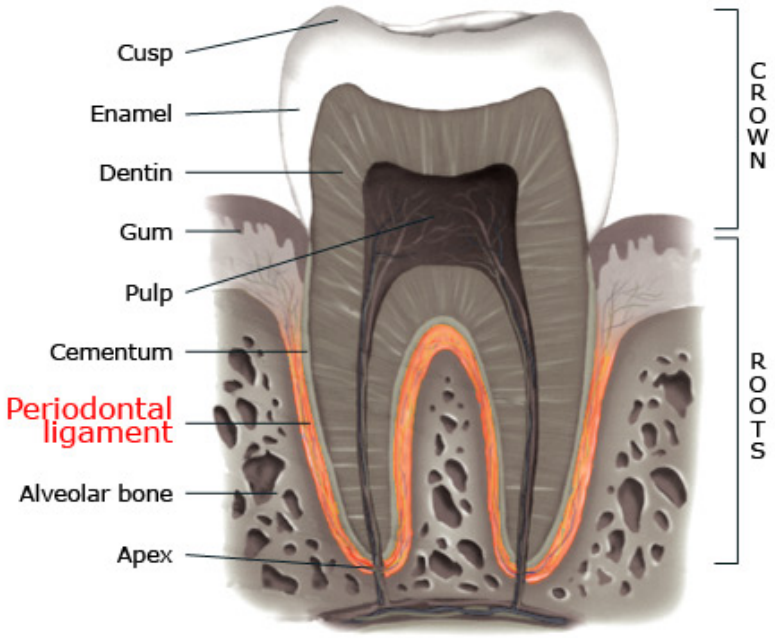

FIGURE 1.1: Location of PDL and Dental Pulp

Additional support for the presence of potential osteogenic forming cells arises from orthodontic forces. There is a remodeling effort that takes place in the PDL and adjacent alveolar bone during the controlled force on teeth [18]. These forces contribute to the shift in vascularity, cellular reorganization, while triggering several growth factors and colony-stimulating factors present in the remodeling of bone. This response is a result of strain present due to the loading. The process by which bone formation occurs in the tooth is by resorption of the alveolar bone on the pressure side coupled with the formation of new bone in the area of tension [22, 40]. These processes interact through cellular communication so that the tooth moves through the alveolar bone.

1.4 Dental Pulp

The inner cavities of teeth are filled with dental pulp. It is a fibrous connective tissue surrounded by dentin. The primary focus of dental pulp is to support the dentin. In addition, this region is where the nerves and blood vessels of the tooth reside [3, 12]. Dental pulp can be divided into 4 layers beginning with the outermost layer and moving in toward the center of the tooth. The outermost layer contains odontoblasts, which produce dentin. The following layer is an extracellular matrix primarily free of cells, however the next layer contains a copious amount of progenitor cells. These cells are especially particular in that they demonstrate pluripotent characteristics [4, 36]. Lastly, the inner layer contains the nervous plexus and the vascular structures.

The dental pulp contains four major cell types that contribute to its resourcefulness in the teeth. Odontoblasts secrete the primary layer of dentin, also known as mantle dentin, and then migrate to the center of dental papilla where the cells begin to construct the dentin matrix [41]. In addition to the mantle dentin, a differentiated

odontoblast can secrete many forms of dentin including intertubular, circumpulpal, and peritubular dentins [41]. As in the PDL, fibroblasts are also prevalent in the dental pulp. Predominantly found in the third "highly-cellular" region, these cells perform maintenance on the pulp matrix. Another function of these fibroblasts is to synthesize Type I and Type III Collagens and additional extracellular matrix constituents such as fibronectin. Not only do they promote the synthesis of pulp tissue, but they are also a part of the pulp remodeling process in which the matrix components are degraded [24, 41]. A third cell type found in Dental Pulp is the undifferentiated mesenchymal cell. These cells are related to the vascularity of Dental Pulp [21]. The fourth of the main Dental Pulp cell types are the immunocompetent cells. These are found in the connective tissue of the Dental Pulp and are involved in the area in which there is harm to the tooth such as in caries and cavities. Alongside the rich neuronal and vascular networks located in the Dental Pulp, these four cell types help to maintain and support the teeth.

1.5 Mechanobiology and Effects of Loading

The cells within our body are remarkable in that they respond to mechanical changes in their environment. Mechanotransduction is the process by which cells convert mechanical signals and transpose them into a biological response [13]. It is important that they possess the ability to adapt and respond to the constant forces applied to them through the changes in their environment. More specifically, these cells are constantly exposed to variations in protein or ion concentrations, along with tensile, compressive, and shear stresses. In some cases bones and joints are frequently subject to large dynamic and static loads that may contribute to injury. While large forces can be harmful, the cells rely on more natural forces within the body for repair and regeneration. Specifically the

cells of the PDL are subject to loads when mechanical stress is applied using orthodontic apparatuses. It has been shown that these procedures promote PDL cells to synthesize active enzymes required for the remodeling of the tissue [22, 32].

1.6 Stem Cells

The human body contains in excess of two hundred different cell types, each one having arisen from a common source and differentiated based on its final end function. Stem cells differ from their counterparts due to two main characteristics. First they are unspecialized and have the ability to constantly renew allowing for a continuous supply. In addition, under certain conditions they can differentiate to tissue-specific cells. There have been extensive research efforts throughout the past three decades in which scientists have learned about the potential of these cells. With the knowledge generated, new studies are being conducted in which these stem cells are included for clinical treatments.

One of the primary issues with stem cells for therapeutic use is the accessibility of a sufficient amount of viable stem cells. While there are multiple locations throughout the body possessing stem cells of various differentiation potential, the targeted site must have a high ratio of stem cells to harvested tissue. Unfortunately, this poses a significant problem to therapeutics because the majority of tissues lack this property. If too much of the tissue were to be collected for the amount of cells required there might be adverse effects such as the dwindling of the tissue. While it is ideal to obtain autologous stem cells, there may be a need for donor cells. Subsequently stem cell transplantations from homologous sources have the ability to cause further harm to the host patient due to the presence of pathogens and most importantly immunorejection.

CHAPTER 2: SIGNIFICANCE AND OBJECTIVES

Injured cartilage and bone are among the most common causes of discomfort affecting millions of people around the world. While remedies do exist in the form of invasive surgeries or prolonged treatment with a cast; new solutions are currently being researched to facilitate the issues. Among these novel solutions being developed, stem cells play a major role. Stem cells obtain the ability to proliferate in culture and differentiate into many types of cell lines. Using this principle, extensive research is currently taking place regarding the potential of various stem cell locations.

Within the tooth reside two locations of interest: the PDL and the Dental Pulp. The potential for chondrogenic and osteogenic differentiation is present in the cells from these two locations proven by growth factors and culture conditions [8, 11]. However, there is limited literature that provides insight into the differentiation potential when subjected to loading conditions. The teeth are constantly subjected to forces in such cases as mastication and tooth remodeling. Since compression is proven to initiate cellular responses, we may assume that these forces can have a direct impact on osteogenesis and chondrogenesis when directly applied. Therefore the objective of this study is to examine the response to dynamic loading conditions as well as static loading conditions of cells derived from the PDL and Dental Pulp.

CHAPTER 3: MATERIALS AND METHODS

3.1 Dental Stem Cell Isolation and Culture

Impacted 3rd molars were obtained from healthy human donors following routine medical procedures requiring their extraction based on the IRB approved protocols at the University of Miami. PDLs and dental pulps were harvested from the molars and stem cells were isolated based on the protocol described in the study of Huang et al. [11]. Briefly, PDLs and dental pulps were enzymatically digested in a collagenase solution overnight and filtered through 40μL cell strainer to obtain a single cell suspension. Cells were then selected for adherent-dependence and cultured in DMEM with 10% FBS and 100U/mL Penicillin-streptomycin (Invitrogen) and 0.1% v/v amphotericin B. Passaging of the cells was achieved by enzymatic digestion of Trypsin/EDTA (Invitrogen) and subcultured. Stem cells of primary culture were frozen at a density of $1x10^6$ cell/ml in a solution of High Glucose Dulbecco's Modified Eagle Medium (HG-DMEM) (Invitrogen Corp., Carlsbad, CA), 20% DMSO, and 20% Fetal Bovine Serum (FBS) (Invitrogen Corp).

Frozen cells of the PDL and Dental Pulp were plated prior to each study. In addition DMEM was prepared accordingly containing 10% FBS and 1% Antibiotic/Antimycotic (Invitrogen Corp.) First, the freezing solution was thawed and immediately placed in a 1.5mL micro-centrifuge tube (VWR, Radnor, PA) and centrifuged for seven minutes at 1,000 rpm to form a pellet. The supernatant was removed and re-suspended in 1mL of DMEM (Invitrogen Corp.). This solution was placed in a culture dish with 10mL DMEM (Invitrogen Corp.) to expand in an incubator

with 5% CO_2 at 37°C. Once the dish reached 80% confluence, cells were passaged to three T-175 flasks (Greiner Bio-One, Monroe, NC) and left to incubate.

3.2 Agarose Gel Preparation

Prior to gel formation, a 4% gel solution was prepared using low-gelling temperature Agarose (Sigma, St. Louis, MO) and Dulbecco's Phosphate Buffered Saline (DPBS) (Invitrogen Corp.). The mixture was sterilized using an autoclave and left to cool for 24 hours.

New gels were prepared for each cell type being tested. As soon as each T-175 flask reached confluence cells were detached and counted, respectively, using a Hemacytometer (Hausser Scientific, Horsham, PA). Following the cell counting procedure, cells were centrifuged and a re-suspension amount was calculated in order to obtain a density of $1x10^6$ cells in each gel. The supernatant was removed and re-suspended in the calculated amount of DMEM containing 10% FBS and1% antibiotics. Gels were prepared by combining 50µL cells mixture and 50µL Agarose and placed in molds. All gels had a diameter of 8mm and thickness of 1.5mm. Twelve gels (6 tested, 6 control) were produced for every experiment. Once all the gels solidified, they were placed in 12-well plates and cultured overnight in 2mL DMEM containing 10% FBS and 1% antibiotics in 37°C and 5% CO_2.

3.3 Bioreactor

A custom bioreactor was built in order to meet the specifications and requirements of this study. The design was built around an already developed mechanical loading device. This design is based around a SmartMotor™ SM23165D motor and

actuator (Moog Animatics, Santa Clara, CA). The motor was controlled by Smart Motor Interface (SMI) software (Moog Animatics) along with a program written to modify movement. By using this software one can alter the frequency as well as displacement of sinusoidal movement. Each step in the motor corresponded to 1μm. To control the motor for static compression experiments the SMI playground was utilized to apply the 15% strain necessary. Aluminum was used for all materials including the stand, top plate, and chamber holder. The chamber holder was bored out of solid 0.5" aluminum and was suspended using 4 1.5" bolts. This design was chosen so that the chamber does not move a horizontal or vertical direction, and so that the gel and medium is visible during testing. A top plate was designed to attach to the linear actuator so that displacement can be transferred to the Agarose disks.

Six compression chambers were designed for the bioreactor allowing for a total of six gels to be tested. The material used for the chamber, top plug, and chamber cap is an impact resistant polycarbonate. Not only does this material have the necessary mechanical properties to withstand the prolonged loading factors without failing, but also it is autoclavable. This property was extremely important in order to prevent contamination of gels from outside factors and between each cell type. Before each study the chambers were autoclaved at 250°F for 15 minutes, and left to dry for 99 minutes. Each specimen was individually placed in a chamber along with 500μL DMEM with 10% FBS and 1% antibiotics. The top plug and cap were carefully placed on top of the gel and a bolt was used to prevent the plug from moving down during experiment set up. The chambers were then placed into the bioreactor which was in an incubator at 37°C with 5% CO_2 to maintain viable conditions for the cells during testing.

Each cell type underwent dynamic compression of 1Hz, 0.1Hz, and static compression for two hours a day, for three consecutive days. The gels were under 15% strain throughout the duration of the experiment. Initially 5% strain was added to ensure sufficient contact between the top plug and the gel. Following this step, either 10% dynamic compression or static compression was applied. For each variable (1Hz, 0.1Hz, static) both cell types were tested in the same day and the chambers were autoclaved between the cell types. Control gels remained in the incubator and medium was changed every two days. Concluding a three-day experiment, gels were placed in a 15mL tube with 2mL Tri Reagent (Molecular Research Center, Cincinnati, OH). The tubes were vortexed for five minutes to ensure homogenization of gel with Tri Reagent and placed in the -20°C freezer.

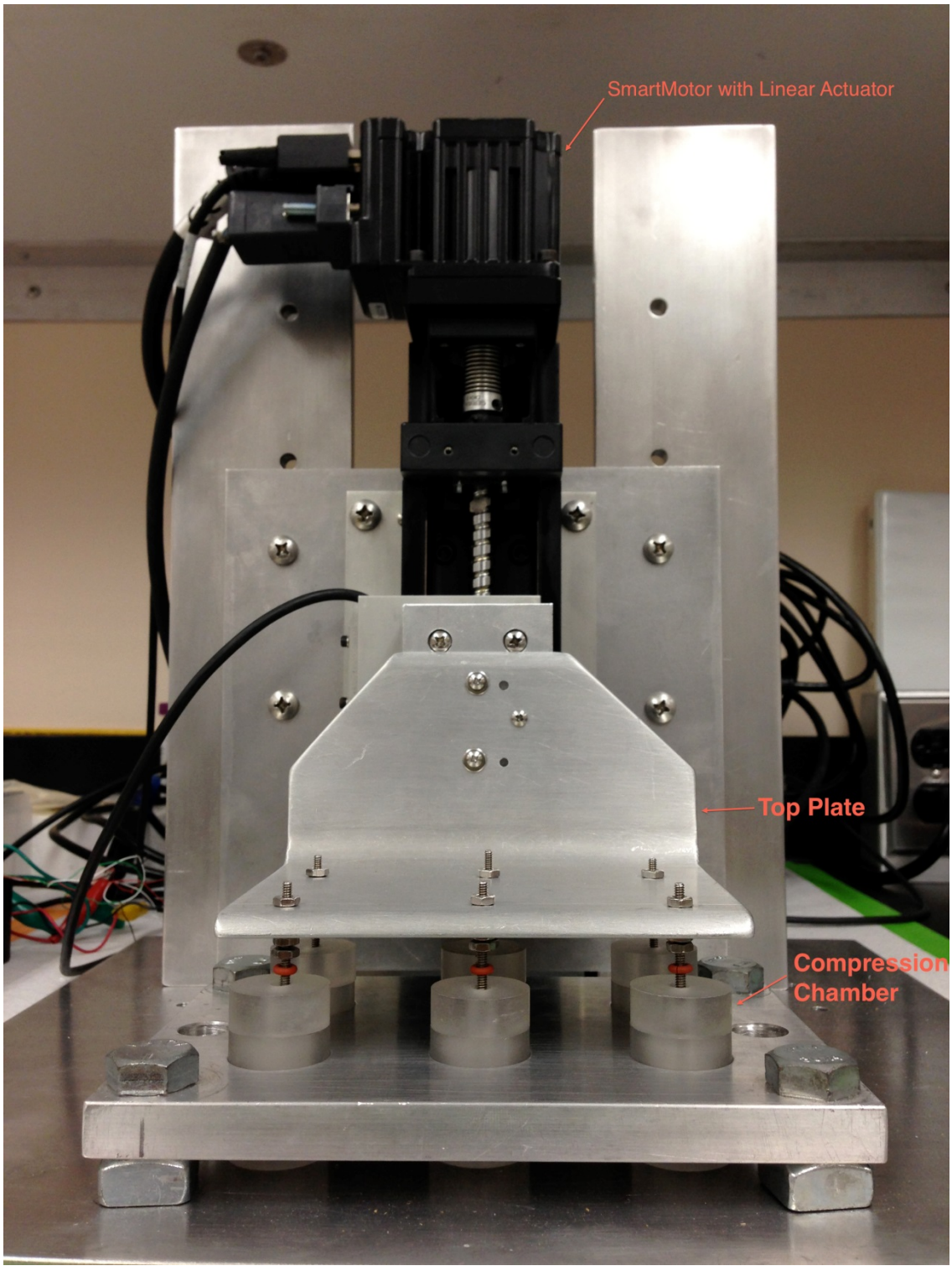

FIGURE 3.1: Custom Designed Bioreactor

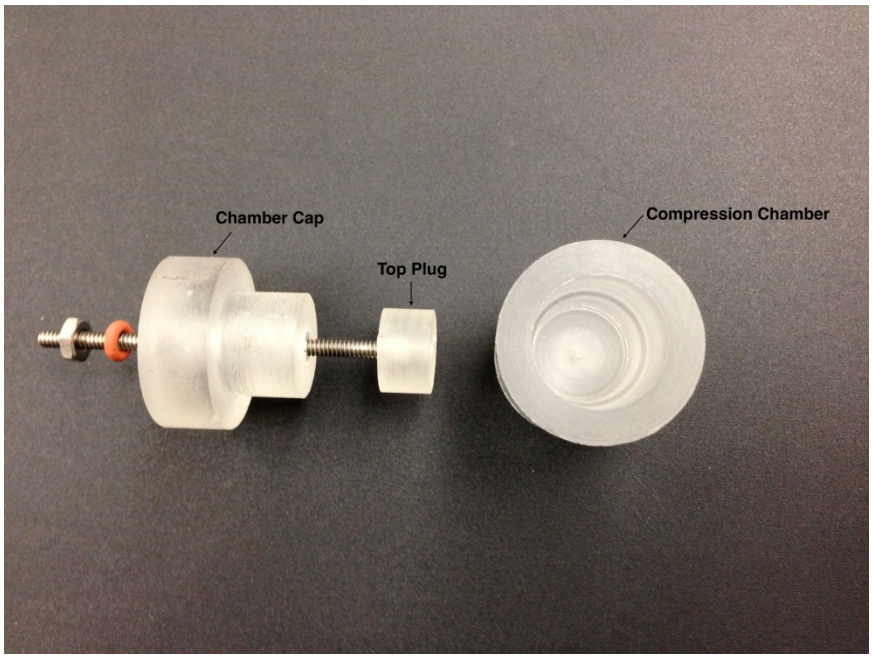

FIGURE 3.2: Compression Chamber with Plug

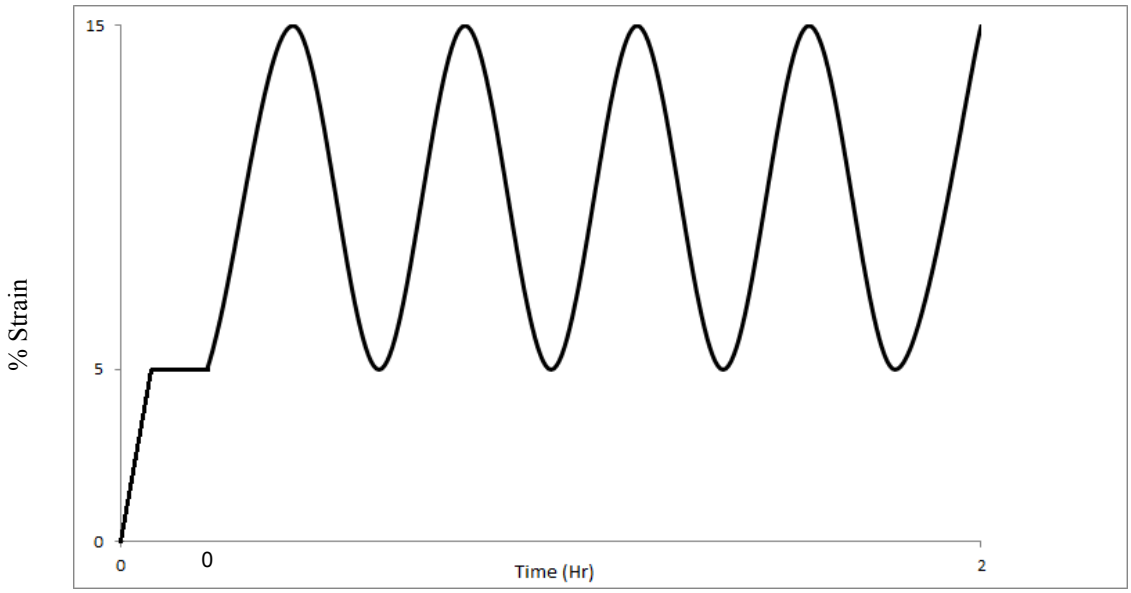

FIGURE 3.3: Graphical Representation of Dynamic Loading Protocol

3.4 RNA Extraction and cDNA Synthesis

RNA extraction is a process required to isolate the RNA from the Agarose. The gels remained in the -20°C freezer for at least 24 hours before starting the RNA extraction procedure. The tubes were thawed and contents were split into two Eppendorph tubes. 200µL of Chloroform (Sigma) was added and vortexed until the solution homogenized. This was then centrifuged for 20 minutes at 13,000 rpm. The aqueous layer was removed and transferred to a sterile Eppendorph tube. Following this step, 500µL of Isopropanol (Sigma) was added to precipitate the RNA and centrifuged for 20 minutes at 13,000 rpm. The pellet was isolated and washed with 75% ethanol and centrifuged for 20 minutes at 13,000 rpm. The final step was to remove the ethanol and re-suspend in 20µL of HyPure Molecular Biology Grade Water (Thermo Scientific, Logan, UT). The tubes with RNA pellet and water were then placed in an -80°C freezer overnight.

On the following day, the RNA and water became thawed and the pellet was homogenized. It was important to only work with one at a time in order to keep the RNA from degrading. RNA samples were quantified using a Quibit fluorometer (Invitrogen Corp.). All samples were normalized to the lowest reading in order to standardize the cDNA synthesis. cDNA was synthesized from all RNA samples using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and a MJ Mini Thermal Cycler (Bio Rad).

3.5 Gene Expression Analysis

Results for these experiments were quantified using Real Time-Polymerase Chain Reaction (RT-PCR). cDNA samples were tested along with SYBR® Green SuperMix (Quanta Biosciences, Inc., Gaithersurg, MD). Gene expression of chondrogenic [Collagen Type II (hColl II) and Aggrecan (hAGG)] and osteogenic markers [Collagen Type I (hColl I), Osteocalcin (OCN), Osteonectin (ONN), Osteopontin (OPN), Alkaline Phosphatase (ALP) and Runt-related transcription factor 2 (hRunX2)] were analyzed and the genes for the PCR are listed in Table 3.1. All genes were ordered from Integrated DNA Technologies, Inc., Coralville, IA. RT-PCR was completed using a StepOnePlus™ Real-Time PCR system (Applied Biosciences) and associated StepOne Software v2.1. The software is utilized for the assignment of associated genes and cDNA to be tested. Once the RT-PCR run was completed, threshold cycle (C_T) results were collected and exported to Microsoft Excel spreadsheets. To analyze the relative gene expression of each gene, the samples were normalized to the reference gene 18s. Following the normalization procedure the R-value was calculated according to Equation 3-1.

$$R = \frac{E_{target}^{\Delta C_T(\text{Control} - \text{Treated})}}{E_{reference}^{\Delta C_T\, reference(\text{Control} - \text{Treated})}}$$

Equation 3.1

R is the Ratio, also known as the fold increase of the control. *E* is the efficiency calculated from a standard curve. Because we knew that our curve was standard we were able to make the assumption that E = 2. Lastly, *Reference* refers to 18s.

Gene		Sequence	BP
18s	Sense	5'-CGGCTACCACATCCAAGGA-3'	
	Antisense	5'-AGCTGGAATTACCGCGGCT-3'	
hALP	Sense	5'-GTTCAGCTCGTACTGCATGTC-3'	286
	Antisense	5'-ATCGCCTACCAGCTCATGCAT-3'	
hRunX2	Sense	5'-TTCATCCCTCACTGAGAG-3'	262
	Antisense	5'-TCAGCGTCAACACCATCA-3'	
hOPN	Sense	5'-TGAAACGAGTCAGCTGGATG-3'	162
	Antisense	5'-TGAAATTCATGGCTGTGGAA-3'	
hOCN	Sense	5'-GGCAGCGAGGTAGTGAAGAG-3'	230
	Antisense	CTGGAGAGGAGCAGAACTGG-3'	
hONN	Sense	5'-GTGCAGAGGAAACCGAAGAG-3'	172
	Antisense	5'-TCATTGCTGCACACCTTCTC-3'	
hColl I	Sense	5'-AATTGGAGCTGTTGGTAACGC-3'	126
	Antisense	5'-CACCAGTAAGGCCGTTTGC-3'	
hColl II	Sense	5'-TGGCCTGAGACAGCATGAC-3'	373
	Antisense	5'-AGTGTTGGGAGCCAGATTGT-3'	
hAgg	Sense	5'-TGAGGAGGGCTGGAACAAGTACC-3'	350
	Antisense	5'-GGAGGTGGTAATTGCAGGGAACA-3'	

Table 3.1: List of Genes Used

3.6 Statistical Analysis

Statistical analysis was performed using a two-sample assuming equal variance t-test. Results are reported as statistically significant if $p<0.05$.

CHAPTER 4: RESULTS

In this study the mechanobiological response of cells derived from the PDL and Dental Pulp were evaluated. A total of 48 samples were tested: 24 were PDL (12 tested, 12 control) and 24 were Dental Pulp (12 tested, 12 control). All samples were assembled and tested according to the developed protocol for 1Hz and 0.1Hz dynamic loading and static loading. Gene expression was then performed in order to assess the difference in change between control and treated samples. For statistical analysis all genes were normalized to an internal control, 18s.

4.1 Effects of 1Hz Dynamic Loading

Agarose gels were placed under 15% strain dynamic loading for two hours a day for three consecutive days. The results show that there is a relative increase in gene expression when 1Hz dynamic loading is applied to Dental Pulp cells. The osteogenic markers hOCN, hONN, and hOPN showed no significant differences in expression, however the treated samples tended to show up-regulation in the presence of 1Hz dynamic loading [Figure 4.1], [Figure 4.2], and [Figure 4.3]. The RunX2 gene expression was significantly increased ($p=0.04$) under 1Hz dynamic loading conditions [Figure 4.4]. For the chondrogenic gene hAGG, there was no significance between the control and treated sample, however results show that the samples tended to be down regulated by 1Hz dynamic [Figure 4.5]. Collagen I expression tended to be up regulated in the presence of 1Hz dynamic loading ($p=0.09$) [Figure 4.6]. However there was no change in expression for Type II Collagen [Figure 4.7].

Results show that there are relatively no statistical differences for 1Hz dynamic loading on PDL samples; however the results show that the genes tend to be up regulated. 1Hz mechanical loading tends to up regulate the osteogenic genes hOCN, hONN, and RunX2 compared to the control gels [Figure 4.8], [Figure 4.9], [Figure 4.10], however there was no significant difference. There was down regulation associated with hOPN [Figure 4.11]. Additionally there were no noticeable differences for the chondrogenic genes Aggrecan, Type I Collagen, and Type II collagen [Figure 4.12], [Figure 4.13], [Figure 4.14].

4.2 Effects of 0.1 Hz Dynamic Loading

Dynamic compression of Dental Pulp cells at 0.1 Hz resulted in no significant changes between treated and control samples with the exception of Type II Collagen ($p=0.009$) [Figure 4.18]. Aggrecan, ALP, Type I Collagen, hOCN, hONN, hOPN, and hRunX2 resulted in no significant change [Figure 4.15], [Figure 4.16], [Figure 4.17], [Figure 4.19], [Figure 4.20], [Figure 4.21], [Figure 4.22].

The majority of results for 0.1Hz dynamic compression for PDL cells showed that mechanical loading at this frequency has no significant effect. Osteocalcin was statistically significant for the up regulation of the gene with a $p=0.02$ [Figure 4.23]. For the osteogenic genes hONN, hOPN, hRunX2, and hALP no significance was observed but they tended be up regulated in result to loading [Figure 4.24], [Figure 4.25], [Figure 4.26], [Figure 4.27]. For the chondrogenic gene Aggrecan there was no significant difference for expression however it tends to be down regulated by 0.1Hz dynamic loading [Figure 4.28]. There is also no significant difference for the chondrogenic genes

Type I Collagen and Type II Collagen expression but it tends to be up regulated as a result of loading [Figure 4.29], [Figure 4.30].

4.3 Effects of Static Compression

Results show there is little to no promotion in regards to static compression on Dental Pulp cells. However, expression of Alkaline Phosphatase was down regulated ($p = 0.005$) [Figure 4.31]. Additionally there was down regulation in the expression of the Type I Collagen gene ($p=0.009$) [Figure 4.32]. The other chondrogenic genes Type II Collagen and Aggrecan were not significantly expressed [Figure 4.33], [Figure 4.34]. The osteogenic genes hOCN, hONN, hOPN, and hRunX2 were relatively unchanged as a result of static compression [Figure 4.35], [Figure 4.36], [Figure 4.37], [Figure 4.38].

PDL samples tended to result in a decrease in expression when under static loading conditions. For osteogenic markers there was significant decrease in expression between the control and treated samples. hOCN ($p=0.02$), hONN ($p=0.03$), hOPN ($p=0.005$), and hRunX2 ($p=0.04$) were significantly down regulated during the exposure to static compression [Figure 4.39], [Figure 4.40], [Figure 4.41], [Figure 4.42]. There was no significance for expression of the gene hALP, but there tends to be down regulation due to static loading [Figure 4.43]. For the chondrogenic genes Aggrecan, Type I and Type II Collagen there are no significant differences for expression however they tend be down regulated by static loading [Figure 4.44], [Figure 4.45], [Figure 4.46].

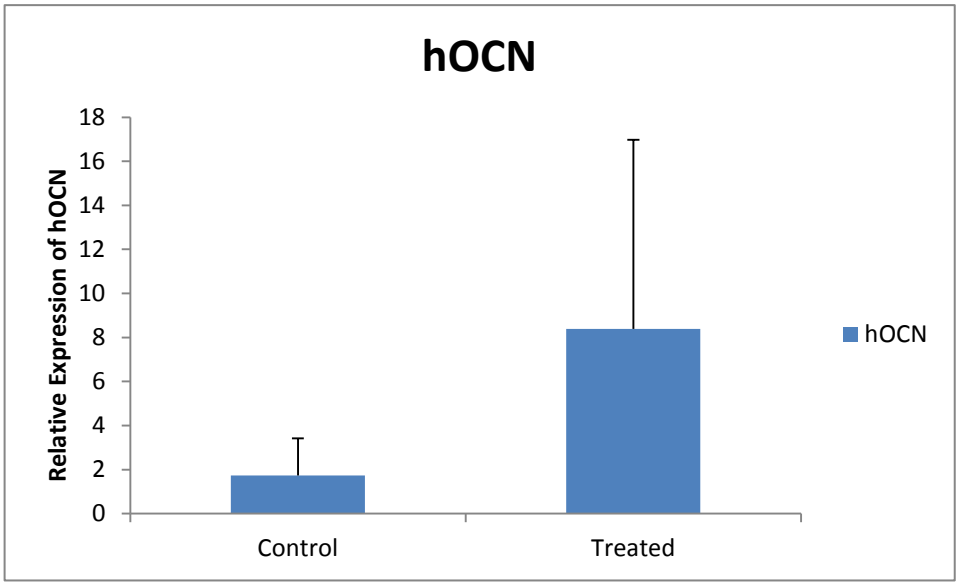

FIGURE 4.1: Relative Expression of hOCN for Dental Pulp 1Hz Dynamic Compression. Results show that is a large increase of expression in compared to the control samples. The results are closely significant with a $p>0.12$. The data was normalized by the expression of 18s.

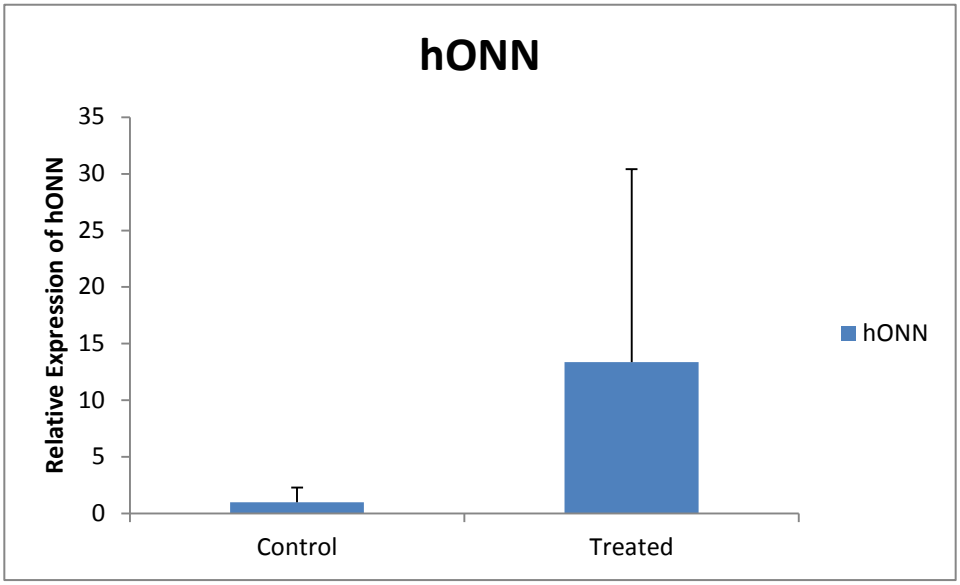

FIGURE 4.2: Relative Expression of hONN for Dental Pulp 1Hz Dynamic Compression. Results show a large difference between the treated and control sample however a high standard deviation is associated. The results are not significant with $p>0.18$. The data was normalized by the expression of 18s.

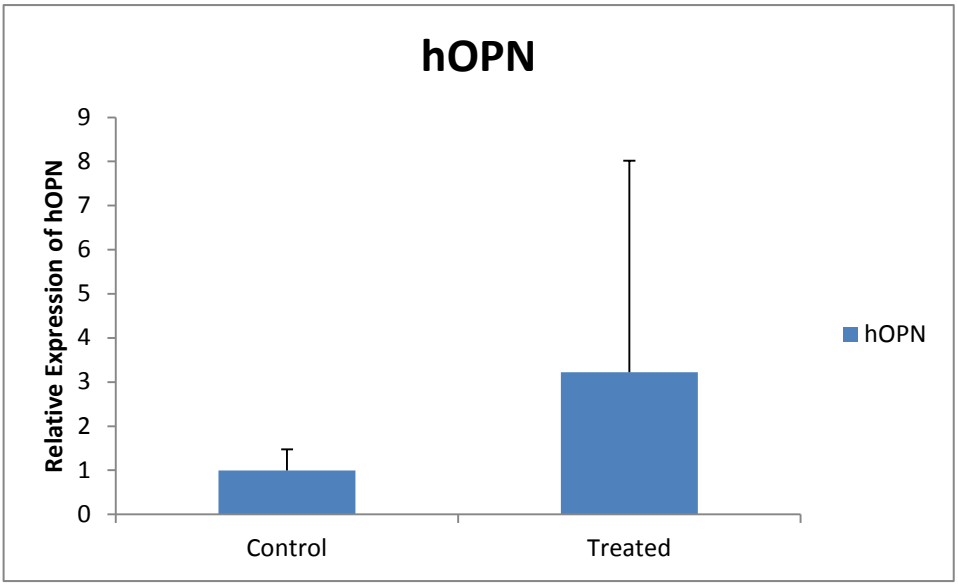

FIGURE 4.3: Relative Expression of hOPN for Dental Pulp 1Hz Dynamic Compression. Results show an increase of expression for treated samples. The results are not significant with a p>0.23. The data was normalized by the expression of 18s.

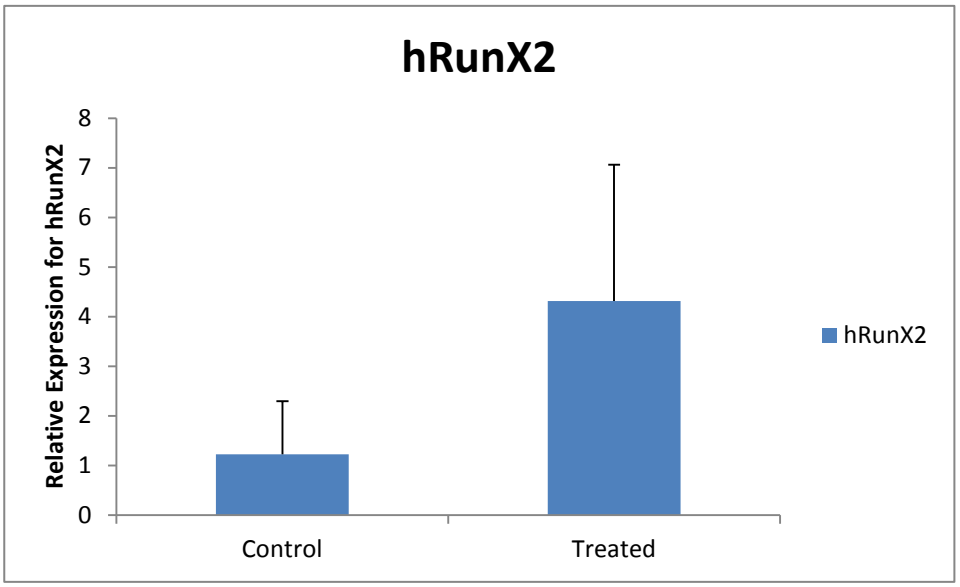

FIGURE 4.4: Relative Expression of hRunX2 for Dental Pulp 1Hz Dynamic Compression. The results show an increase of up regulation for treated samples. The data is significant with a p>0.04. The data was normalized by the expression of 18s.

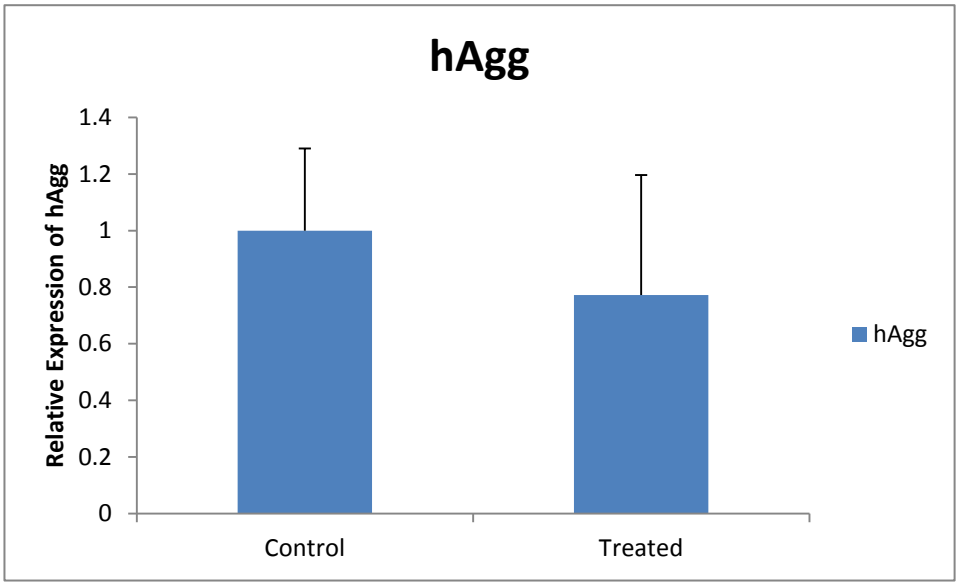

FIGURE 4.5: Relative expression of hAgg for Dental Pulp 1Hz Dynamic Compression. The results show that there is slight down regulation for treated samples. The results are not significant with a p>0.35. The data was normalized by the expression of 18s.

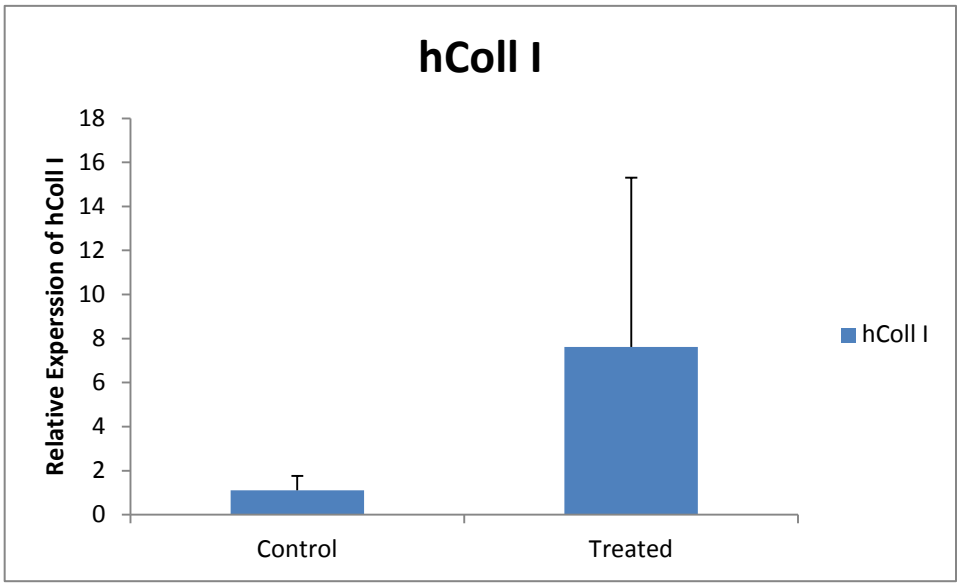

FIGURE 4.6: Relative Expression of Type I Collagen for Dental Pulp 1Hz Dynamic Compression. The results show that there is a large difference expression compared to the control samples. The results are closely significant with a p>0.09. The data was normalized by the expression of 18s

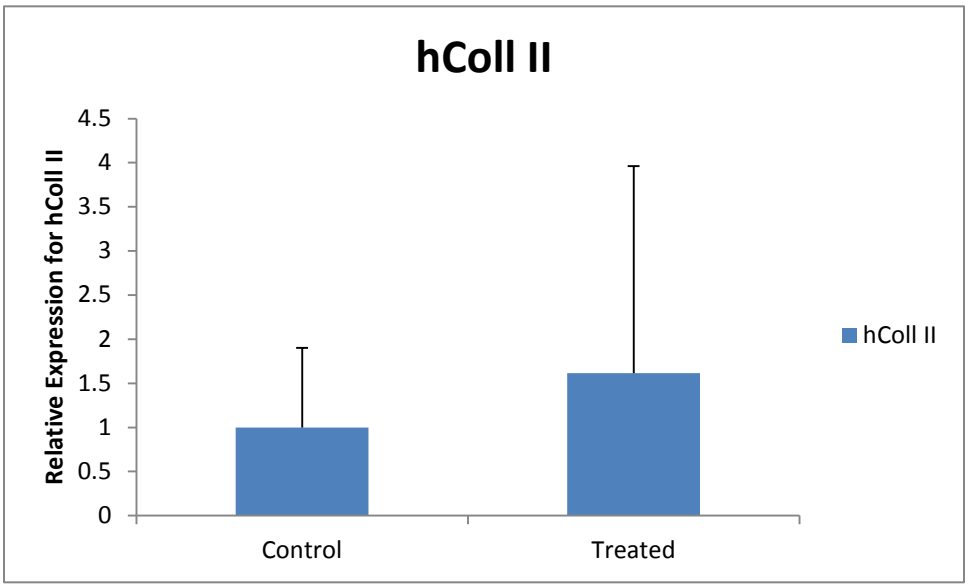

FIGURE 4.7 Relative Expression of Type II Collagen for Dental Pulp 1Hz Dynamic Compression. The data was normalized by the expression of 18s.

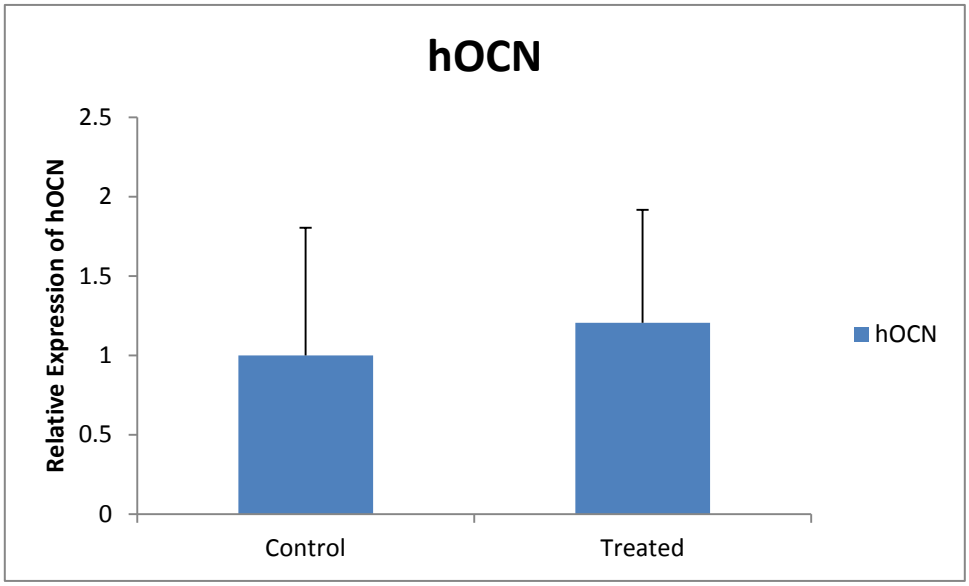

FIGURE 4.8: Relative Expression of hOCN to PDL 1Hz Dynamic Compression. There is a slight increase in expression between the treated and control samples. All samples are normalized by the expression of 18s.

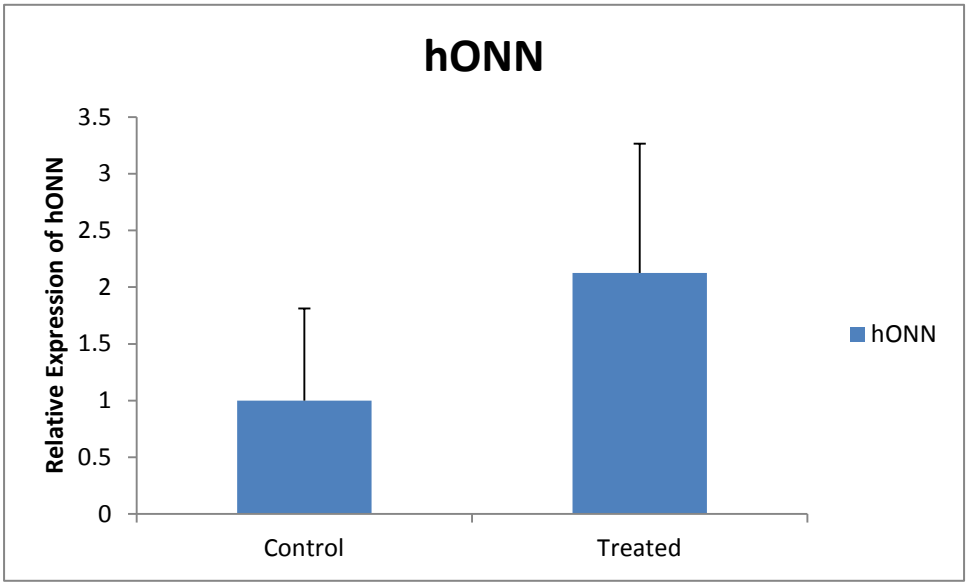

FIGURE 4.9: Relative Expression of hONN to PDL 1Hz Dynamic Compression. There is a noticeable increase of expression between the treated and control samples. The results are closely significant with a $p>0.2$. All samples are normalized by the expression of 18s.

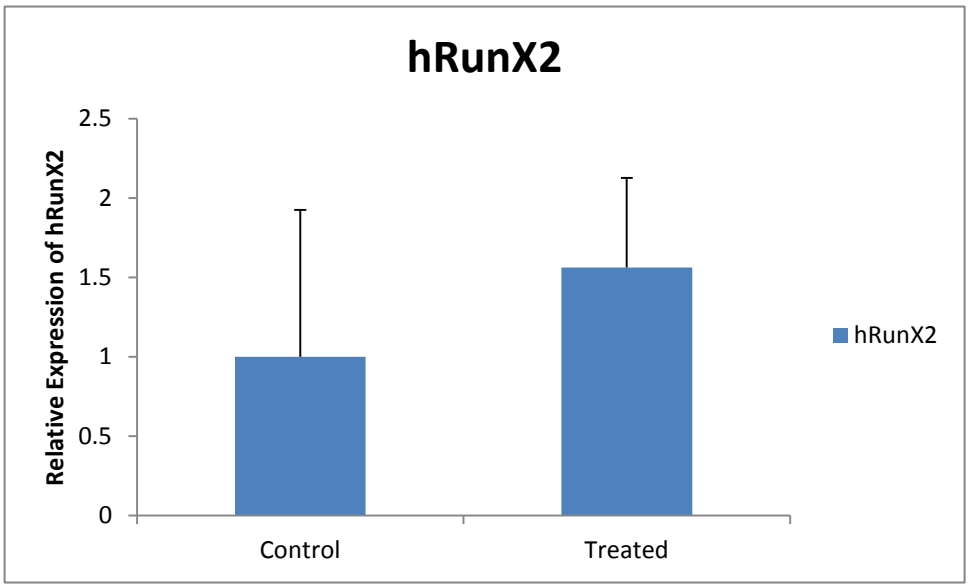

FIGURE 4.10: Relative Expression of RunX2 to PDL 1Hz Dynamic Compression. There is an increase of expression between treated and control samples. The results are closely significant with a p>0.2. All samples are normalized by the expression of 18s.

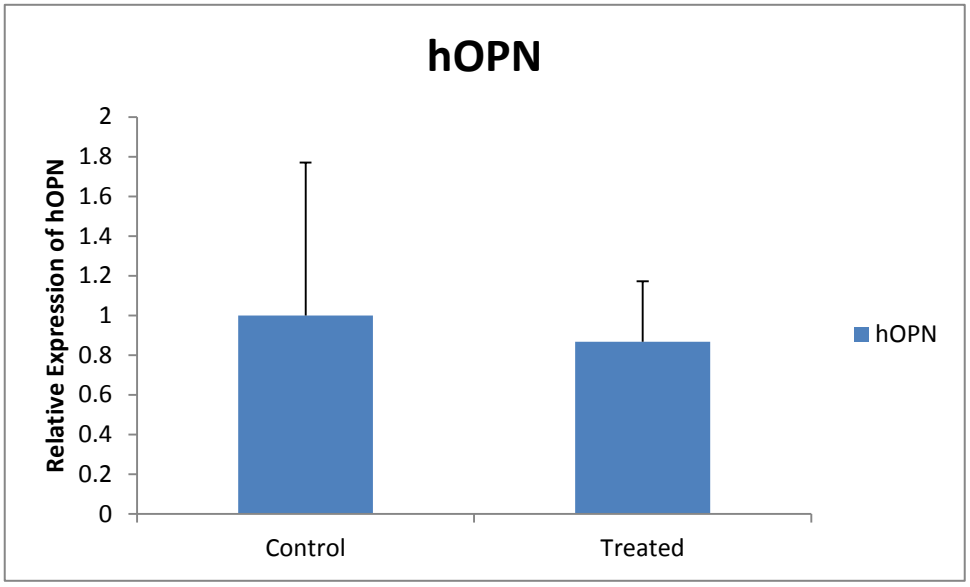

FIGURE 4.11: Relative Expression of Osteopontin to PDL 1Hz Dynamic Compression. There is a very small noticeable difference between the treated and control sample however down regulation does appear. All samples were normalized by the expression of 18s.

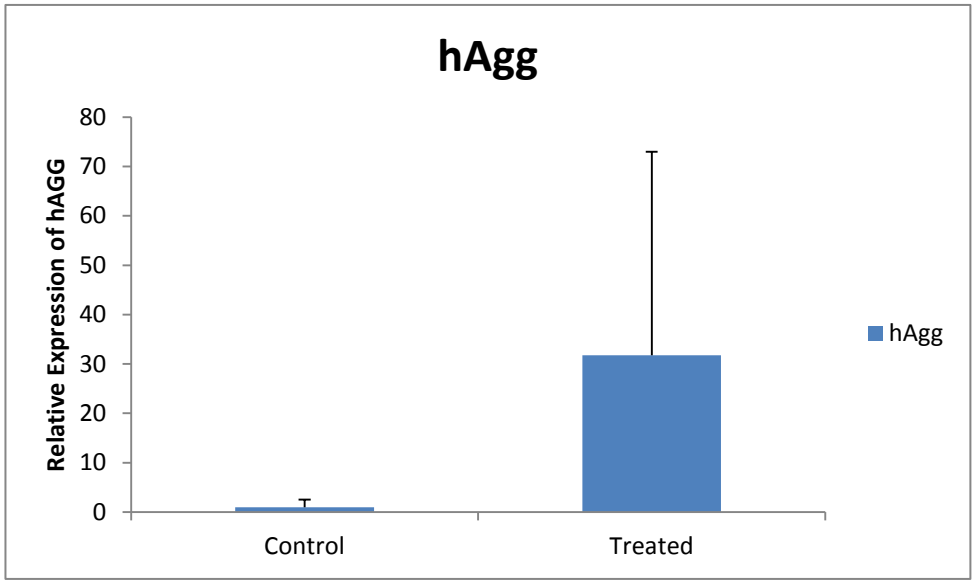

FIGURE 4.12: Relative Expression of Aggrecan to Static Compression on Dental Pulp. There is a large variation between the treated and control samples indicating some differentiation potential. All samples were normalized by the expression of 18s.

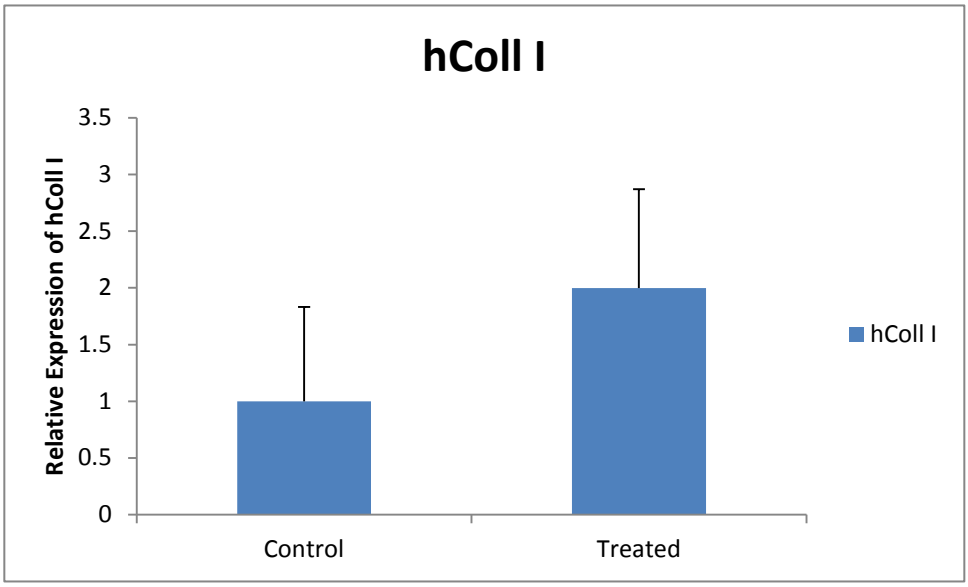

FIGURE 4.13: Relative Expression of Type I Collagen to PDL 1Hz Dynamic Compression. There is a noticeable increase in expression for treated samples. All samples were normalized by the expression of 18s.

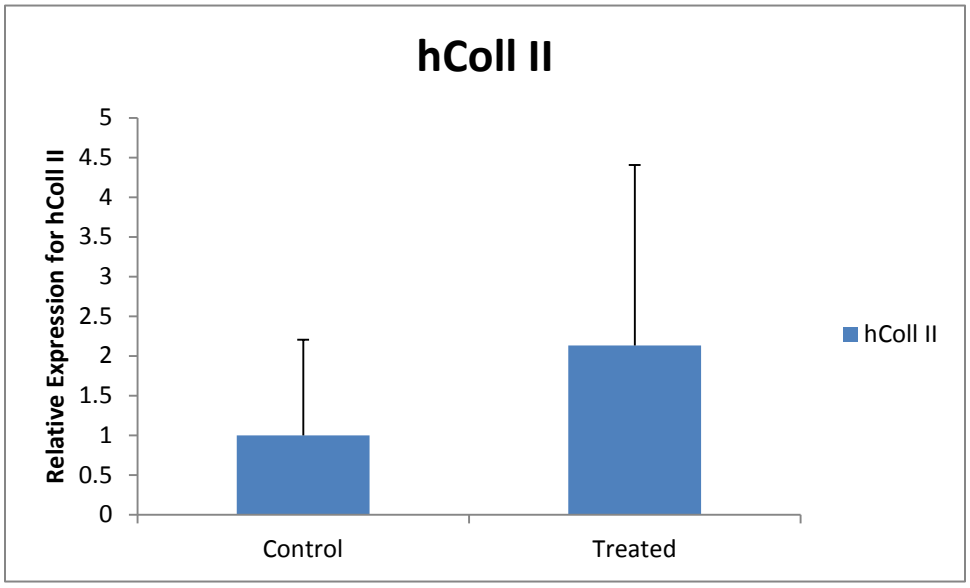

FIGURE 4.14: Relative Expression of Type II Collagen to PDL 1Hz Dynamic Compression. All samples were normalized by the expression of 18s.

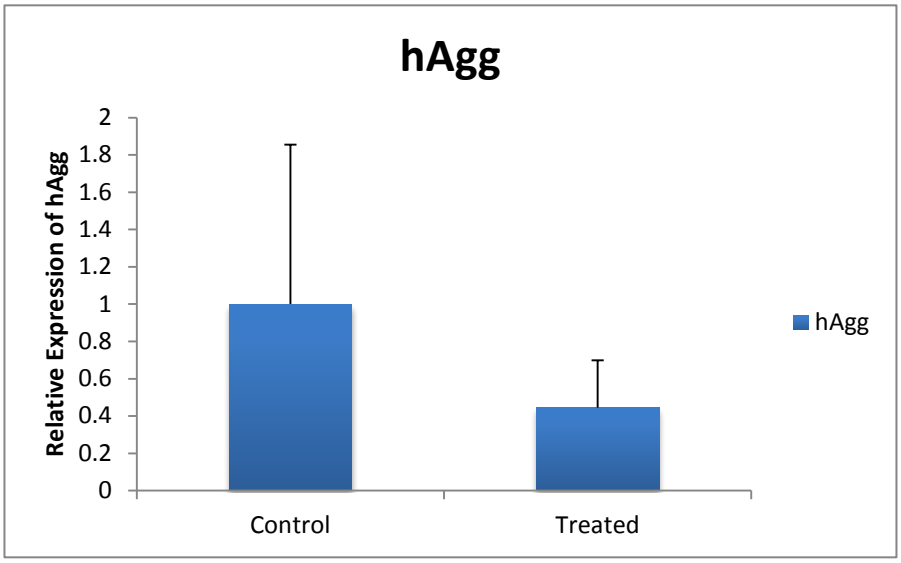

FIGURE 4.15: Relative Expression of hAgg to 0.1Hz Dynamic Compression on Dental Pulp cells. There is a noticeable decrease in expression for treated samples. All samples were normalized by the expression of 18s.

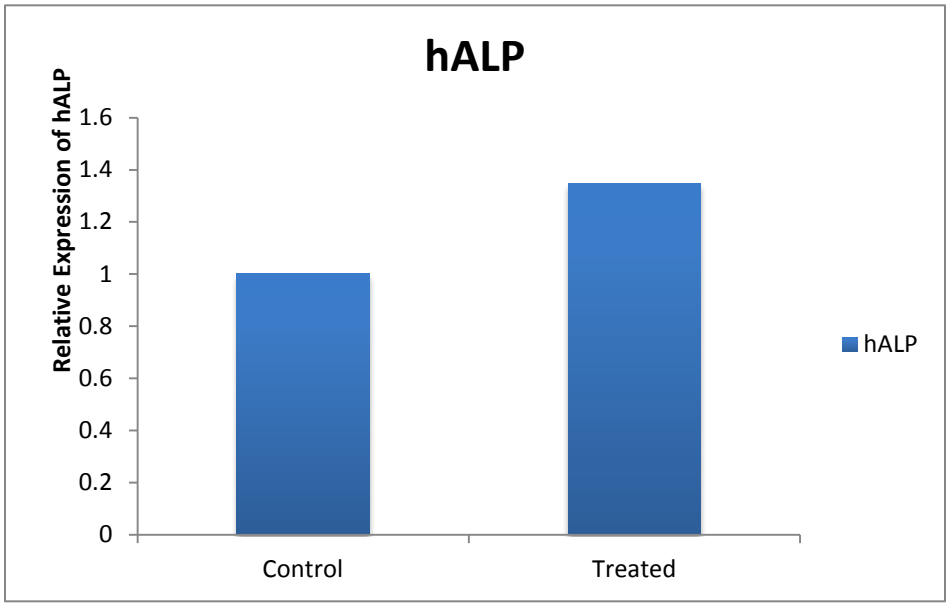

FIGURE 4.16: Relative Expression of hALP to 0.1Hz Dynamic Compression on Dental Pulp cells. There is a slightly higher expression amount for treated samples but result is not statistically significant. All samples were normalized by the expression of 18s.

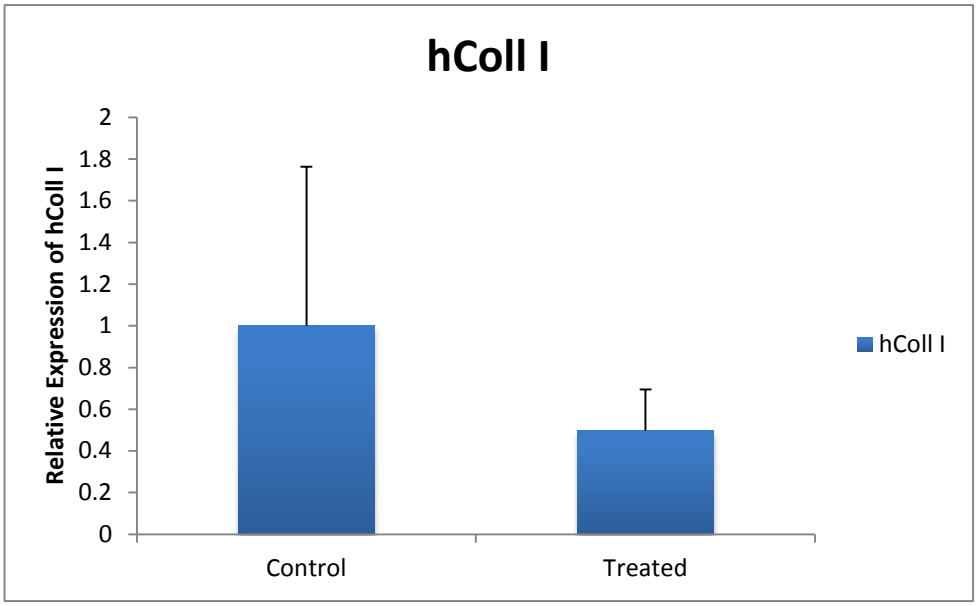

FIGURE 4.17: Relative Expression of Type I Collagen to 0.1Hz Dynamic Compression on Dental Pulp cells. There is a decrease in expression for treated samples. All samples were normalized by the expression of 18s.

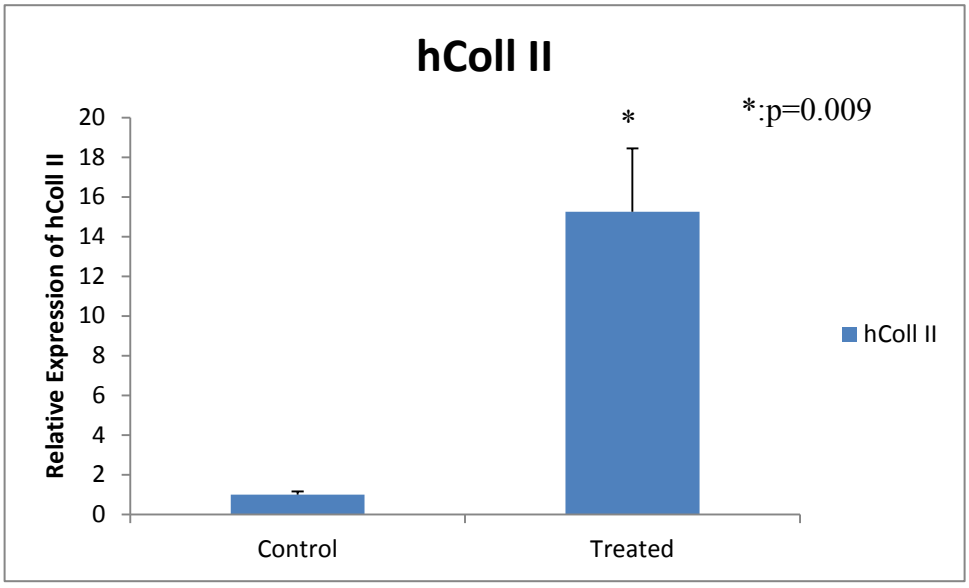

FIGURE 4.18: Relative Expression of Type II Collagen to 0.1Hz Dynamic Compression on Dental Pulp cells. There is a significant increase in expression, p=0.009

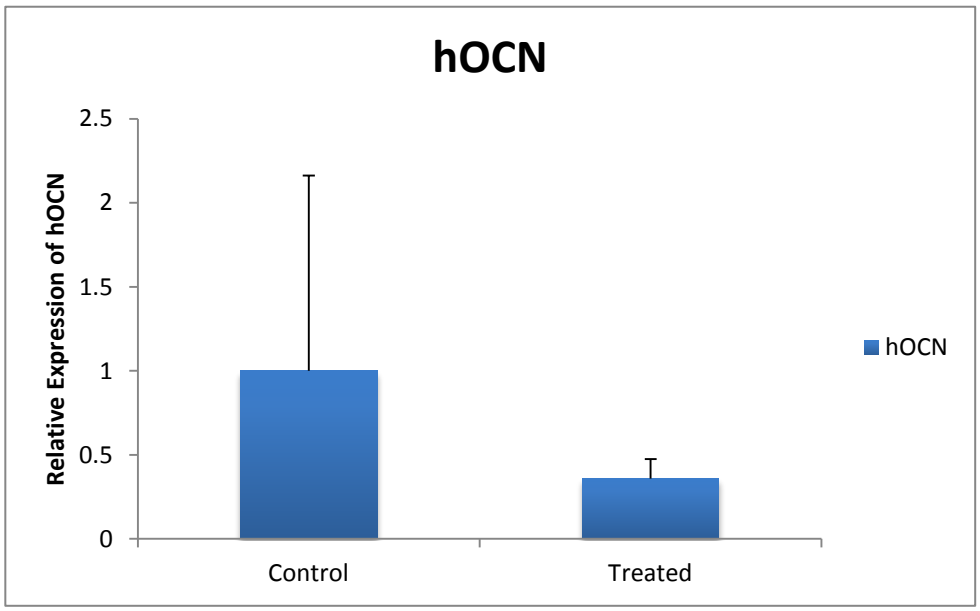

FIGURE 4.19: Relative Expression of hOCN to 0.1Hz Dynamic Compression on Dental Pulp cells. There is a decrease in expression for treated samples. All samples were normalized by the expression of 18s.

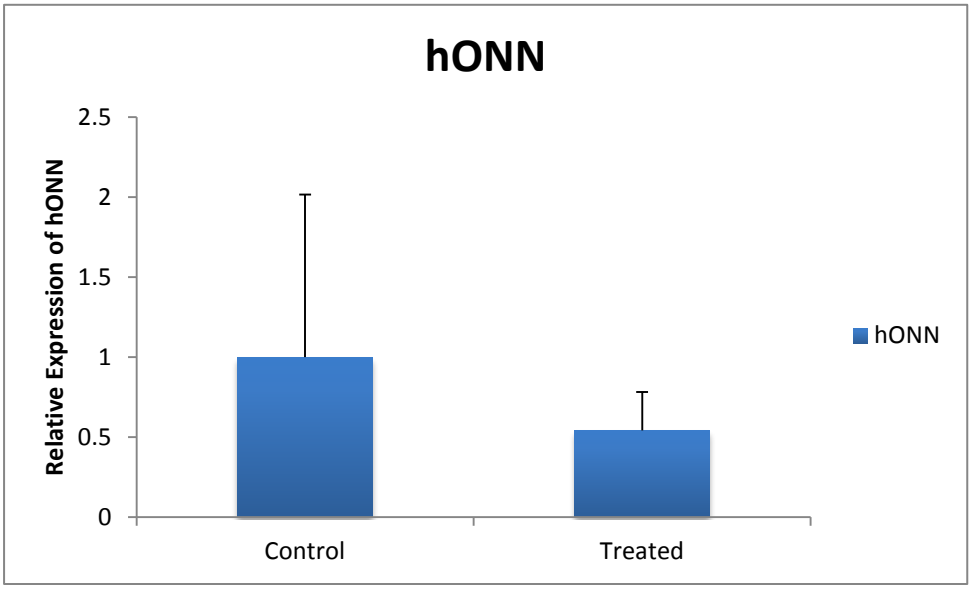

FIGURE 4.20: Relative Expression of hONN to 0.1Hz Dynamic Compression on Dental Pulp cells. There is a decrease in expression for treated samples. All samples were normalized by the expression of 18s.

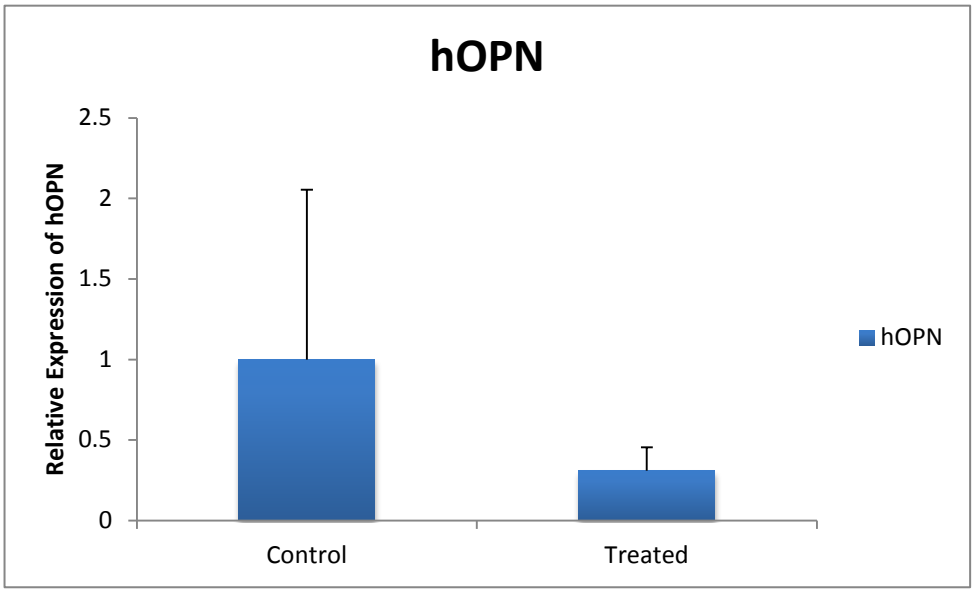

FIGURE 4.21: Relative Expression of hOPN to 0.1Hz Dynamic Compression on Dental Pulp cells. There is a decrease in expression for treated samples. All samples were normalized by the expression of 18s.

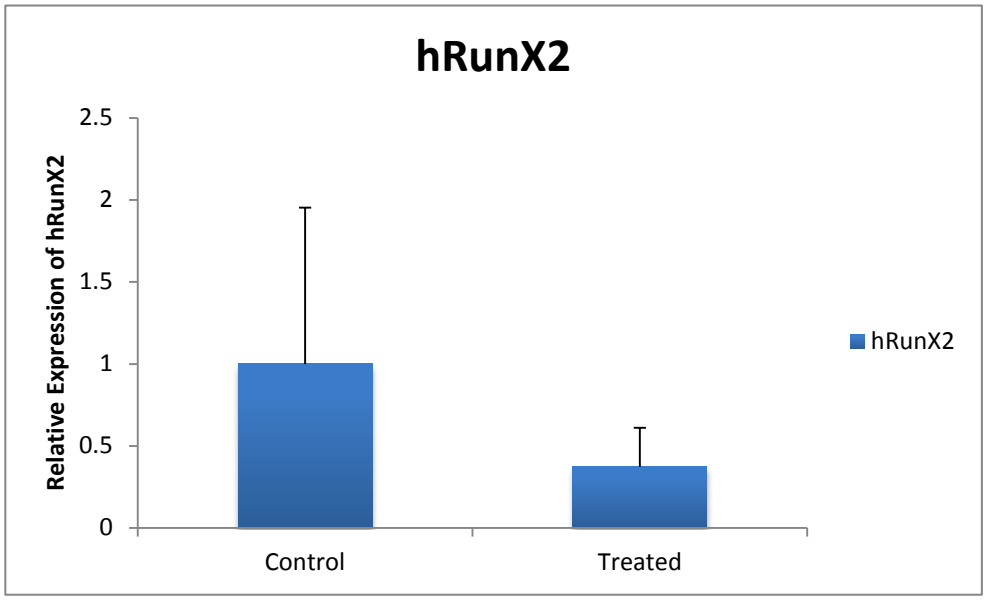

FIGURE 4.22: Relative Expression of hRunX2 to 0.1Hz Dynamic Compression on Dental Pulp cells. There is a decrease in expression for treated samples. All samples were normalized by the expression of 18s.

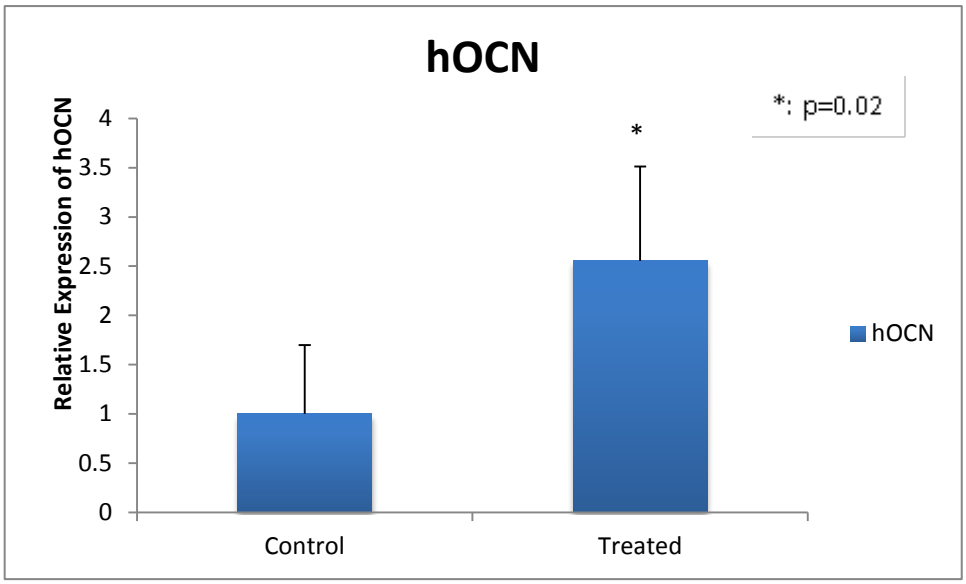

FIGURE 4.23: Relative Expression of hOCN to 0.1Hz Dynamic Compression on PDL cells. There is a significant increase in expression for treated samples (p=0.02). All samples were normalized by the expression of 18s.

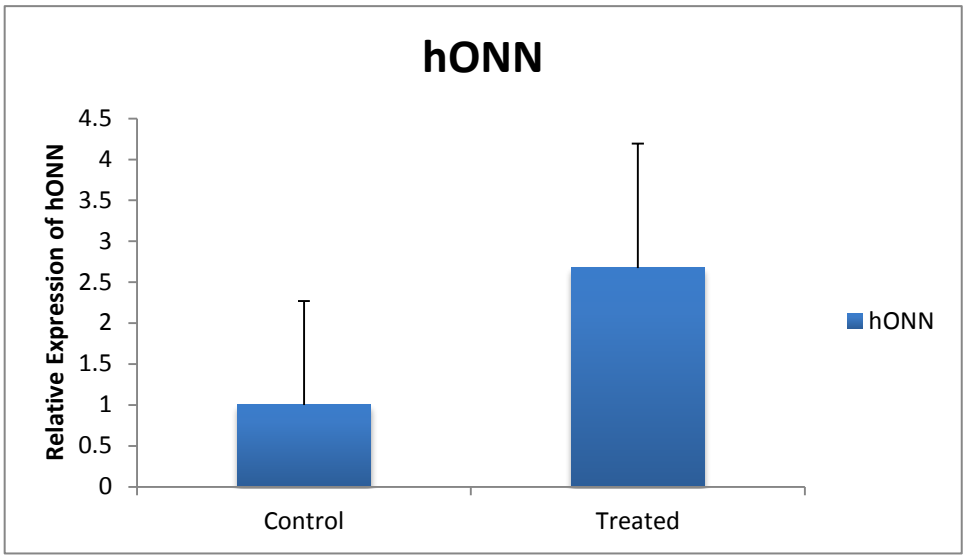

FIGURE 4.24: Relative Expression of hONN to 0.1Hz Dynamic Compression on PDL cells. There is an increase in expression for treated samples. All samples were normalized by the expression of 18s.

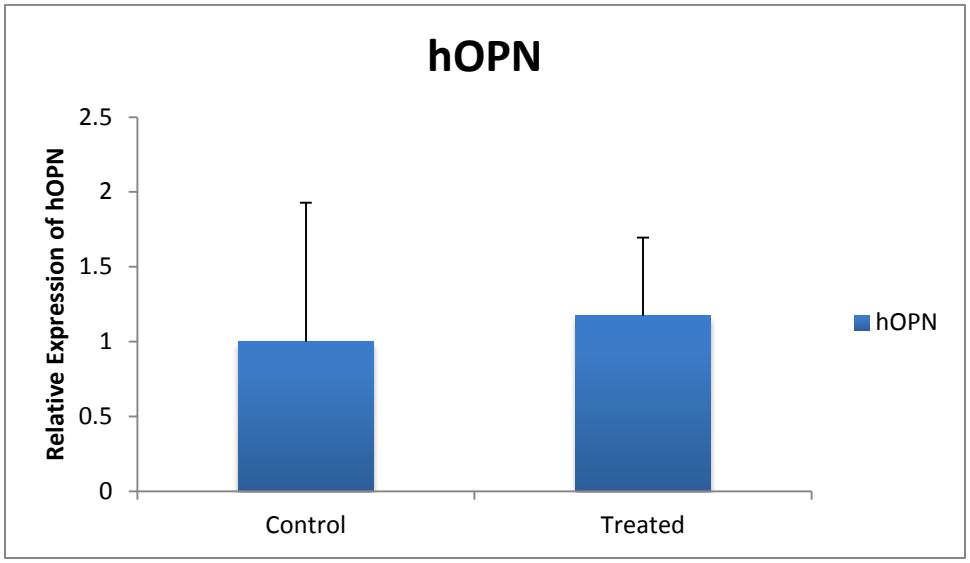

FIGURE 4.25: Relative Expression of hOPN to 0.1Hz Dynamic Compression on PDL cells. There is relatively no change between treated and control samples. All samples were normalized by the expression of 18s.

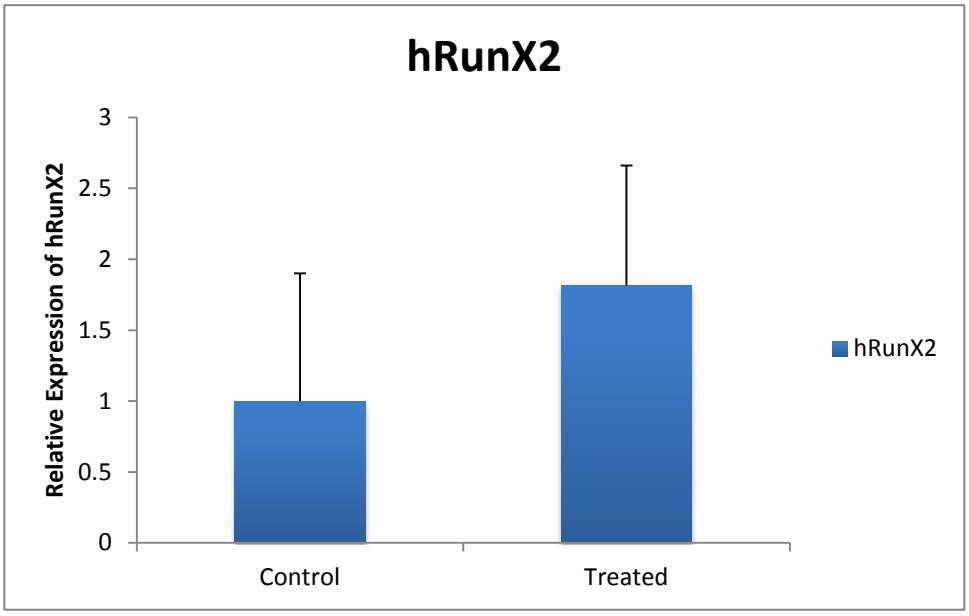

FIGURE 4.26: Relative Expression of hRunX2 to 0.1Hz Dynamic Compression on PDL cells. There is a noticeable increase in expression for treated samples. All samples were normalized by the expression of 18s.

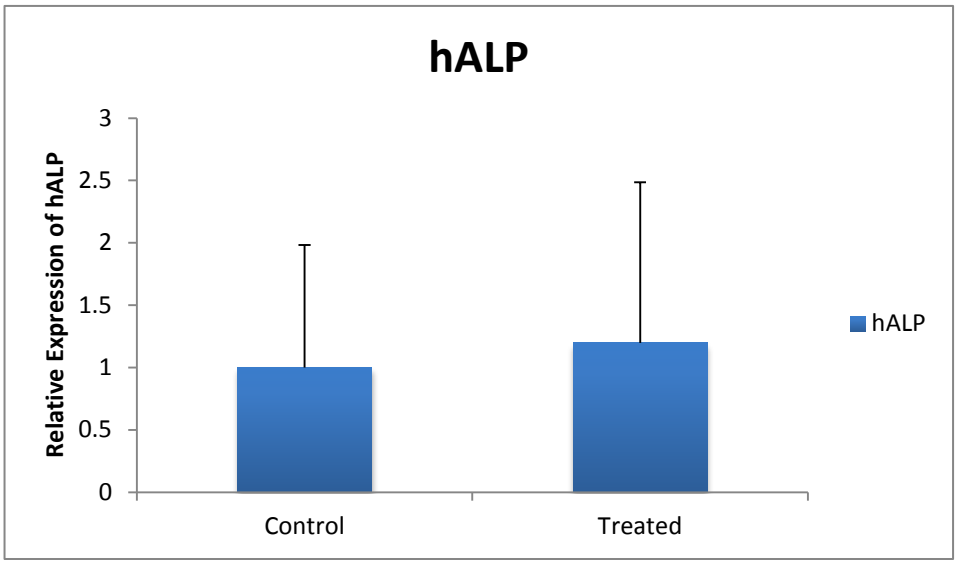

FIGURE 4.27: Relative Expression of hALP to 0.1Hz Dynamic Compression on PDL cells. There is a slight increase in expression for treated samples. All samples were normalized by the expression of 18s.

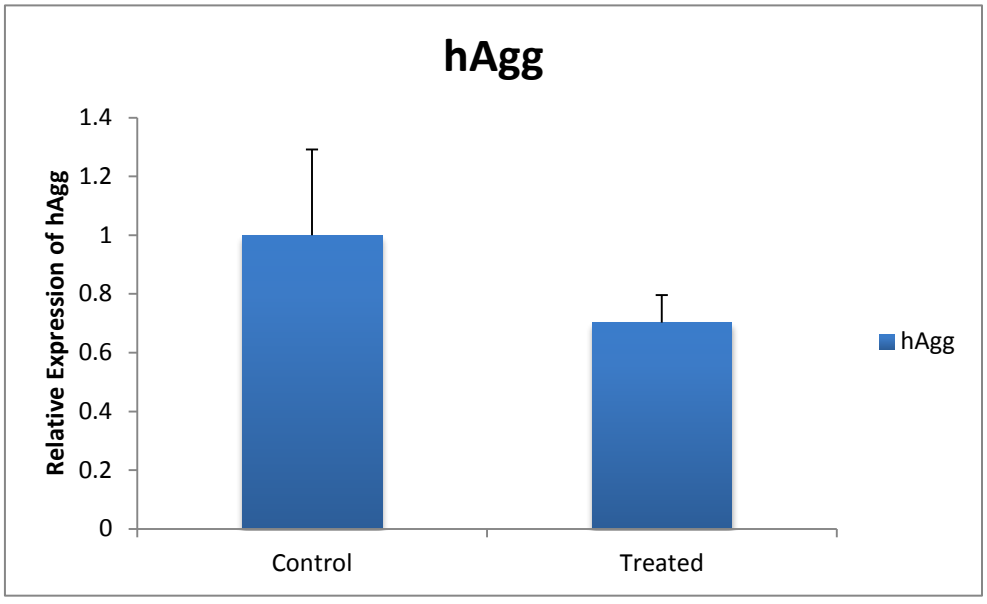

FIGURE 4.28: Relative Expression of hAgg to 0.1Hz Dynamic Compression on PDL cells. There is a minor decrease in expression for treated samples. All samples were normalized by the expression of 18s

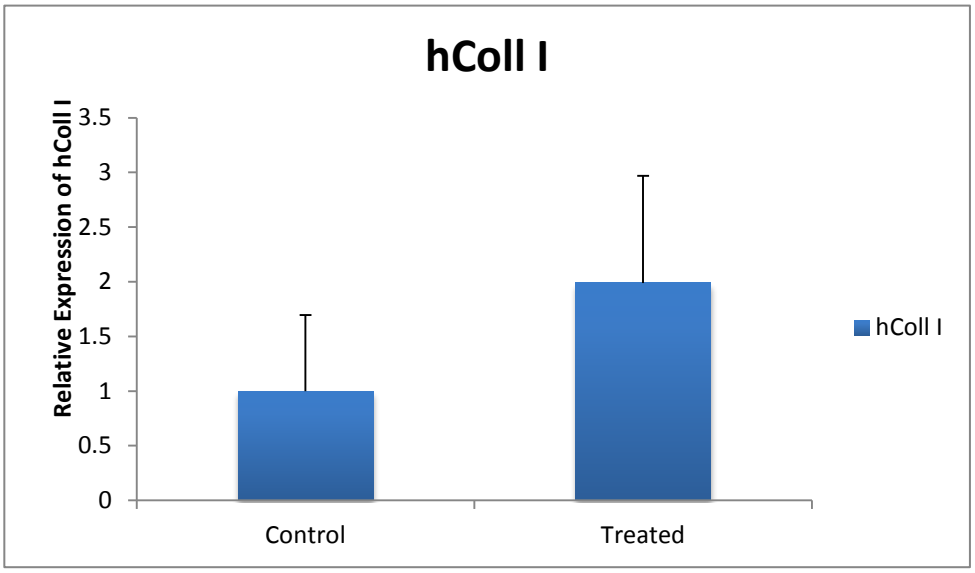

FIGURE 4.29: Relative Expression of Type I Collagen to 0.1Hz Dynamic Compression on PDL cells. There is an increase in expression for treated samples. All samples were normalized by the expression of 18s.

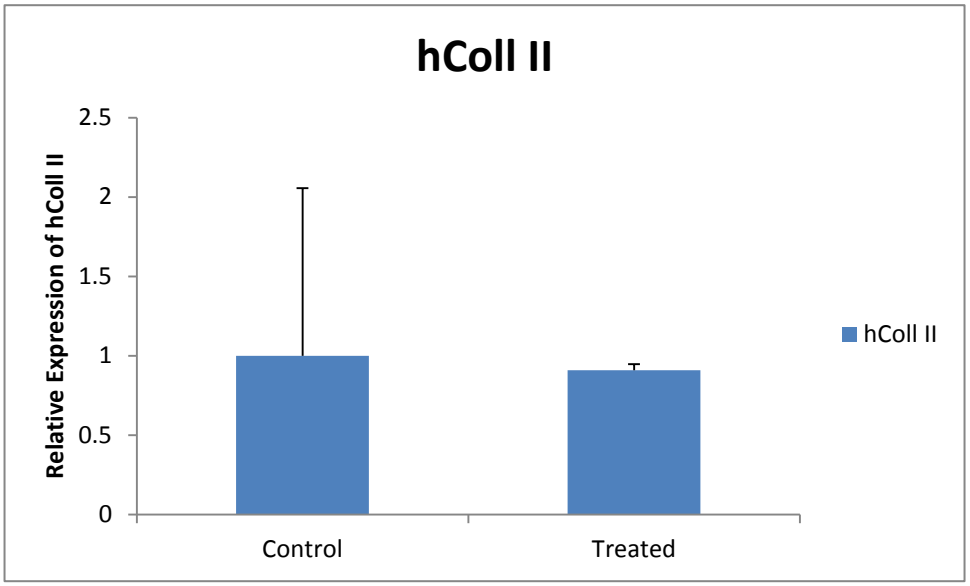

FIGURE 4.30: Relative Expression of Type II Collagen to 0.1Hz Dynamic Compression on PDL cells. All samples were normalized by the expression of 18s.

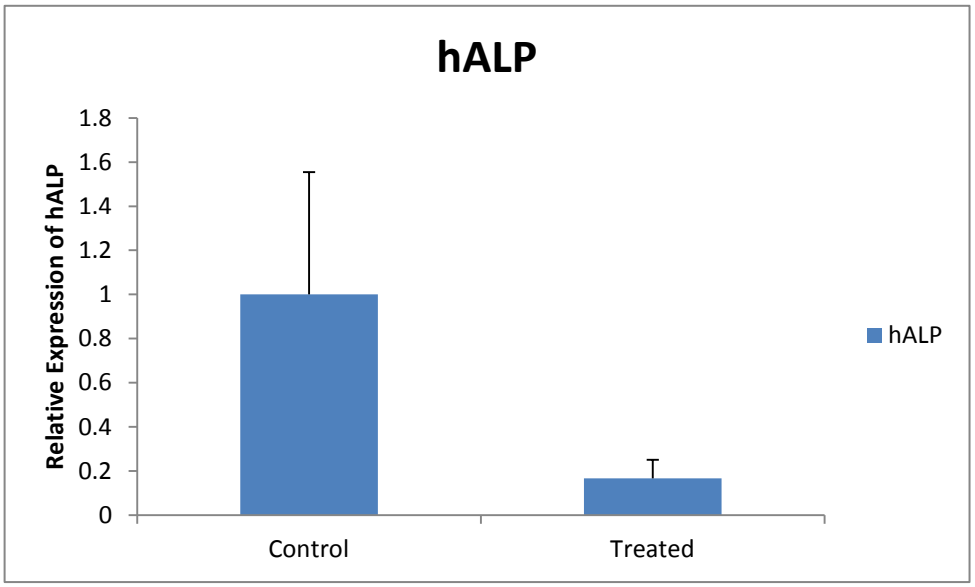

FIGURE 4.31: Relative Expression of hALP to Static Compression on Dental Pulp. There is considerable down regulation of the ALP marker when samples are subjected to static compression. All samples were normalized by the expression of 18s.

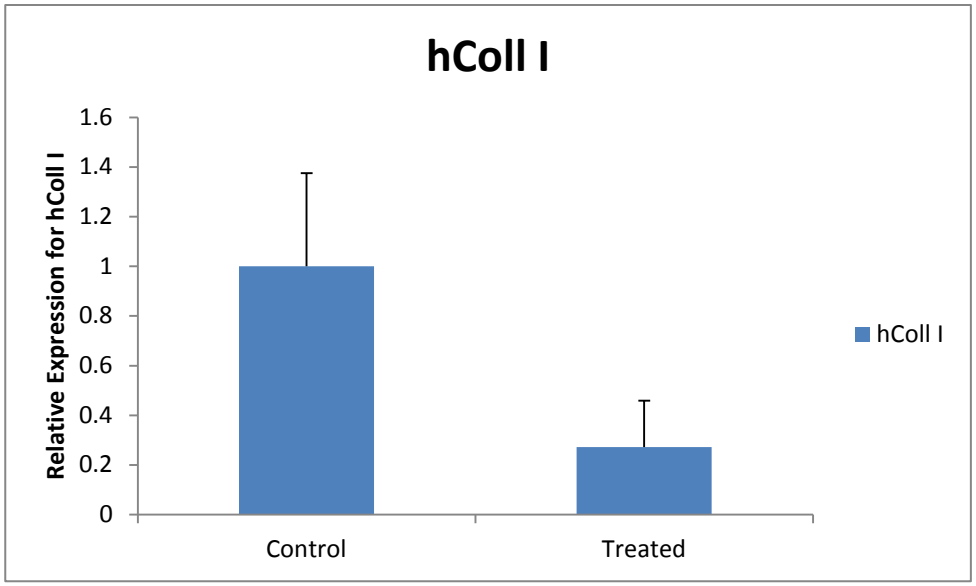

FIGURE 4.32: Relative Expression of Type I Collagen to Static Compression on Dental Pulp. There is a considerable amount of down regulation associated with static compression for the type I collagen marker. All samples were normalized by the expression of 18s.

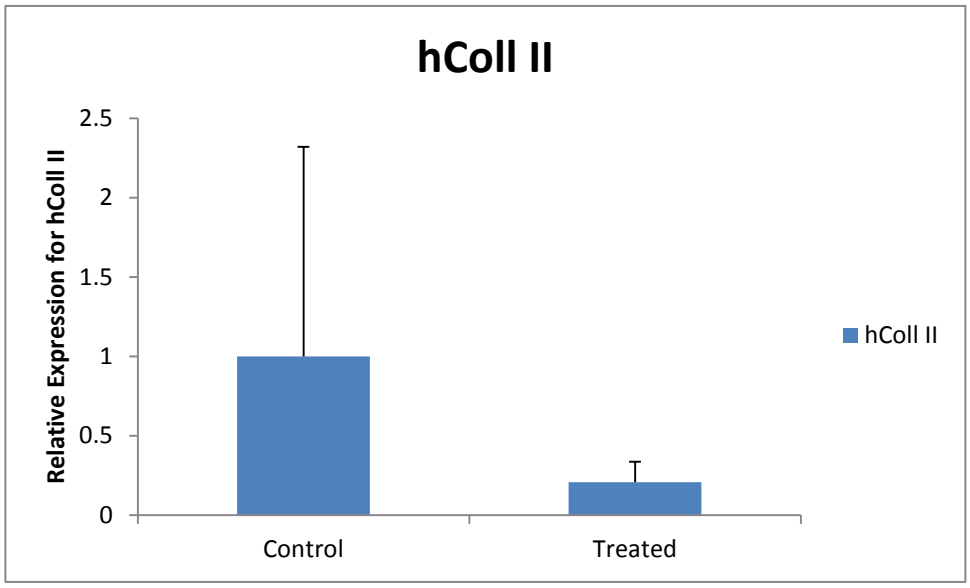

FIGURE 4.33: Relative Expression of Type II Collagen to Static Compression of Dental Pulp. All samples were normalized by the expression of 18s.

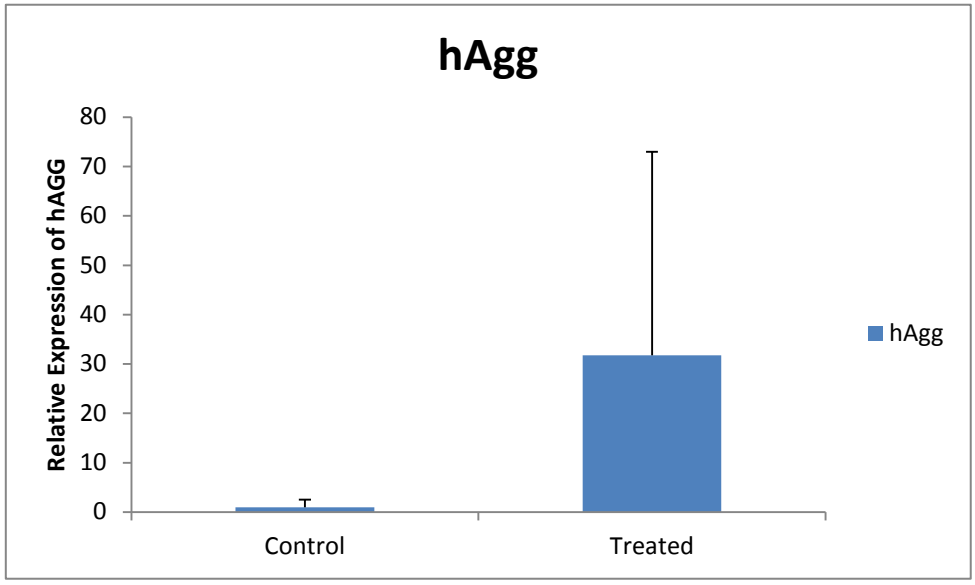

FIGURE 4.34: Relative Expression of hAgg to Static Compression on Dental Pulp. There is a large variation between the treated and control samples indicating some differentiation potential. All samples were normalized by the expression of 18s.

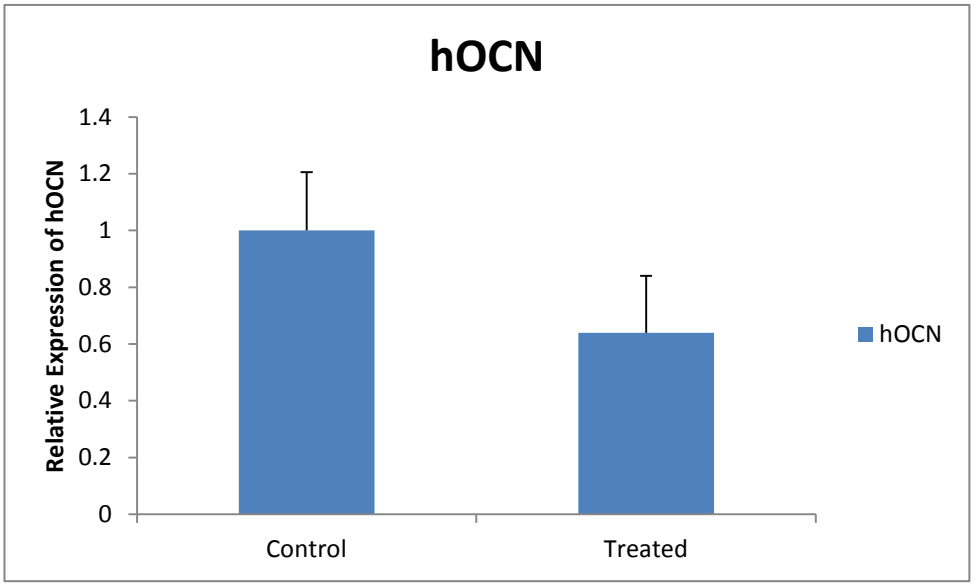

FIGURE 4.35: Relative Expression of hOCN to Static Compression on Dental Pulp. Down regulation is present for the OCN marker when samples are subjected to static compression. All samples were normalized by the expression of 18s.

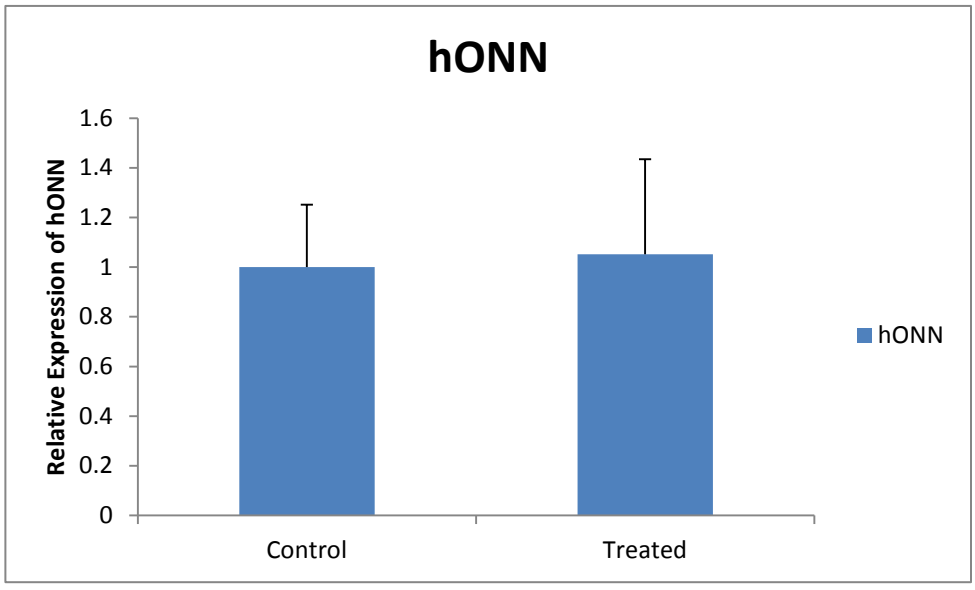

FIGURE 4.36: Relative Expression of hONN to Static Compression on Dental Pulp. There is no difference between the control and treated samples. All samples were normalized by the expression of 18s.

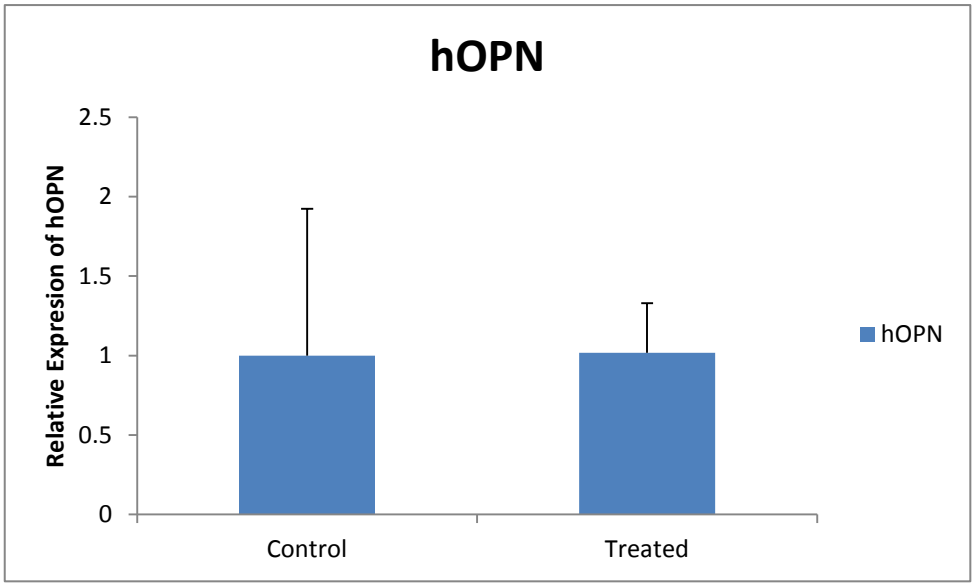

FIGURE 4.37: Relative Expression of hOPN to Static Compression on Dental Pulp. There is no difference between treated and control samples in regards to the expression of OPN. All samples were normalized by the expression of 18s.

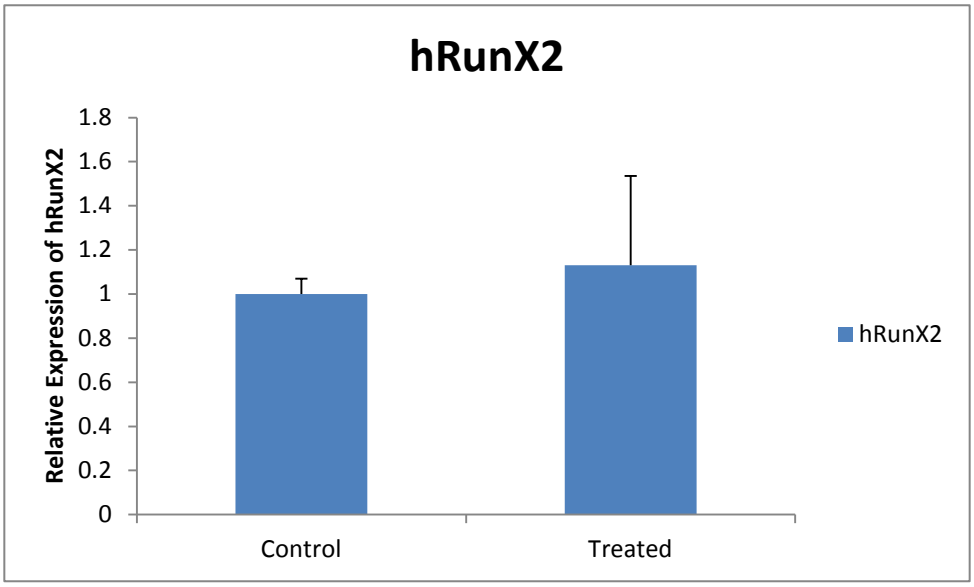

FIGURE 4.38: Relative Expression of hRunX2 to Static Compression on Dental Pulp. Only a miniscule difference was observed for the expression of RunX2 with regards to static compression. All samples were normalized by the expression of 18s.

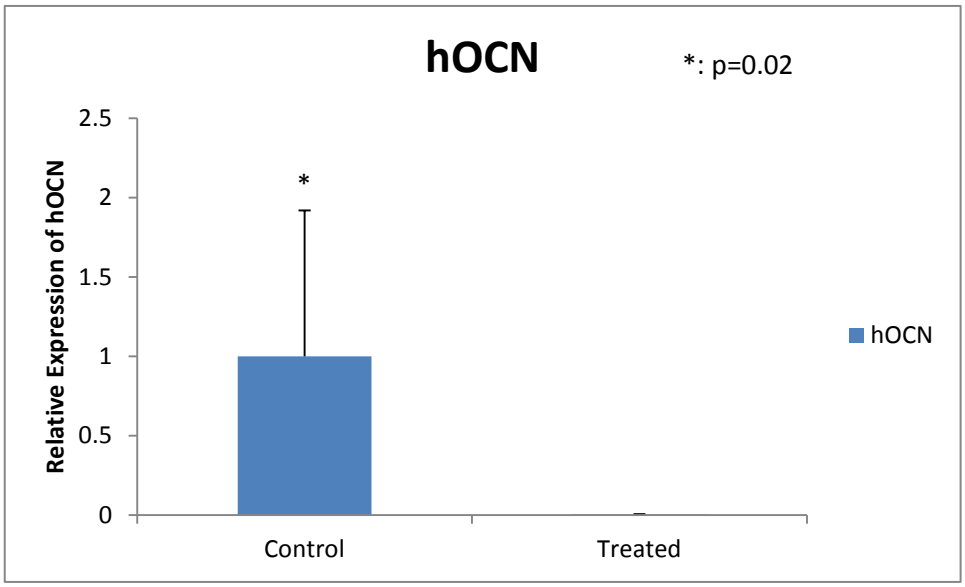

FIGURE 4.39: Relative Expression of hOCN to Static Compression on PDL cells. There is a significant decrease in expression between the control and treated groups (p=0.02). All samples were normalized by the expression of 18s.

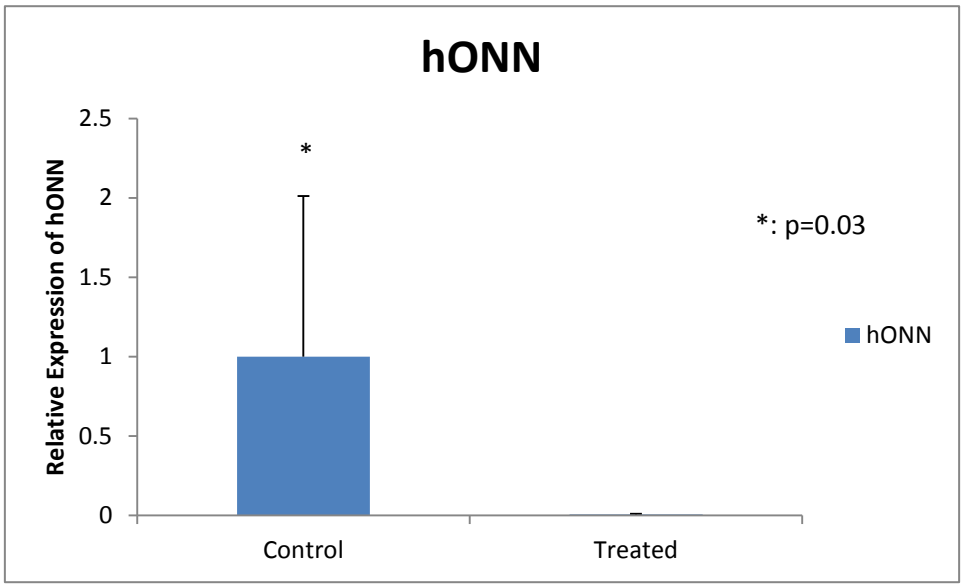

FIGURE 4.40: Relative Expression of hONN to Static Compression on PDL cells. There is a significant decrease in expression between the control and treated groups (p=0.03). All samples were normalized by the expression of 18s.

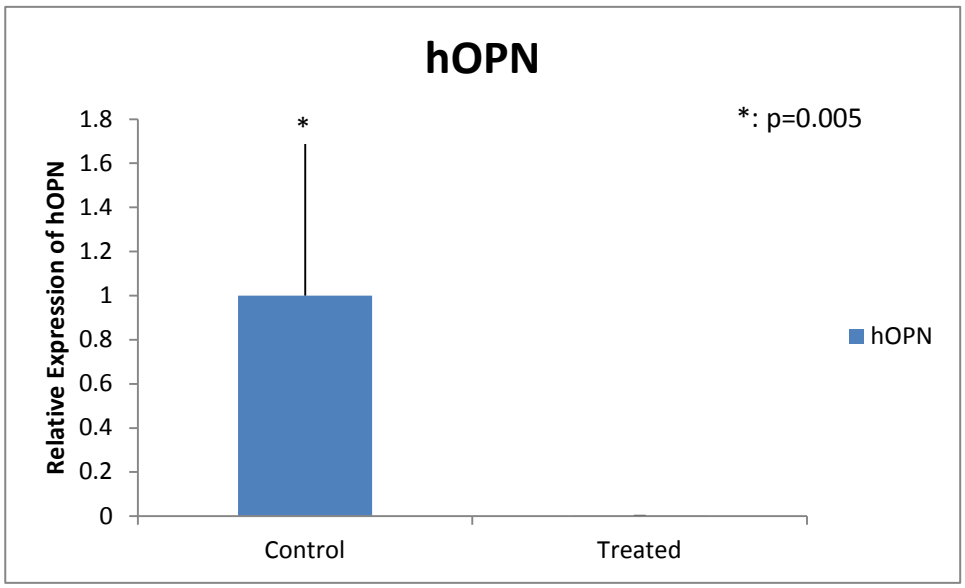

FIGURE 4.41: Relative Expression of hOPN to Static Compression on PDL cells. There is a significant decrease in expression for treated samples (p=0.005). All samples were normalized by the expression of 18s.

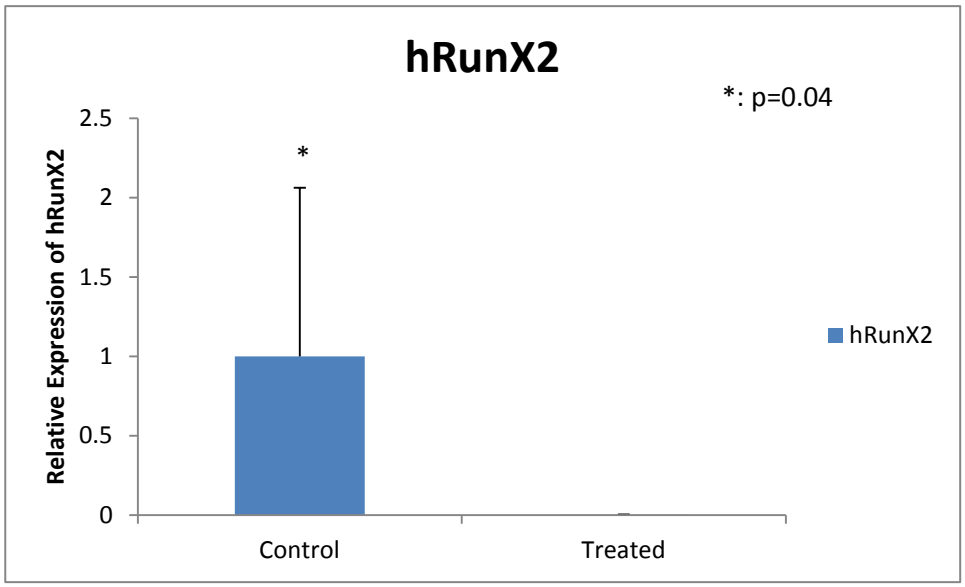

FIGURE 4.42: Relative Expression of hRunX2 to Static Compression on PDL cells. There is a significant decrease in expression for treated samples (p=0.04). All samples were normalized by the expression of 18s.

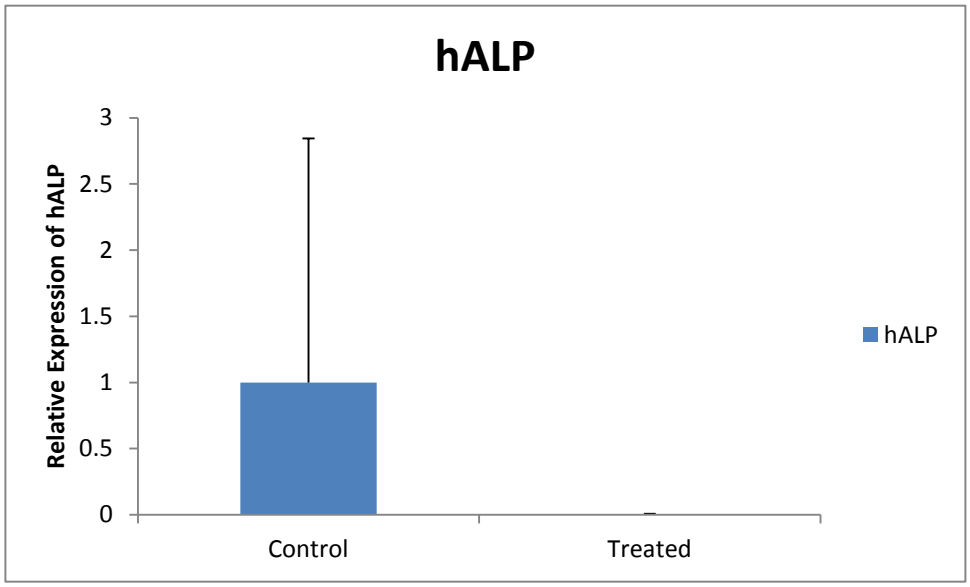

FIGURE 4.43: Relative Expression of hALP to Static Compression on PDL cells. There is down regulation for treated samples under static conditions. All samples were normalized by the expression of 18s.

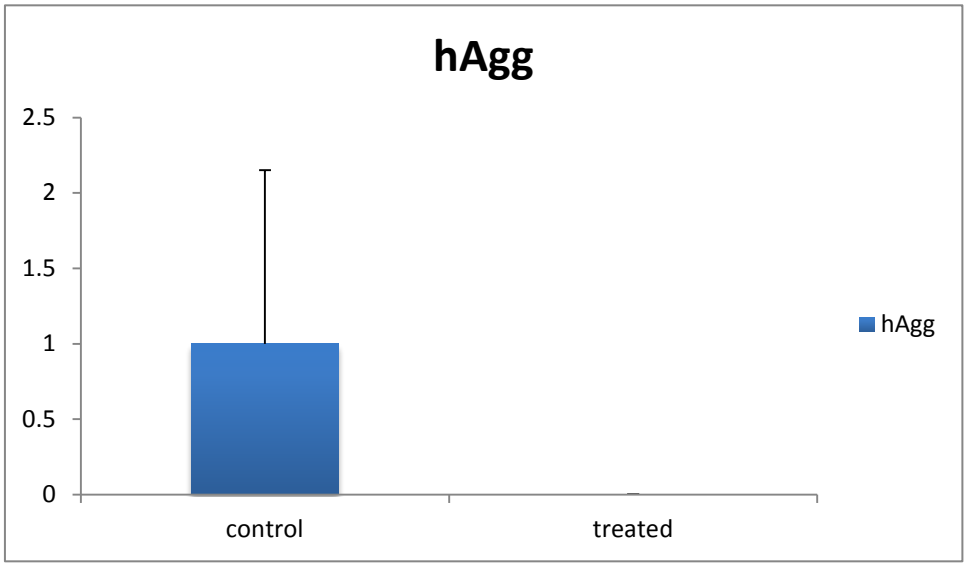

FIGURE 4.44: Relative Expression of hAgg to Static Compression on PDL cells. There is down regulation present due to the mechanical loading. All samples were normalized by the expression of 18s.

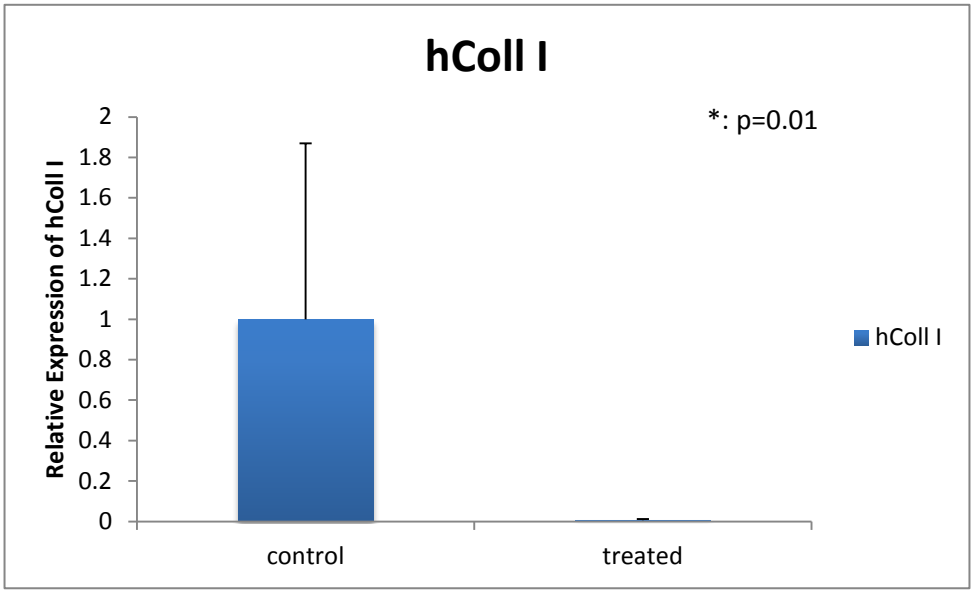

FIGURE 4.45: Relative Expression of Type I Collagen to Static Compression on PDL cells. All samples were normalized by the expression of 18s.

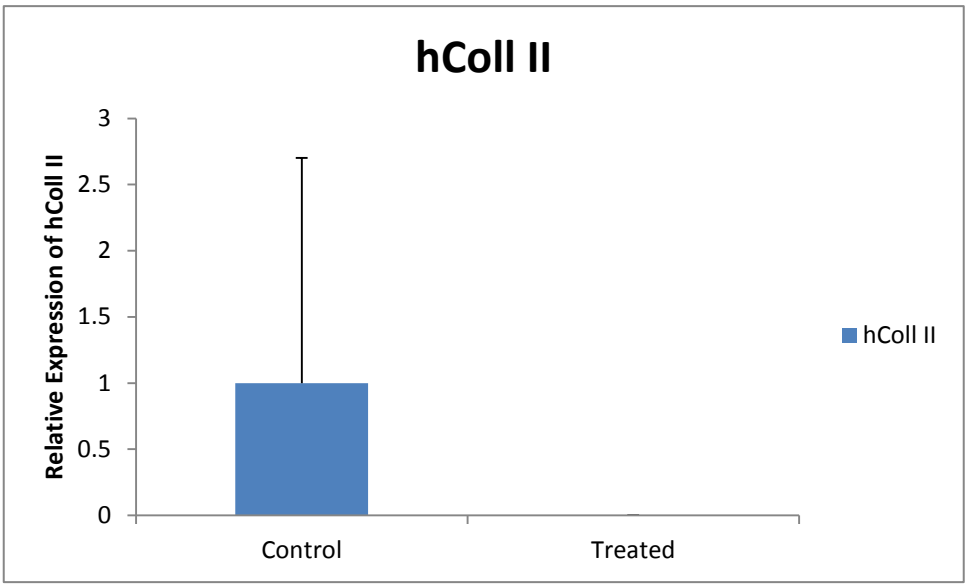

FIGURE 4.46: Relative Expression of Type II Collagen to Static Compression of PDL cells. All samples were normalized by the expression of 18s

CHAPTER 5: DISCUSSION

In this study it was shown that mechanical loading has the potential to affect the differentiation of cells derived from the PDL and Dental Pulp. In the mouth, loading conditions are constantly present and it is these variables that promote the synthesis and release of remodeling factors to maintain a healthy tissue. By understanding how the cell lines respond to either everyday or orthodontic mediated conditions, the cell lines can be further investigated for the use in regenerative tissue engineering therapies. This study utilized the stem cell properties of the PDL and Dental Pulp to examine how different compression factors can affect the mechanobiological response.

5.1 Effects of Dynamic Loading on PDL and Dental Pulp

Cells from the PDL and Dental Pulp were subjected to two different dynamic loading conditions: 1Hz and 0.1Hz. Dental pulp cells showed the highest response of up regulation in regards to 1Hz dynamic compression. Conversely, there was an opposite reaction for the cells when exposed to 0.1Hz. The tested genes showed that there was a significant decrease in differentiation when comparing the treated samples to the control. All of the osteogenic genes such as hOCN, hONN, and hOPN were largely down regulated signifying no osteogenic differentiation. This is similar with the tested chondrogenic genes such as Type I Collagen, Type II Collagen and Aggrecan. The PDL on the other hand exhibited consistent up regulation when exposed to both 1Hz and 0.1 Hz dynamic loading. Two particular genes that stood out for both conditions are hONN and hOPN. hONN is a glycoprotein that is released by osteoblasts in the stages of bone formation. It is responsible for regulating mineralization and cell matrix interactions, and

can bind collagen [5]. hOPN is a non-collagenous protein that also plays a significant role in bone remodeling. These results are also seen in a study conducted by Pavasant and Yongchaitrakul in which they concluded that mechanical stimuli influence the hOPN expression of PDL cells [26].

With the exception of dental pulp samples test at 0.1Hz dynamic loading, no other samples resulted in significant chondrogenic up regulation. However, conclusions from previous studies conducted under similar conditions showed the promise of chondrogenesis. Huang et al., conducted an experiment in which human MSC's in fibrin gel scaffolds were placed under dynamic loading. These results suggested a significant chondrogenic response [27]. Additionally Huang et al., conducted a loading study on rabbit bone-marrow derived MSC's, which resulted in similar chondrogenic findings [10]. The main difference between those findings and the ones conducted in this study is the cell type. The PDL has been shown to contain several of the self-renewal and multipotency characteristics of MSC's however they are not truly classified under the category [20]. Cells from the PDL and dental pulp tissue are highly differentiated and are involved mainly in the formation and remodeling of bone. This may suggest the reason for the lack of chondrogenic ability due to dynamic loading.

One explanation for the difference between 1Hz loading and 0.1Hz is that the pulp resides within the tooth and is largely a neural and vascular network. It is not susceptible to the same amounts of forces as the PDL. Because of this, the PDL cells may be programmed to differentiate in the presence of a higher mechanical loading such as forces on the tooth that promote bone resorption and formation. Additionally the 0.1Hz frequency is very minimal and does not have a relevant clinical application. This

frequency is unlike that seen in the mouth and this variable was tested as a medium between dynamic movement and static placement.

5.2 Effects of Static Loading on PDL and Dental Pulp

Static loading conditions did not result in a positive effect on either cell type. There were no signs of up regulation for any genes tested. In fact for the PDL samples all genes were down regulated. It can be seen that there is relatively no expression of any genes. While the Dental Pulp cells did not have any positive reactions, they were not as severe as the PDL. For the Dental Pulp there was little to no change in expression between the control and treated samples. In a study conducted by Mathes et al., it was shown that static conditions might not be the most ideal way to investigate the natural interaction of cells. While a collagen sponge in which human gingival fibroblasts were suspended, they were able to conclude that mechanical stimulation is needed in order to transfer the naturally occurring forces to the cells [16]. While these results may seem counterproductive to the study, they may be resultant of the physiological conditions in the tooth. Forces placed on the tooth initiate bone remodeling. When the load is applied, a site of tension and a site of compression are present in order to play a significant role in the formation of new tissue. On the pressure side there is resorption of the alveolar bone, while there is bone formation on the tension side. When a static load is applied it can be equivalent to constant pressure being exerted on the cells, which signal them to begin the resorption processes. This relates to the results of static compression, especially of the PDL, in that there was a down regulation of differentiation for all genes tested. Dental Pulp may not be exposed to these certain forces because they reside within the tooth, which can explain the lack of change between the control and treated samples. The forces

that are encountered by the teeth, whether they are natural or orthodontically promoted, are typically not static forces applied for long periods of time.

CHAPTER 6: CONCLUSION

In summary, this study provided understanding of how cells derived from the PDL and dental pulp respond in the presence of dynamic and static loading conditions. The PDL is constantly exposed to mechanical loading factors that help to support the overall health of the teeth. Dynamic loading proved to have a more profound effect on the overall outcome of differentiation. Cells from the PDL emitted an increased response to the two dynamic loading conditions while dental pulp cells primarily responded to 1Hz dynamic loading. Subsequently static loading did not elicit any positive response to differentiation for either cell type. In fact, the dental pulp cells remained relatively unchanged in the presence of static loading. The PDL samples on the other hand exhibited down regulation; however, this may be explained by the fact that in teeth remodeling bone is resorbed on the side of compression allowing bone formation on the tension side.

6.1 Study Limitations

While gene expression using RT-PCR is a proven way to assess the differentiation of the samples, additional experiments must be performed to reaffirm these conclusions. There is an extensive list of genes that can be used to help verify these findings. Subsequently, it would be helpful to conduct studies to discover whether the tested cells would proliferate in culture following loading, and to evaluate whether mineral deposits such as calcium are present. Such considerations can be investigated in future studies.

6.2 Future Studies

The need for novel tissue regeneration procedures is becoming more prevalent in today's society. In the future it may be helpful to conduct these experiments for a longer duration. By exposing the samples to increased loading times there may be an increased effect for differentiation.

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