The Response of Preosteoblasts to Combined Shear and Thermal Stress for Bone Tissue Engineering

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ABSTRACT

Due to the fact that bone cells are highly responsive to mechanical stimuli, shear stress has been extensively studied for its ability to enhance osteogenic differentiation through mechanotransduction. In addition, thermal stress has also been explored as a conditioning method to stimulate cellular proliferation, differentiation, and cytoprotection through heat shock protein induction. Despite the beneficial effects observed with individual stress on cells, there has been little focus on the potential of a combination of stresses to improve cellular response. Therefore, the aim of this study was to investigate the effect of combined shear and thermal stress on preosteoblasts to stimulate an enhanced osteogenic response. To achieve this, MC3T3-E1 cells were exposed to one of the following protocols for an hour: no stress (control), shear stress at 1 dyne/cm^2 using a parallel plate flow chamber, thermal stress in a 42°C incubator, or combined shear and thermal stress (1 dyne/cm^2 at 42°C). Stress treatments were applied on Day 2, Day 6, and Day 10. To assess the early response of cells to stress treatments, we measured metabolic activity, expression of signaling factors, and HSPs following stress on Day 2. Despite an initial decrease in metabolism, combined stress stimulated a strong response in VEGF (12.49 RFI) COX-2 (12.32 RFI), HSPs (2-4 RFI) and increased PGE accumulation. The long-term cellular response to stress treatments was measured on Day 15 by evaluating the ability of combined stress to stimulate late stage markers of differentiation. Combined stress increased OPN gene and protein expression, yet OCN was minimally affected by stress treatments. However, mineralization was significantly decreased with combined stress. Overall, combined stress was able to stimulate an enhanced effect across a majority of the bone-related markers measured, whereas individual shear stress or thermal stress were limited in their response. This suggests that combined stress can provide the appropriate cues to modify osteoblast differentiation and generate an enhanced osteogenic response.

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Chapter 1: Introduction

1.1 Significance and Motivation

In the US alone, more than 1 million fractures occur annually, costing an estimated $10 billion [1]. Furthermore, 5% of all fractures result in delayed healing due to critical-sized defects that have minimal capacity to heal on their own [2]. For this reason, bone defects remain a significant clinical problem affecting [3]. The clinical need for tissue engineered bone substitutes is evident in the 4 million operations performed annually that require bone grafting [1]. Tissue engineered constructs, which combine three dimensional biomaterials and cells to provide a platform for tissue growth, have great potential to facilitate bone regeneration. The possibility of manufacturing functional bone substitutes is a promising notion that would greatly alleviate the burden associated with current bone grafts, which include autografts, allografts, and synthetic grafts. Although autografts are currently the gold standard in bone grafting, they require multiple surgeries, their extraction leads to donor site morbidity, and they are in limited supply. On the other hand, allografts obtained from cadavers provide an abundant source of bone tissue, yet they pose a risk of disease transmission and infection. Finally, synthetic grafts can be tailor-made with a specific biomaterial and possess unlimited availability, but are missing the necessary components to facilitate osteogenesis. Bone tissue engineering can be an important approach to overcome these limitations. However, despite advances in tissue engineering, there has been limited success in maturation and integration of these engineered bioactive scaffolds *in vivo* to provide efficient bone regeneration on a level similar to autografts. Continued research is necessary to find the appropriate osteoinductive cues that will facilitate bone healing.

1.2 Fluid Flow-induced Shear Stress on bone

The use of external mechanical force, such as shear stress, has been considered one method for producing bone-like constructs. *In vivo*, cells are inherently exposed to shear stresses predicted to range from 8-30 dynes/cm^2 as a result of interstitial fluid flow through lacunar-canalicular spaces during bone mechanical loading [4]. Previous studies have shown that these hydrodynamic shear stresses can direct cell behavior through the activation of different signaling cascades and initiate synthesis of proteins essential for bone development [5-7]. These stresses can also influence cell proliferation, extracellular matrix (ECM) deposition, and scaffold maturation [7]. However, understanding the mechanism of this stimulatory response is key to regulating cellular behavior. When exposed to mechanical forces like shear stress, cells are able to convert that macroscale force into a biochemical response through a process known as mechanotransduction [8].

Although the exact mechanism is not clear, mechanotransduction involves the activation of multiple signaling pathways, that are regulated by early signaling events, such as increases in intracellular calcium, nitric oxide (NO) release, prostaglandin (PGE) secretion, and activation of mitogen activated protein kinases (MAPKs), like extracellular regulated kinase (ERK), p38, and c-jun kinase (JNK). Local signaling factors, such as PGE, cyclooxygenase 2 (COX-2), and vascular endothelial growth factor (VEGF), have been investigated for their role in mediating the transduction of shear stress into meaningful cellular responses. PGE_2 is a lipid-based hormone enzymatically derived from arachidonic acid, while COX-2 is a highly inducible enzyme necessary for the synthesis of PGE_2. Both these factors have been shown to stimulate increased alkaline phosphatase (ALP) activity and bone nodule formation [9]. The upregulation of VEGF due to fluid flow may not only facilitate angiogenesis, but also interact with osteoblasts to promote cell proliferation, differentiation, and enhanced mineralization [10]. Important regulatory factors, like COX-2, PGE_2, and VEGF, ultimately influence the progression of osteoblastic differentiation by modifying the expression of bone-related proteins. As stem cells differentiate along the osteoblast lineage, they progress through three distinct phases characterized by a decrease in cell proliferation, ECM development and maturation, followed by mineralization. In fact, matrix proteins like type I collagen and non-collagenous proteins like osteocalcin (OCN), osteopontin (OPN), and bone sialoprotein (BSP) are typical indicators of bone development [6, 11-13]. Mineralization is another late stage marker that is often used as a measure of osteoblast differentiation and ECM maturation [14].

1.3 Thermal Stress and the Stress Response

Although bone cells have been extensively studied for their high sensitivity to mechanical stimulation, hyperthermic responsiveness can also play an important role in modifying osteogenic differentiation. This sensitivity has been demonstrated in previous studies by increased HSP expression, ALP activity, calcium deposition, OPN, and bone morphogenetic protein 2 (BMP-2) expression following mild thermal stress of MSCs [15, 16]. In addition, our prior work has shown that thermal stress has potential to induce osteogenic effects [17]. Mild thermal stress (40 – 42°C) has been shown to stimulate a beneficial response in a variety of tissues, including muscle [18], cartilage [19] and bone [20]. Improved metabolic responses and cell proliferation have also been reported with heat stress [19].

Some of the beneficial effects observed with thermal stress are associated with the stress response, which is a rapid induction of heat shock proteins (HSP) following exposure to environmental stress, such as hyperthermia, hypoxia, ischemia, and free radical synthesis [21]. HSPs function as molecular chaperones that refold, repair or remove denatured proteins following stress. Increased HSP expression has been associated with anti-apoptotic properties, increased cell proliferation and differentiation [22]. HSPs are classified according to molecular

weight and are involved in normal cellular functions. HSP47 is a procollagen-binding protein localized in the endoplasmic reticulum and involved in the biosynthesis of type I collagen, a major bone extracellular matrix protein [23]. HSP60 is associated with mitochondrial transport, while HSP90 and 70 are highly conserved proteins. HSP70 is highly inducible under thermal stress and has been extensively studied for its cytoprotective roles. The upregulation of these proteins is often times dependent on the magnitude and duration of stress [24]. The ability to manipulate cellular response by regulating HSP expression can be a potential strategy for influencing bone development.

Although ample research has been conducted on the effect of individual stress on cells, there is little focus on cellular response to a combination of stresses. The application of multiple stimuli on cells may have a synergistic effect on protein upregulation through the activation and integration of several signaling pathways. In particular, since shear stress and thermal stress each stimulate different biochemical responses, a combination of these two stresses may initiate a cellular response that leads to enhanced osteogenesis. Therefore, we aim to determine the osteogenic-inducing effects of combined thermal and shear stress on pre-osteoblastic cells through *in vitro* analysis of bone-related protein expression and secretion.

Chapter 2: Effect of Combined Shear and Thermal Stress on Bone Development

2.1 Introduction

The inability of critical sized bone defects to adequately heal remains a significant problem in bone regeneration [2]. The substantial loss of tissue and signaling factors within the defect greatly impair bone's intrinsic ability to regenerate. Recent tissue engineering approaches have focused on overcoming these limitations through stem cell implantation, gene therapy, extracellular matrix (ECM) modifications of scaffolds, exogenous delivery of growth factors, or a combination of these techniques to facilitate enhanced bone development [25, 26]. However, these methods can be difficult to control, costly, pose safety risks, and have undesired side effects.

Alternatively, stress conditioning offers a more controlled, non-pharmacological means of promoting osteogenesis by directing cellular behavior. *In vitro* stress conditioning can mimic physiological stimuli that cells naturally experience and provides dynamic culture conditions that stimulate cells [27]. Sub-lethal stress has been shown to beneficially affect tissue development in a variety of tissues, including blood vessels [28], muscle [29], and bone [30, 31], by initiating cellular differentiation and modifying gene and protein expression [32]. In particular, the regulation of bone growth and repair is heavily influenced by external mechanical stimuli, where the balance between bone resorption and bone deposition is dependent on the response of cells to external cues. The ability of bone cells to recognize and adapt to stimuli make stress conditioning a promising technique for modifying cellular behavior to promote bone development.

Hydrodynamic shear stress has been extensively investigated as a stress conditioning method since bone cells are highly sensitive to mechanical stimuli and naturally experience these stresses within their physiological environment. As the bone matrix deforms, interstitial fluid is forced through lacunar-canalicular channels and bone cells experience shear stresses theoretically estimated to range from 8 - 30 dynes/cm^2 [4]. Shear stress is able to influence cell activity through a mechanism known as mechanotransduction, where the stimuli is transmitted through a series of biochemical signaling pathways into a cellular response. Previous studies show shear flow affects proliferation [7, 33] and stimulates osteogenic differentiation resulting in maturation of the ECM [14, 34]. Using either steady or dynamic fluid flow, studies have observed a significant increase in bone proteins, such as osteocalcin (OCN), collagen Type I, bone sialoprotein (BSP), and osteopontin (OPN), which are essential components of the bone extracellular matrix and important markers of osteogenic differentiation [35, 36]. An improved osteogenic response due

to shear flow was also shown in a study where Fassina et al observed enhanced calcium deposition and mineralization following shear stress [37]. Similarly, Sikavistas et al demonstrated that increased mineralization is shear stress dependent by isolating the contributory effects of flow rate and nutrient transport [38]. Although the cellular response to shear flow can be complex and the exact mechanism is still unclear, the upregulation of secondary messengers like cyclooxygenase (COX-2) [39, 40], prostaglandins (PGE) [39, 41], and nitric oxide (NO) [26, 42-45], are among the most immediate responses of shear flow that may have a huge influence on downstream changes in cell behavior, gene expression, and bone matrix protein production. In addition, shear flow induced expression of vascular endothelial growth factor (VEGF) has been investigated due to its importance as a key regulator of bone growth and potential to initiate angiogenesis [46].

Thermal stress has also been investigated as a stress conditioning method for its potential to modify cell activity. Although long periods of hyperthermic exposure at extreme temperatures (>45°C) cause apoptosis, the application of mild heat stress has shown positive outcomes. Previous studies have demonstrated that thermal stress ranging from 41°C to 45°C can have beneficial effects in a variety of tissues and pathological conditions. *In vivo* studies applying local heat treatments to bones have shown that hyperthermia can facilitate the healing process and provide therapeutic effects for musculoskeletal diseases [47, 48]. In one study, mild thermal stress of 42°C for 30 minutes was shown to make myocardial cells more tolerable to subsequent periods of ischemia [49]. These cytoprotective effects can be attributed to the rapid induction of heat shock proteins (HSP), which act as molecular chaperones to protect cells after stress. In addition to cytoprotection, thermal stress can initiate a mitogenic response [16] and promote synthesis of proteins important to bone tissue development. For example, Kirkpatrick *et al* observed increased angiogenesis when thermally stressed bone cells were co-cultured with endothelial cells [50], while Ye et al observed enhanced osteogenesis when conditioned media from heat stress cells was added to bone marrow stromal cells (BMSCs) [51]. Although the mechanism is unknown, the osteogenic response of cells to thermal stress is demonstrated across multiple studies. Our own group has shown that thermal stress at 44°C for short durations (<10 min) can induce HSP expression and important bone matrix proteins like OCN and OPN [17]. Similarly, Wang et al observed that weekly exposure at 41°C for 1 hour significantly increased bone-related proteins, such as alkaline phosphatase (ALP) activity, bone morphogenetic protein 2 (BMP-2), OPN, and calcium deposition [15].

Despite the beneficial effects observed with individual shear stress and thermal stress treatments, few studies have investigated what effect the combination of these stresses could have on increasing the osteogenic potential of cells and promoting enhanced bone growth. The influence of both shear flow in combination with thermal stress could produce synergistic effects

through the integration and activation of additional biochemical signaling pathways that enhance the bone regeneration process. Therefore, the objective of this study was to investigate the effects of combined shear and thermal stress on preosteblasts and evaluate the short- and long-term cellular response during osteogenic differentiation. The cellular response to combined stress was evaluated for changes in metabolic activity, HSP expression, accumulation of bone matrix proteins, and mineralization. The potential of this combined shear and thermal stress regime to integrate the fundamental mechanotransduction effects of shear flow and the beneficial aspects of the thermal stress response may provide an enhanced method of stress conditioning. This study will be useful in tissue engineering by developing a dynamic culture protocol that will expose cells to the appropriate stimuli to increase production of proteins and factors associated with bone growth. Based on the success of individual stress treatments, combined shear and thermal stress conditioning may have the potential to accelerate bone development by stimulating cell differentiation, inducing expression of essential bone-related proteins, and promoting maturation of bone ECM.

2.2 Methods

2.2.1 Substrate Preparation

The substrates used for experimental studies were glass slides coated with proteins, such as fibronectin and vitronectin, to facilitate cell adhesion. Briefly, glass slides (35 x 75 mm) (Fisher Scientific) were sterilized by soaking in 70% ethanol overnight. Prior to cell seeding, slides were washed with sterile deionized water and placed in 100 mm polystyrene petri dishes (Fisher Scientific) to air dry. Then, 1 ml of media supplemented with 10% fetal bovine serum (FBS, Corning) was placed on top of slides for 1 hour at room temperature to allow proteins in the media to adhere to the slides. The media was removed and slides were washed with phosphate buffered saline (PBS, Life Technologies) and stored in PBS until use.

2.2.2 Cell Culture

A mouse preosteoblast cell line (MC3T3-E1, subclone 4, ATCC) was cultured in Minimum Essential Medium (α-MEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS, Corning), and 1% penicillin/streptomycin (Life Technologies). Once cells reached 80% confluence, they were rinsed with PBS, detached using 0.25% Trypsin-EDTA (Life Technologies), and a cell suspension of 2.5×10^5 cells/ml was made. Then, 2 ml of the cell suspension was seeded onto protein-coated glass slides and allowed to attach overnight in a 37°C incubator. Day 0 is denoted as the day cells were seeded onto slides. The next day, 6 ml of osteogenic media (complete growth media supplemented with β-glycerol phosphate [4mM] and ascorbic acid [50ug/ml]) was added to stimulate osteoblast differentiation. Cells were cultured in a humidified incubator (5% CO_2, 95% air) at 37°C until application of stress treatment.

2.2.3 Stress Limit Studies

A preliminary study to determine an appropriate stress treatment protocol was performed, in which the cellular response to a range of conditions for shear stress and thermal stress was evaluated. The duration and intensity for each stress was varied to determine the limit for stress treatments. For shear stress, the time of treatment was adjusted (1 hr, 3 hr or 5 hr) for a constant shear stress of 1 dyne/cm^2. The limits of thermal stress were investigated by exposing cells to temperatures of 42, 43.5 or 45°C for 1 hr. Then the duration of thermal stress was varied at 42°C for 1 hr, 3hr, or 5hr. Metabolic activity normalized to cell number as well as cell viability (displayed as the percentage of cells normalized to the control) were used to assess cellular response.

2.2.4 Stress Conditioning Protocol

On Day 2 of culture, cells were treated with one of the following protocols: control, thermal stress, shear stress, or combined shear and thermal stress. Control samples experienced no stress, but the media was changed at the same time as stressed samples. Thermal stress was applied by adding media preheated at 42°C to cells placed in a humidified incubator (5% CO_2, 95% air) set to 42°C. For shear stress, cells were exposed to continuous shear flow using a parallel flow plate chamber (PPFC) (Figure 2.1a). PPFCs have been extensively studied and frequently used in *in vitro* studies to create well-characterized shear flow across cells [32, 35, 42]. In this study, the glass slide with the cell monolayer was placed within a PPFC and connected to a media reservoir filled with 60ml of media. A gear pump (BVP-Z, ISMATEC) was used to flow media across the cell monolayer at a constant flowrate of 60ml/min (τ = 1 dynes/cm^2). To apply combined shear and thermal stress, the PPFC system was placed in an incubator preheated to 42°C where cells were exposed to simultaneous shear and thermal stress, as described previously. The setup of the combined shear and thermal stress system is shown in Figure 2.1b. To maintain consistency across experimental groups and to minimize any effects from the PPFC device itself, control and thermal samples were also briefly placed within the PPFC, but no shear flow was applied. During stress experiments, a specialized α-MEM containing Glutamax® (Gibco, Invitrogen), a more stable version of L-glutamine, was used to limit compound degradation and prevent accumulation of cytotoxic ammonia that can occur at hyperthermic temperatures [52, 53]. All stress treatments were applied for 1 hour, then fresh osteogenic media was added, and cells recovered in a 37°C incubator (5% CO_2, 95% air) until analysis or subsequent stress application. For all groups, the media was replaced every 3-4 days.

2.2.5 Shear Stress (Parallel Plate Flow Chamber)

The parallel-plate flow chamber, PPFC, used to exert shear stress on cell layers consisted of a polycarbonate base, a 0.06in thick silicone gasket (Special Manufacturing Inc), a 35 × 75 mm glass slide containing a cell monolayer, and a metal top (Figure 2.1a). Screws were used to hold the device together. Due to its ability to produce well-controlled, homogenous fluid flow, the PPFC

has been widely used in various applications to study the effect of shear stress on bone cells [5, 35, 54], endothelial cells [55], and various other cell types [32]. To calculate the shear stress, τ, on the cells the following equation was developed by Frangos et al [26].

$$\tau = \frac{6\mu Q}{bh^2} \tag{1}$$

where Q is the flow rate (Q = 60ml/min), μ is the viscosity of culture medium (μ = 0.0089 g/cm-s), the height (h = 0.14 cm), and width (b = 2.5 cm) of the PPFC, which produced a shear stress of 1 dynes/cm^2.

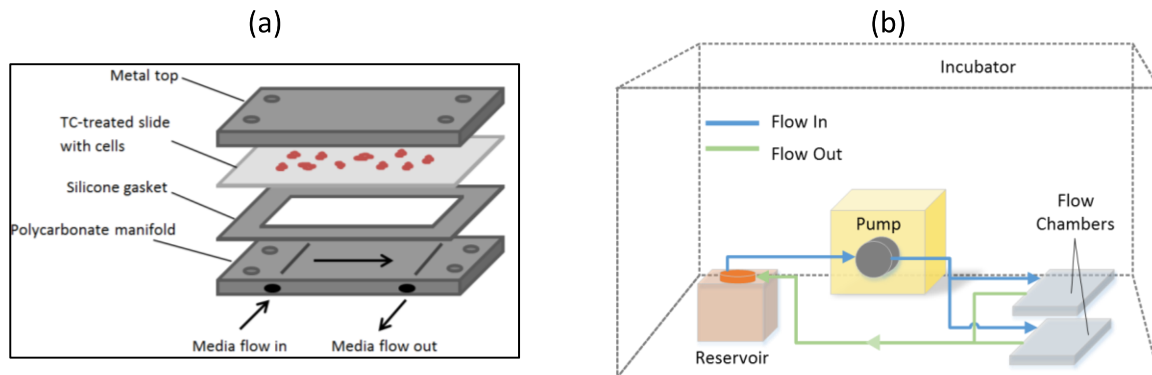

Figure 2.1: (a) Schematic of the Parallel Plate Flow Chamber and (b) the setup of the PPFC system, consisting of a media reservoir, a pump, and two PPFCs inside of an incubator. During experiments, the incubator was set to 37°C for shear stress and set to 42°C for combined shear and thermal stress.

2.2.6 Repeated Stress Treatments

To determine whether repeated stress treatments had an enhanced effect on late stage markers of differentiation, cells were exposed to stress for 1 hr on Day 2, 6, and 10 in the same manner as described previously for single stress treatments. Then, cells were cultured statically until Day 15, where cells were analyzed for calcium deposition and OPN/OCN gene expression and protein secretions in the media. Figure 2.2 illustrates when stress treatments were applied and the type of analysis performed to evaluate cellular response.

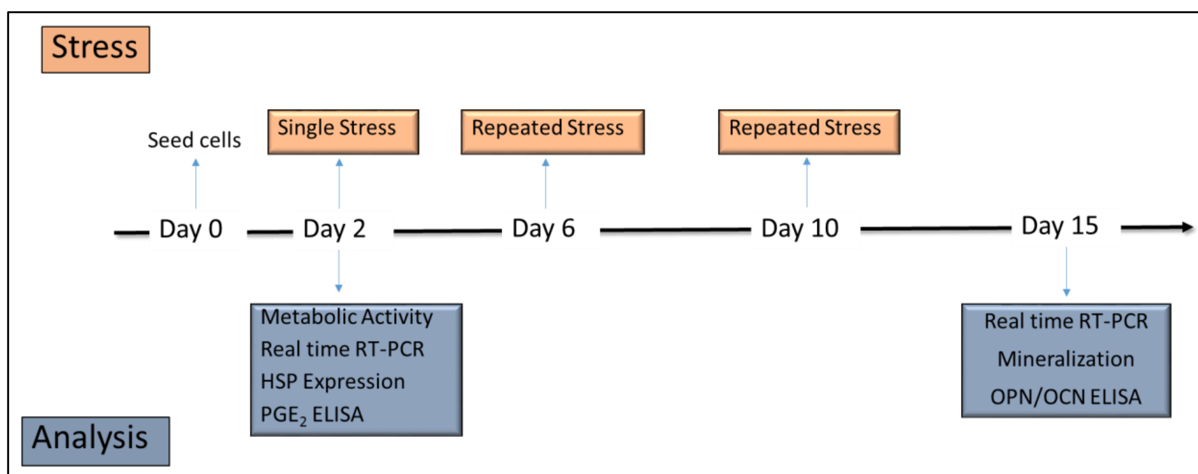

Figure 2.2: Timeline of stress treatments and analysis of cellular response. For single stress treatments, cells were exposed to stress on Day 2, then cultured statically until analysis. For repeated stress treatments, cells were exposed to stress on Day 2, Day 6, and Day 10, then cultured statically until analysis on Day 15.

2.2.7 Analysis of Metabolic Activity

The influence of stress on cellular metabolic activity was determined using an alamarBlue® (Invitrogen) assay, which measures the percentage of cellular conversion of nonfluorescent alamarBlue® to a red, fluorescent product. Twenty-four hours following stress, 10% v/v alamarBlue® reagent in osteogenic media was added to the cells. After a 2 hour incubation at 37°C, 100 µl samples of media were transferred to a 96-well plate and the optical density was measured at 570 nm and 600 nm using a spectrophotometer (SpectraMax M2e, Molecular Devices). A greater degree of alamarBlue® conversion corresponds to increased metabolic activity of the cells.

2.2.8 Analysis of Cell Number

To determine the short- and long-term effects of stress on cell density, a PicoGreen assay was used to measure dsDNA concentrations. Following stress treatments, cell lysates were collected on Day 3 (24 hours after stress treatments), Day 6, and Day 9 of culture. For studies used to determine the maximum tolerable stress, cell number was measured using an automated cell counter (Vi-CELL).

2.2.9 Measurement of Heat Shock Proteins (HSP) - Immunoflourescent Staining

The accumulation of heat shock proteins was measured for each stress treatment due to their cytoprotective properties and ability to increase cell proliferation. Immunofluorescent staining was used to measure HSP47, 70, 60, and 90. Briefly, 12 hours after stress, cell monolayers were fixed in HistoChoice MB Fixative (Electron Microscopy Sciences), then permeabilized using 0.5%

Triton X-100 (Sigma Aldrich). Non-specific binding was prevented using a blocking solution of 5% donkey serum (Santa Cruz Biotechnology) in 6% bovine serum albumin (BSA, Fisher Scientific). Then, primary polyclonal antibodies (Santa Cruz Biotechnology) were applied for 1 hr at room temperature followed by multiple washes with PBS, and 1 hr incubation with the secondary antibodies (Life Technologies) to visualize protein expression. Table 2.1 lists the primary and secondary antibodies used. Samples were preserved in ProLong® Diamond Antifade Mountant with DAPI (Molecular Probes, Invitrogen) to counterstain the nuclei blue.

Table 2.1: Primary and secondary antibodies and dilutions used for immunoflourescent staining

Primary Antibody		**Secondary Antibody**	
HSP60 (goat)	1:150	Donkey anti-goat Alexa Flour 488	1:200
HSP70 (goat)	1:100		
HSP47 (rabbit)	1:100	Donkey anti-rabbit Alexa Flour 594	1:50
HSP90 (rabbit)	1:100		

2.2.10 Osteocalcin (OCN) and Osteopontin (OPN) accumulation

To analyze the effect of stress treatments on late stage bone markers of osteoblast phenotype development, osteocalcin and osteopontin concentrations were measured using a "sandwich" enzyme-linked immunoabsorbant assay (ELISA). Conditioned media from cell monolayers was collected on Day 15 and incubated in 96-well microplates coated with antibodies specific to OCN (Biomedical Technologies) or OPN (Quantikine ELISA, R&D Systems). For each experimental group, there were four or six replicates and all samples were performed in duplicate. Optical density was measured at 405 nm with a spectrophotometer and protein concentrations were calculated using a standard curve of known concentrations. Concentrations were normalized to total protein measured with a Pierce BCA Protein Assay Kit (Thermo Scientific).

2.2.11 PGE_2 Accumulation

The concentration of PGE_2 secreted into the media was quantified using a competitive binding, ELISA (R&D Systems). Immediately following stress treatments, cell monolayers were placed in 8 ml of fresh osteogenic media, incubated for an additional 1 hour or 24 hour post stress, then media was collected and stored at -20°C until use. Upon analysis, frozen samples were thawed and particles removed by centrifugation. PGE_2 within samples competitively binds to antibody coated wells and the optical density was read at 450 nm using a spectrophotometer. For each experimental group there were four replicates and all samples were performed in duplicate. PGE_2 concentrations were determined from curves generated using known PGE_2 concentrations.

2.2.12 Gene Expression

Gene expression was evaluated in early and late stages of bone development using real time RT-PCR to determine the effect of stress treatments on heat shock proteins, phenotypic markers of

osteogenic differentiation, and important signaling markers. On Day 2 (six hours following stress treatments) and on Day 15, total RNA was isolated from cells using the RNeasy Plus Mini kit (Qiagen), according to the manufacturer's instructions. Genomic DNA was removed with a DNAse eliminator column to prevent interference with downstream applications. Then, RNA concentrations were measured using the Quant-iT RiboGreen® RNA Assay Kit (Invitrogen, Life Technologies), a highly sensitive assay that quantifies the amount of flourescent reagent bound to nucleic acid in the sample. Then, cDNA was synthesized through reverse transcription of 1ug of RNA using reagents of the Reverse Transcription System (Promega). Finally, real-time RT-PCR was performed in a 7300 Real-Time PCR System (Applied Biosystems) through an amplification reaction that contained cDNA, Taqman® PCR Master Mix (Applied Biosystems), and a specific primer for the target protein (Taqman® Gene Expression Assay, Applied Biosystems). The specific primers used are shown in Table 2.2. In addition, isolated RNA samples were used during PCR as a negative control and to confirm the removal of genomic DNA. Data was analyzed using the comparative threshold cycle ($2^{-\Delta\Delta C_T}$) method described by Livak et al. The relative fold induction (RFI) represents relative changes in gene expression for each specific marker that was normalized to the housekeeping gene GAPDH and the average of the control group. For each experimental group, there were four replicates.

Table 2.2: Primers used for quantitative real-time RT-PCR.

Protein	Size	Assay ID
HSP47	73	Mm01307330_g1
HSP60	138	Mm00849835_g1
HSP70	141	Mm03038954_s1
HSP90	167	Mm00833431_g1
COX-2	73	Mm01307330_g1
Osteocalcin	89	Mm00649782_gH
Osteopontin	114	Mm00436767_m1
Collagen	89	Mm00801666_g1
VEGF	81	Mm01281449_m1
GAPDH	107	Mm99999915_g1

2.2.13 Mineralization

The degree of mineralization of cell cultures was analyzed on Day 15 with an Osteogenesis Quantitation Kit (Millipore) that uses Alizarin Red to stain calcium deposition within the cell layers. Alizarin Red was quantified by extracting the dye from the stained cultures at a low pH using acetic acid, then neutralizing the samples with ammonium hydroxide. The absorbance was measured at 405 nm using a spectrophotometer. A standard curve of known Alizarin Red concentrations was used to determine the unknown concentrations of Alizarin Red in stressed samples.

2.2.14 Statistics

Using JMP Pro 11.0.0 statistical software (SAS Institute Inc), data was statistically analyzed using a one-way ANOVA and Tukey's HSD multiple comparisons test to determine significant differences among experimental groups ($p<0.05$). Data is expressed as mean ± standard deviation (SD).

2.3 Results

The two-fold goal of this study was to (1) determine the effect of combined shear and thermal stress compared to individual stress and (2) evaluate whether repeated applications of stress would enhance the osteogenic response of cells by inducing late-stage markers of differentiation. To achieve this, cells were exposed to no stress (control), shear stress, thermal stress, or combined shear and thermal stress protocols for 1 hr on Day 2 of culture. For repeated stress experiments, stress was applied on Day 2, Day 6, and Day 10, then analyzed on Day 15. These specific days were chosen to periodically stimulate cells to enhance expression of late stage markers of differentiation, while allowing a 4-day recovery period after stress to avoid overstimulation. Following stress treatments, the cellular response was analyzed at specific time points for changes in metabolic activity, HSP expression, signaling factors, and phenotypic expression of osteogenic differentiation.

2.3.1 Stress Limit Studies

An initial study was performed to determine the appropriate stress conditioning protocols for shear and thermal stress. A range of conditions for shear stress and thermal stress were tested as shown in Table 2.3, then metabolic activity normalized to cell number was measured to evaluate cellular response. For shear stress, metabolic activity was enhanced as the duration of shear stress increased, but a significant difference was only observed for 5 hr of shear stress (Figure 2.3a). However, cell viability was greatly reduced after 5 hr of continuous flow due to a significant amount of cells being detached from the substrate. For shorter durations of 1 hr and 3 hr stress, the viability was similar to the control (Figure 2.3b). Contrary to shear stress, metabolic activity decreased in proportion to increased durations of thermal stress (Figure 2.4a). In addition, cell death was induced with longer periods of thermal stress, which was observed by a significant decrease in cell viability at 3 hours and 5 hours of thermal stress compared to 1 hour and the control (Figure 2.4b). The variation in thermal stress temperature from 42°C to 45°C showed a temperature-dependent reduction in metabolic activity (Figure 2.4c). Based on these results, longer durations of stress (3hr and 5hr) caused a significant loss of cells and higher temperatures (43.5°C and 45°C) resulted in a reduction in metabolic activity. Therefore, to maximize cell viability and maintain metabolic processes, the appropriate protocol chosen for thermal stress was 42°C for 1 hour and for shear stress was 1 dyne/cm^2 for 1 hour. For the

combined stress protocol, the parameters for independent shear and thermal stress were used and a duration of 1 hour was also chosen to maintain consistency across experimental groups.

Table 2.3: The range of parameters evaluated for shear stress and thermal stress to determine the appropriate stress protocol

Type of Stress	Parameter Varied	Duration and Temperature
Shear Stress	Duration	1 hr 3 hr at 1 dyne/cm^2 5 hr
Thermal Stress	Temperature	42°C 43.5°C for 1 hr 45°C
	Duration	1 hr 3 hr at 42°C 5 hr

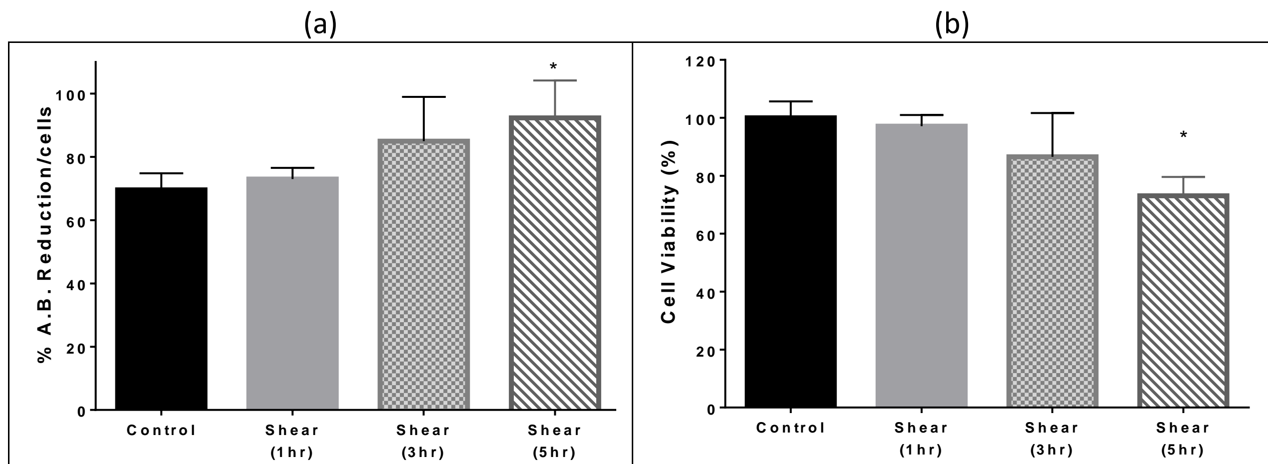

Figure 2.3: (a) Metabolic activity and (b) cell viability (% of the control) were measured 24 hours following shear stress. Cells were exposed to shear stress (τ = 1 dyne/cm^2) for various durations (1hr, 3hr, and 5hr) (n=4). Metabolic activity data is represented as the percentage of alamarBlue (A.B.) reduction and was normalized to cell number. Asterisk (*) indicates a significant difference from control (no stress) (p < 0.05).

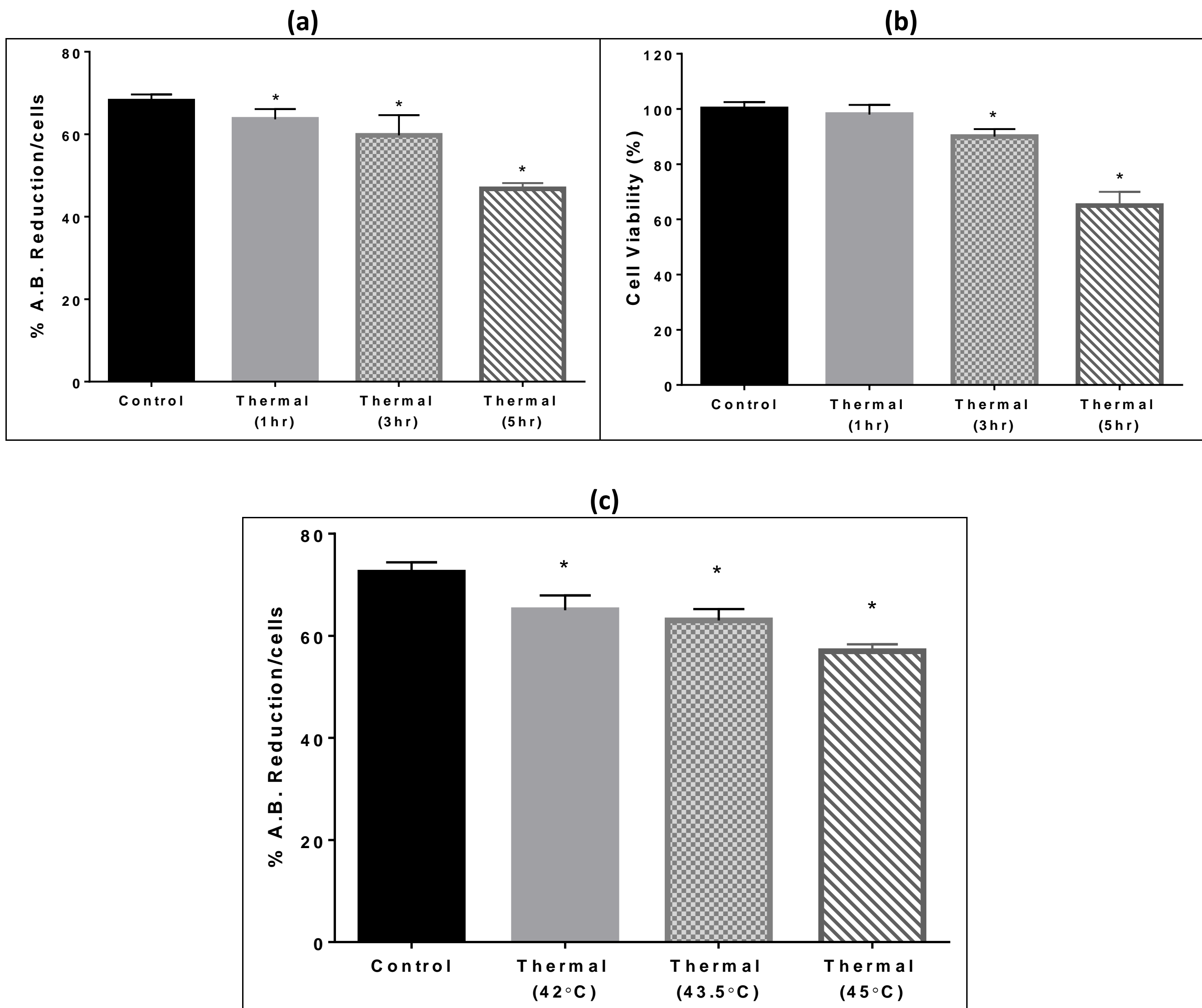

Figure 2.4: (a) Metabolic activity and (b) cell viability (% of the control) was measured 24 hrs following thermal stress when the (a-b) duration of stress was varied (1hr, 3hr, 5 hr) and the (c) temperature was varied at 42, 43.5, and 45°C (n=3). Metabolic activity data is represented as the percentage of alamarBlue (A.B.) reduction and was normalized to cell number. Asterisk (*) indicates a significant difference from control (no stress) ($p < 0.05$).

2.3.2 Cell Number

Following a single application of stress treatments on Day 2, the effect on cell number was evaluated by PicoGreen assay on Day 3, Day 6, and Day 9. These time points were chosen in order to assess the immediate and long term effects of stress. Figure 2.5 shows that for all groups the dsDNA concentrations increased initially until Day 6, then began to plateau around Day 9. Cell number was not significantly different among stress groups.

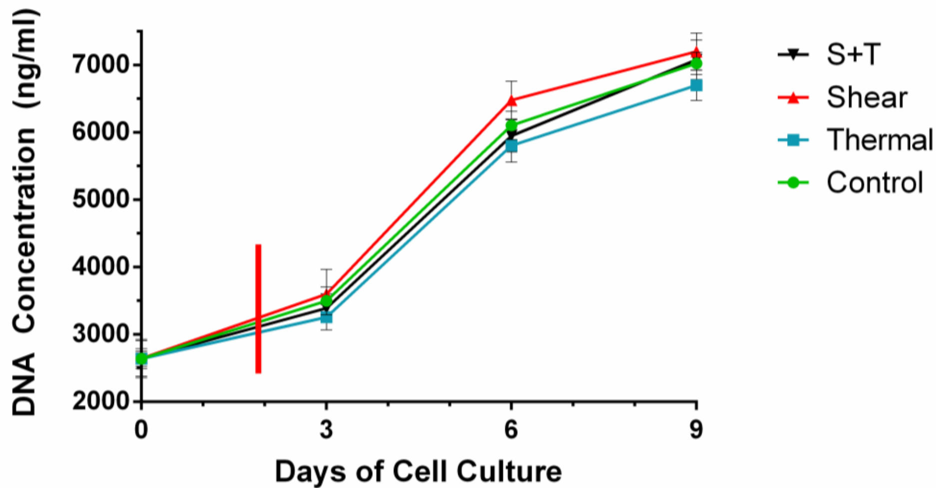

Figure 2.5: DNA concentrations were measured on Day 3, 6, and 9 to evaluate cell number following stress treatments (n=6, 4, and 3, respectively). The vertical red line indicates the time stress treatments were applied on Day 2.

2.3.3 Metabolic Activity in Response to Stress Treatments

As an indicator of cellular response to stress, the metabolic activity of cells was evaluated 24 hrs following stress treatments by measuring the percentage reduction of alamarBlue. As shown in Figure 2.6, all stress treatments had a significant effect on cellular metabolism. Compared to the control, shear stress increased metabolic activity, while thermal and the combined stress showed similar decreases in metabolic response. Although there was a reduction in metabolic activity for thermal stress and combined stress, it was less than a 10% decrease which may not detrimentally affect cellular behavior.

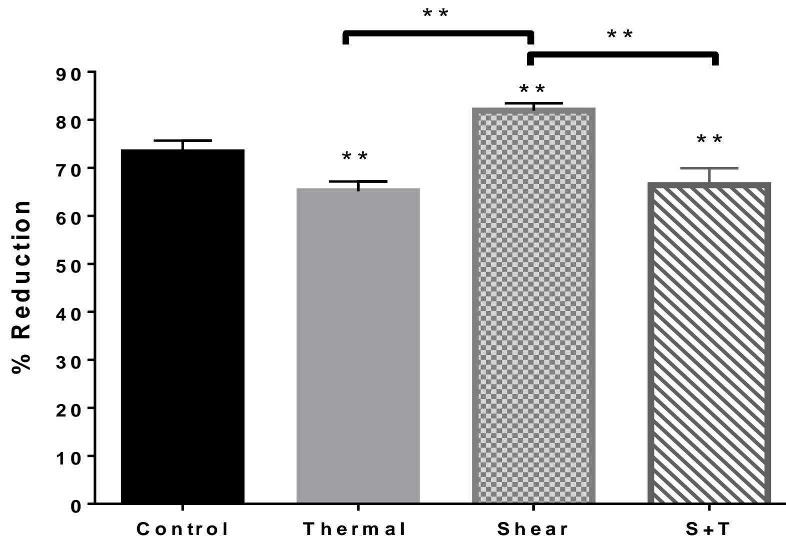

Figure 2.6: Metabolic activity measured 24 hours following stress treatments (n=4). Metabolic activity data is represented as the percent of alamarBlue reduction. Asterisk (*) indicates a significant difference from control (no stress). Bars indicate significant differences between stressed groups (**$p < 0.01$).

2.3.4 HSP Gene Expression

To determine the stress response and subsequent induction of cytoprotective proteins, mRNA expression of HSP47, 60, 70, and 90 was measured 6 hrs following stress treatments on Day 2. As shown in Figure 2.7, for all HSPs, except HSP90, mRNA expression was significantly upregulated in response to combined shear and thermal stress (2.84 RFI for HSP47; 3.18 RFI for HSP60; and 4.56 RFI for HSP70). Similarly, thermal stress initiated a significant increase in HSP47 gene expression (3.87 RFI) and HSP70 gene expression (3.36 RFI). However, shear stress treatments did significantly affect the gene expression of any of the HSPs measured, although there was a slight increase in HSP47 and HSP60 mRNA levels. HSP90 gene expression levels were not affected by any of the stress treatments applied. Due to its transient nature, stress induced HSP expression may be more prominent at earlier time points than the ones used in this study, since HSPs can be upregulated within minutes of stress exposure [56].

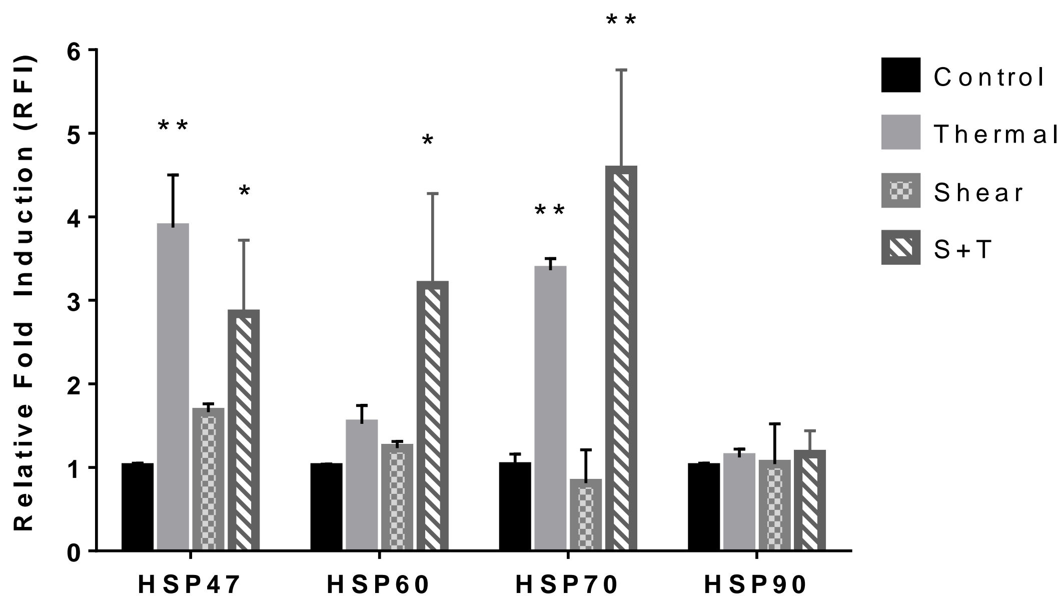

Figure 2.7: HSP gene expression measured 6 hrs after stress treatments on Day 2 (n=6). Asterisk (*) indicates a significant difference between control (no stress) and stressed groups (* = p < 0.01 and ** = p<0.001).

2.3.5 HSP Expression

The protein expression levels of HSP47, 60, 70, and 90 were measured 12 hrs following stress treatments. The time points chosen for this study were based on previously published data that showed HSP expression peaks around 12-16 hours, depending on the magnitude and duration of the heat stress [56, 57]. Based on the protocol used in our study (42°C for 1 hr), it was expected that HSP expression would peak approximately 12 hours following stress treatments. However, since no studies have previously evaluated HSP expression for combined shear and thermal stress, the peak concentrations for this may be at earlier or later time points due to the transient nature of HSP expression. To determine the peak expression for each HSP, it may be necessary to measure HSP expression over time immediately following stress treatments up to 48 hours later [57]. For HSP47 (Figure 2.8 a-d), it was difficult to detect changes of expression between stress treatment groups. For HSP60 (Figure 2.8 e-h), significant increases in protein synthesis were observed for thermal stress and combined stress, with slight changes in shear stress compared to the control. For HSP 70, increased fluorescence intensity in the nucleus indicates the greatest upregulation in combined stress and thermal stress, whereas there was minimal nuclear localization for shear stress and the control (Figure 2.8 i-l), despite basal levels of HSP70 in the cytoplasm. With HSP90, all treatment groups showed increase expression, however it was difficult to determine differences between stress groups. Localization of HSPs was also evaluated.

HSP60 accumulated in the mitochondria. HSP 70 was localized in the cytoplasm and the nucleus, while HSP90 was only observed in the cytoplasm.

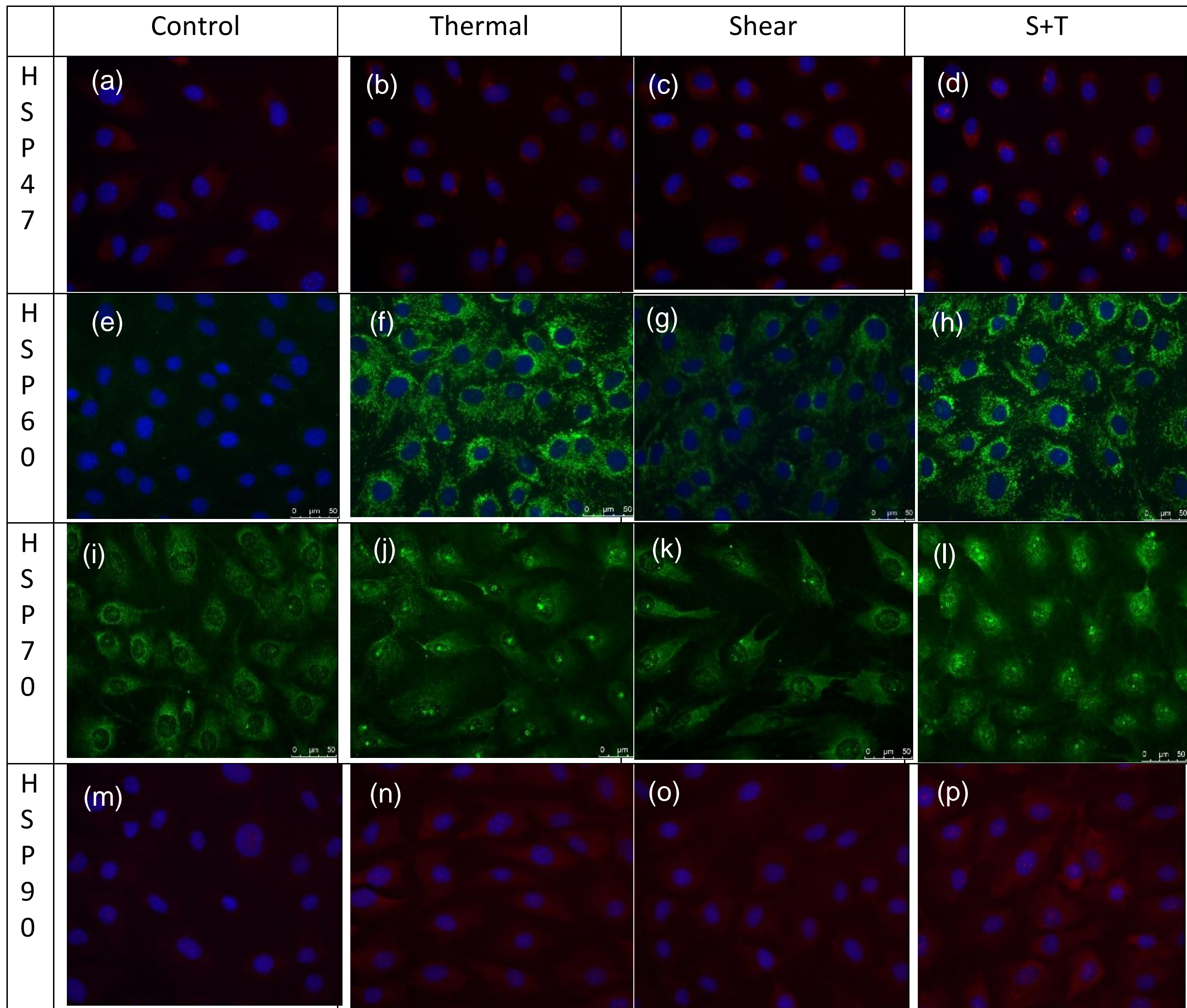

Figure 2.8: Immunoflourescent staining of (a-d) HSP47, (e-h) HSP60, (i-l) HSP70, and (m-p) HSP90 measured 12 hrs after stress treatments on Day 2. The nucleus was counterstained with DAPI (blue) in all groups except for HSP70 in order to show nuclear protein accumulation (Scale bar = 50um).

2.3.6 COX-2 mRNA expression and PGE_2 Accumulation

Due to their involvement in mechanotransduction pathways, COX-2 gene expression and subsequent release of PGE_2 was evaluated. Gene expression of COX-2 was measured 6 hr following stress on Day 2. There was a significant upregulation of COX-2 in response to shear stress (5.83 RFI) and combined stress (12.32 RFI), but thermal stress did not stimulate a response (Figure 2.9a). Since COX-2 is an inducible enzyme necessary for the production of PGE_2, the

accumulation of PGE_2 in the media was measured at 1 hr and 24 hrs following stress treatments. As shown in Figure 2.9b, there was no difference in the concentration of PGE_2 collected within 1 hour post stress. However, 24 hours following treatments, shear stress and combined stress induced a significant increase in PGE_2 synthesis and accumulation. Combined stress released a lower concentration of PGE_2 compared to shear stress.

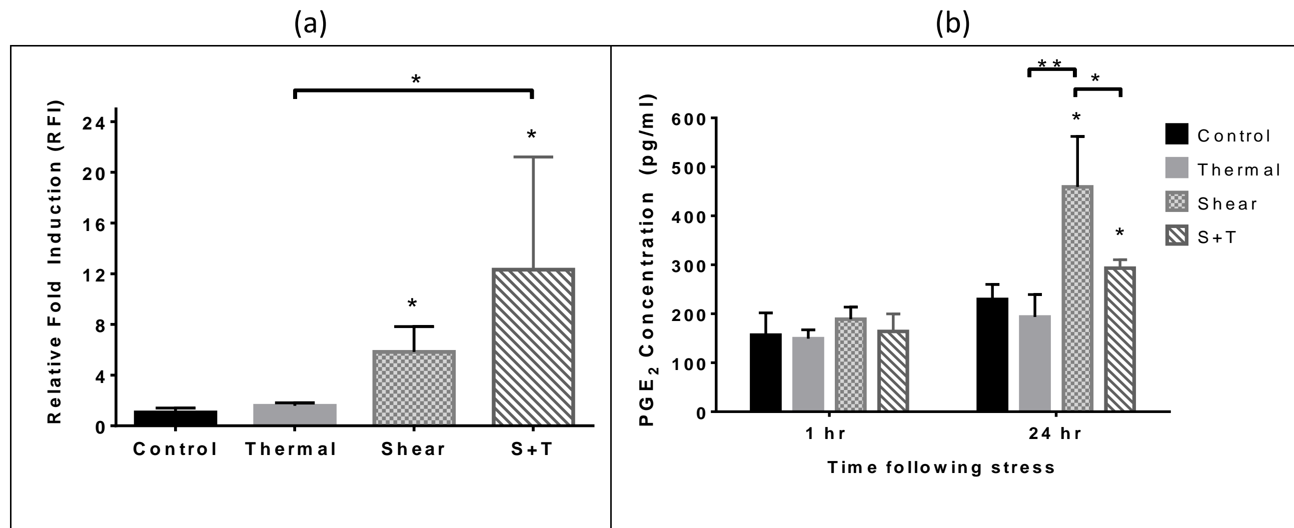

Figure 2.9: (a) Gene expression of COX-2 measured 6 hrs following stress treatments on Day 2 (n=4). (b) Concentration of PGE_2 in the media collected 1 hr and 24 hrs following stress treatments on Day 2 (n=6). Asterisk (*) indicates a significant difference from control. Bars indicate significant differences between stressed groups (* = $p < 0.01$ and ** = $p<0.001$).

2.3.7 Gene Expression of VEGF

Due to its angiogenic potential, VEGF mRNA expression levels were evaluated 6 hrs following stress treatments on Day 2. Figure 2.10 shows that combined shear and thermal stress induced the highest upregulation of VEGF (12.49 RFI), followed by a 2.62 fold induction with shear stress. Thermal stress alone had no influence on VEGF levels compared to the control.

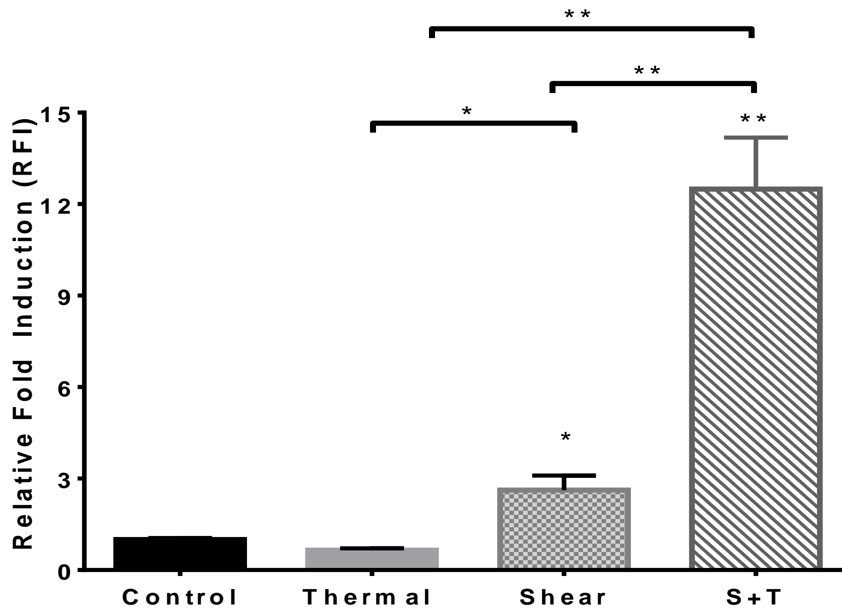

Figure 2.10: Gene expression of VEGF 6 hrs following stress treatments (n=4). Asterisk (*) indicates a significant difference from control (no stress). Bars indicate significant differences between stressed groups (* = p < 0.05 and ** = p<0.01).

2.3.8 Gene expression and protein secretion of OPN and OCN

To investigate the effect of stress treatments on late stage phenotypic markers, OPN and OCN gene expression and protein accumulation in the media were measured on Day 15. For a single application of stress treatments on Day 2, only shear stress treatments induced an increase in OPN mRNA levels and none of the stress treatments affected OCN gene expression (Figure 2.11a). However, this stimulatory effect in gene expression was not apparent in OPN and OCN protein secretions in the media, where no significant differences from the control were observed (data not shown). In order to stimulate enhanced expression of late stage markers, repeated stress treatments were investigated by exposing cells to stress on Day 2, 6, and 10.

As shown in Figure 2.11, all stress treatments increased OPN gene expression, but the greatest induction occurred with combined shear and thermal stress (5.64 RFI), followed by thermal stress (3.47 RFI) and shear stress (2.76 RFI). For OCN gene expression, although all stress treatments increased compared to the control, there was no significant difference among stress treatments (Figure 2.11b). For OPN secretions, combined stress stimulated the greatest secretion, followed by similar levels of accumulation in shear stress and thermal stress (Figure 2.12). Repeated stress treatments were not able to stimulate a significant change in OCN protein secretion (Figure 2.12b).

(a) (b)

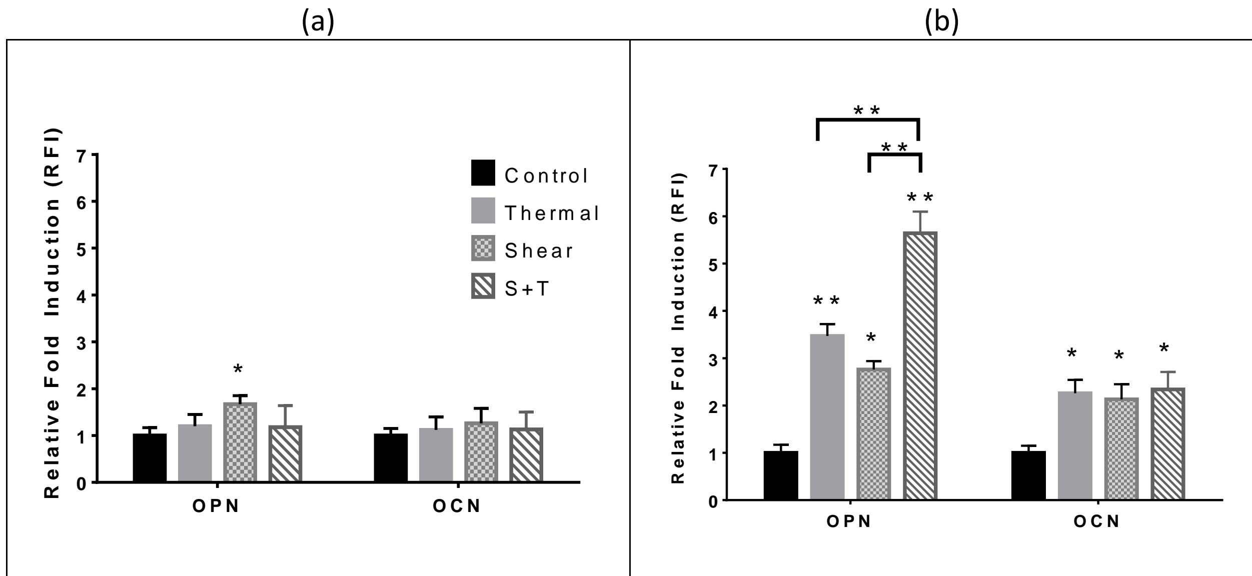

Figure 2.11: OPN and OCN gene expression measured on Day 15 following (a) single and (b) repeated stress treatments. Asterisk (*) indicates a significant difference from control (no stress). Bars indicate significant differences between stressed groups (*p < 0.05 and ** = p<0.01).

(a) (b)

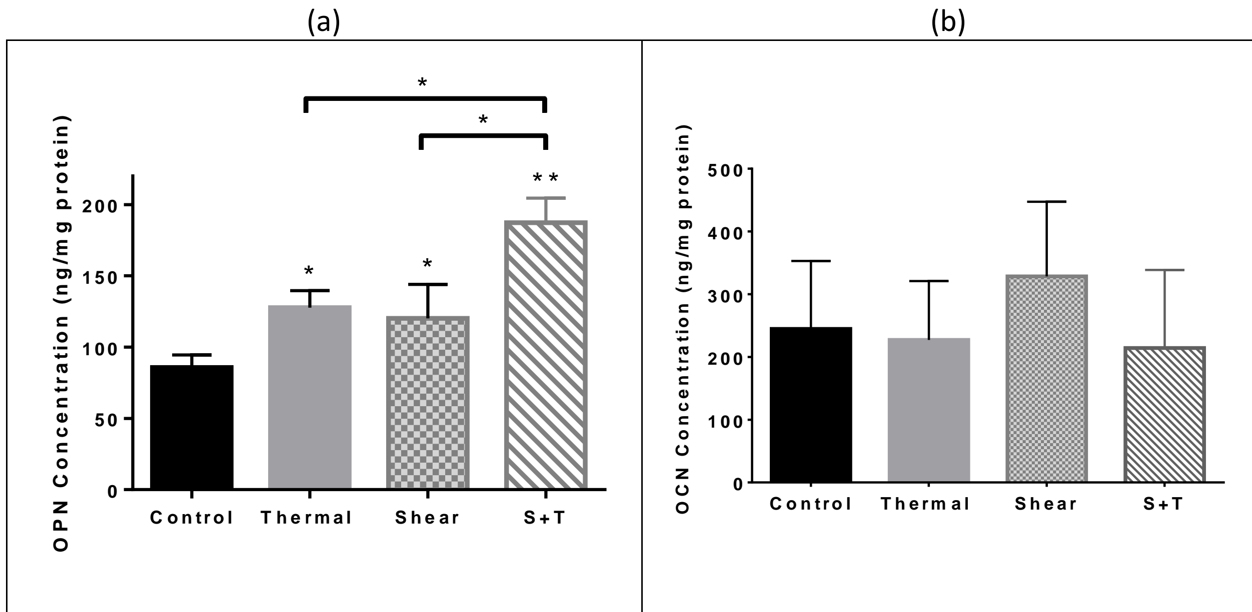

Figure 2.12: (a) OPN and (b) OCN protein secretion measured on Day 15 following repeated stress treatments. Asterisk (*) indicates a significant difference from control (no stress). Bars indicate significant differences between stressed groups (* = p < 0.05 and ** = p<0.01).

2.3.9 Mineralization

Calcium deposition was used as a late-stage marker of osteoblast differentiation and maturation. Figure 2.13 shows that only a single stress treatment application on Day 2 was not able to induce a significant osteogenic response, since all stress treatments showed similar calcium deposition as the control. This may be due to the long recovery period from stress treatment on Day 2 until mineralization analysis on Day 15, which may have allowed any stress-induced cellular response to dissipate. Many previous studies have also shown that repeated stress enhances osteogenesis, while a single application of stress has minimal or no effect for late stage markers of osteoblast differentiation [15]. Therefore, stress treatments were repeated on Day 2, Day 6, and Day 10 to determine if multiple stress applications could enhance osteoblast differentiation and modify ECM development. Repeated stress induced a significant change in calcium deposition for all stress groups compared to the control (Figure 2.13b), where thermal stress treatments enhanced calcium deposition, but repeated application of shear stress and combined shear and thermal stress caused a significant reduction in calcium deposition.

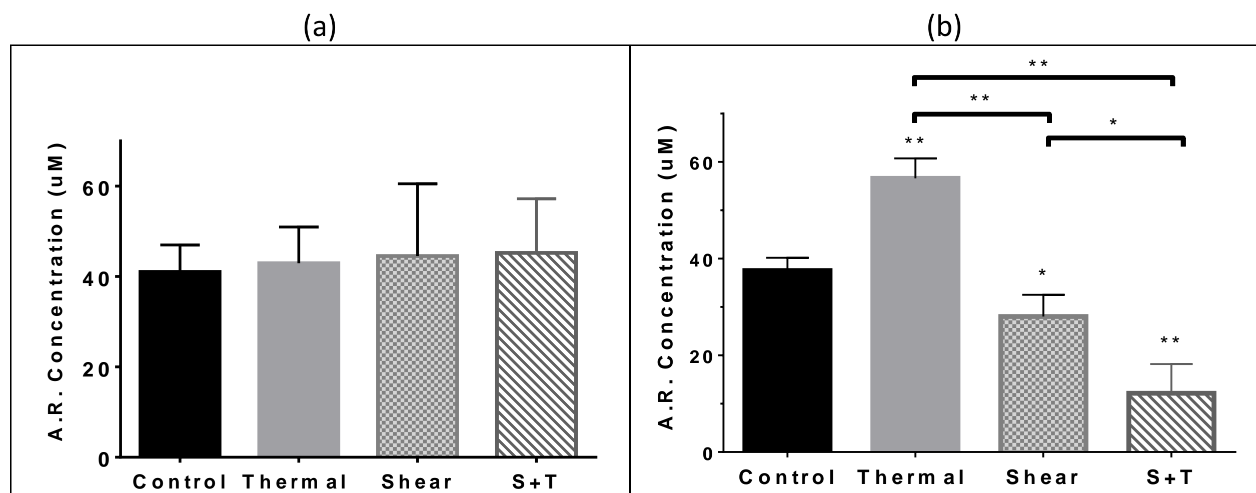

Figure 2.13: Calcium Deposition measured on Day 15 following (a) single or (b) repeated stress treatments (n = 6 for single stress treatment, n = 4 for repeated stress treatments). Asterisk (*) indicates a significant difference from control (no stress). Bars indicate significant differences between stressed groups (* = p<0.05 and ** = p<0.01).

2.4 Discussion

In this study, a preliminary objective was to evaluate cellular response to various stress treatment parameters to determine maximum tolerable thresholds for shear and thermal stress. Higher magnitudes and longer durations lead to reduced cell viability and decreased metabolic activity. Therefore, the parameters chosen for thermal stress was 42°C for 1 hour and for shear stress was 1 dyne/cm^2 for 1 hour in order to maintain viability following stress. The parameters chosen were also supported by previous studies [5, 15, 16, 24, 36, 56-58]. For example, Rylander et al

observed that cell viability was significantly compromised at higher temperatures (>44°C) and longer heating times (>10 min), concluding that a threshold exists where cell death is eminent because the cellular injury exceeds reparative capabilities [56]. Also, multiple studies have investigated mild heat stress using 41°C - 42°C for 1 hour and observed an enhanced cellular response [15, 16, 57]. For shear stress, although 1 dyne/cm^2 used in this study is on the lower end of the predicted range of mechanical stress cells experience *in vivo*, previous studies have shown that low magnitudes of shear stress (0.036 - 2.7 dynes/cm^2) are sufficient to stimulate a cellular response and promote osteoblast differentiation[5, 36, 58]. Therefore, the parameters chosen for this study have the potential to adequately stimulate cells, without significant consequences on cell viability and metabolic activity. More importantly, no other studies have investigated the effect of combined shear and thermal stress on cellular behavior or its potential to improve osteoblast differentiation.

In this study, cell number was not significantly affected by any of the stress treatments. This may suggest that osteogenic differentiation is occurring since it is well established that as differentiation progresses, cell proliferation decreases. These events typically occur within the first week on culture, prior to onset of ECM maturation [31]. Other studies have shown that even without significant increases in cell proliferation, shear stress and thermal stress were still capable of influencing osteogenic progression. Scaglione et al saw a decline in hBMSC growth following 10 days of shear stress at 0.012 dynes/cm^2, yet increased mineralization was still observed [59]. Another study saw no effect on cell proliferation when MSCs were exposed to shear flow every 2 days for 20 days, however, there was significant upregulation of bone specific genes, like OPN and BSP [36]. Alternatively, in studies that have shown increased proliferation due to shear stress, the magnitude of shear stress was at least an order higher (10 dynes/cm^2) than what was used in this study [7, 33, 60]. Although cell type (stem cells vs osteoblast-like cell lines) can influence the response to stress, magnitude and duration of stimuli appear to have a more potent effect on metabolic activity and cell proliferation.

In order to gauge the reaction of cells to stress, this study also evaluated fluctuations in metabolic activity. Increased cellular metabolism was observed following shear stress, which is consistent with previous studies that described an anabolic response to fluid flow stimulation, including early gene upregulation of bone specific proteins, like ALP [7] and type I collagen [37] as well as rapid induction of signaling factors like PGE [43], NO [43, 44], IP_3 [41], and cAMP [41], within minutes. In this study, thermal stress and combined stress caused a reduction in metabolic activity. However, this decrease is not uncommon and may be associated with a temporary cessation in cellular processes. Mizzen and Welch demonstrated that translational arrest occurs within the cell after heat shock and correlates to the severity of stress [61]. Furthermore, synthesis of HSPs was necessary prior to the recovery of translational activities for other, non-

stress proteins [61]. Another study describing the relationship between thermal exposure and cell viability, observed a decline in metabolic activity with increasing magnitude and duration of thermal exposure [62]. Overall, considering that stress treatments in this study did not negatively affect cell denisty over 9 days, we can assume that reductions in metabolic activity were temporary and did not significantly impact cell viability.

In response to stress treatments, this study also measured HSP gene and protein expression to determine the relative degree of stress cells experience as well as any implications they may have on osteogenic differentiation. There has been extensive research investigating the stress response, characterized by a rapid induction of HSPs that act as molecular chaperones to refold and repair damaged proteins [21]. Although HSPs are involved in normal physiological functions, additional HSP induction under stress has also been shown to elicit cytoprotective functions, enhance cell viability and proliferation, and stimulate differentiation [21, 56, 63, 64]. In this study, shear stress had a limited ability to induce HSP gene expression above basal levels, yet thermal and combined stress caused up to 3- and 4-fold differences, respectively. This is consistent with previous studies that show HSP induction is highly temperature sensitive, despite the fact that a variety of stresses can stimulate HSP upregulation [21, 56, 65]. In particular, one study measured a 29% increase in HSP70 expression at hyperthermic temperatures of 39°C, while hypothermic temperatures of 33°C resulted in a 56% decrease in expression [16].

The different responses for induction of each specific HSP may be due to their various chaperone responsibilities. For example, in this study, the large upregulation in HSP47 gene expression resulting from thermal stress and combined stress appear to be correlated with relatively high amounts of collagen mRNA levels, indicated by a low C_T value of 14 measured during RT-PCR (data not shown). Due to the involvement of HSP47 in regulating collagen biosynthesis, the significant induction of this protein in response to thermal stress and combined stress may be beneficial to enhancing collagen synthesis and ultimately ECM development. HSP60 is involved in synthesis and translocation of mitochondrial proteins from the cytoplasm to the mitochondrial matrix [66] and in this study, HSP60 expression was localized in the mitochondria. HSP60 has also been implicated in preventing bone loss by initiating cytoprotection within skeletal tissue [67]. Also given that HSP70 and HSP90 are both highly conserved and that stress inducible proteins have similar roles in inhibiting apoptosis, the overexpression of HSP 70 may be attributed to the minimal response in HSP90 induction. A similar trend was observed by Chen et al where thermal stress exposure for 1 hr at 42°C induced expression of HSP70, but not HSP90 [15]. Although the mechanism is unclear, in this study stress treatments had a greater effect on post-translational modifications of HSP90 since mRNA expression levels had a limited response, but protein expression was increased compared to basal levels. The high basal level expression in the control of HSPs may be associated with a highly expressed constitutive form of HSPs as well as

stimulation from typical culture methods that may impose stress, or influence from the serum due to serum response elements found on HSPs. Considering that the translation of genes to proteins is a complex process facilitated by heat shock factors (HSF) and regulated by feedback control mechanisms, additional experiments would be necessary to determine the true reasoning for some of the differences in HSP expression.

However, we can conclude from the results of this study that HSP induction is more sensitive to thermal stress than shear stress, given that the latter did not induce any statistically significant changes from basal HSP levels, but the former increased gene or protein expression for all HSPs. In addition our results show in some cases, this temperature-sensitive effect can be enhanced when thermal stress is combined with shear stress to generate a greater response than either individual stress. In addition, since it is well known that HSP induction is dependent on the magnitude of stress [21], we can speculate that the enhanced response seen with combined stress may be due to cells simply experiencing a greater degree of stress, instead of any specific contributory effects from thermal or shear stress. Either way, combined stress had a strong stimulatory effect that induced expression across multiple HSPs. Determining the appropriate stress magnitude and duration to regulate HSP expression kinetics can be an effective therapeutic strategy for various applications. Although currently unknown, it is also important to explore whether HSP expression directly influences osteogenesis. Chen et al investigated HSP expression in response to periodic thermal stress and the influence HSPs may have on thermal stress-induced osteogenesis [15]. They observed that the conditions that induced HSP also correlated to enhanced expression of bone related proteins, although HSPs did not directly act on these proteins. However, the beneficial aspects associated with HSP induction, including cytoprotective properties, enhanced cell proliferation, and stress tolerance, suggests their usefulness in bone development. Based on these findings, the ability of combined stress to induce HSP expression may indirectly promote an enhanced osteogenic response.

In addition to HSPs, other factors that have an early response to stress include COX-2 and PGE_2. PGE_2 plays an important role as an extracellular signaling molecule mediating the transduction of shear stress into a biochemical response [41]. PGE_2 has been shown to cause an anabolic response and is involved in regulating ECM development and maturation when shear flow is applied to bone cells [41, 68]. COX-2 has also been investigated because it is a highly inducible enzyme that is necessary for the production of PGE_2 [69]. In agreement with previous studies [43, 58], our shear flow protocol upregulated COX-2 expression and induced the highest levels of PGE_2 release compared to static conditions. It was expected that combined stress would stimulate similar or greater levels of PGE_2 accumulation, but the combination of thermal stress with shear flow actually caused a reduction in PGE_2 concentration compared to shear stress alone. Although combined stress had the highest induction of COX-2 mRNA levels, this did not directly translate

into the highest concentration of PGE_2 because of the many factors involved in the synthesis and release of PGE_2. For example, a possible explanation for the reduced PGE_2 levels observed with combined stress is that thermal stress prevented the release of arachidonic acid, inhibited the activity of COX-2, decreased the production of enzymes like PGE synthase, or prevented the release of PGE_2 from the cell. Since these proteins are typically investigated in association with shear flow, there is little evidence explaining the effect of thermal stress.

Vascular endothelial growth factor (VEGF) has been recognized as an important autocrine and paracrine signaling factor involved in osteoblast differentiation. In this study, shear stress induced a 6-fold change in VEGF expression. However, combined stress initiated the greatest response in VEGF gene expression, with a 12-fold increase in mRNA levels. In agreement with these results, previous studies have described the importance of VEGF upregulation during osteoblast differentiation [10, 70, 71] and shown enhanced VEGF gene expression and protein secretion in response to shear stress [46]. In addition, studies have demonstrated that cytokines, such as PGE_2, can influence VEGF expression [70]. This is in agreement with our study, where significant increases in PGE accumulation due to shear stress and combined stress were also observed in VEGF gene expression. Although additional experiments may be necessary to confirm that this effect is translated to proteins synthesis and secretion, it is important to note that in this study, thermal stress alone did not alter VEGF gene expression, but thermal stress in combination with shear stress produced a significant upregulation of VEGF mRNA levels, greater than that of shear stress. This substantial induction of VEGF gene expression suggests the importance of combined stress to promote angiogenesis, which is essential in bone fracture healing.

Calcium deposition, OPN, and OCN are commonly used as a late-stage markers of osteoblast differentiation and maturation. In this study, a single stress treatment application on Day 2 was not able to induce a significant osteogenic response in these markers, except for shear-induced upregulation of OPN gene expression. This response is most likely due to the long recovery period from stress treatment on Day 2 until analysis on Day 15 which may have diminished any stress-induced cellular response. Previous studies agree that repeated stress enhances osteogenesis, while a single application of stress has minimal or no effect on late stage markers of osteoblast differentiation [15, 16, 36]. Repeated stress treatments can provide periodic stimulation of cells, which is essential to prolonging the cellular response to stress.

Therefore, in this study stress treatments were applied on Day 2, Day 6, and Day 10 to determine if multiple stress applications could enhance osteoblast differentiation and modify ECM development. Repeated thermal stress treatments enhanced mineralization, however, periodic applications of shear stress and combined stress decreased calcium deposition. Our results

showing thermal stress-induced mineralization are comparable to previous studies that investigated the effect of heat shock on osteoblast differentiation of MSCs [15, 16]. One study observed increased calcium deposition following repeated thermal stress exposure (41°C for 1 hr) every 3 days for 21 days [16]. Another study by Chen et al demonstrated that weekly thermal stress applications for 1 hr at 41°C increased calcium deposits in 2D culture on Day 19 and Day 27, compared to non-stressed cell cultures [15].

Our results showing decreased mineralization due to shear stress and combined stress were contradictory to some previous studies. Fassina et al showed a 10-fold increase in calcium deposition by applying shear stress within a human osteoblast-seeded polyurethane scaffold [37]. Another study applying perfusion flow at low shear stresses (0.05 - 0.15 dynes/cm^2) to a beta-tricalcium phosphate scaffold saw increased mineralization as long as mass transport was not high (<3 ml/min) [30]. The discrepancies in mineralization between these studies and the current study may stem from the longer durations of shear stress exposure, as well as different substrates used since 3D substrates may better facilitate cell-matrix interactions than 2D substrates. Decreased mineralization observed in this study may also result from shear flow preventing multicellular bone nodule formation or fluid flow washing away necessary components of mineralization. Kreke et al observed decreased mineralization and formation of multicellular aggregates on Day 20 after repeated exposure to shear stress every 2 days, despite an increase in OCN [5]. Although many components are involved in facilitating mineralization, multicellular aggregates are necessary to provide nucleation sites for mineralization [72]. In addition, Li et al described the influence of fluid-induced shear stress and mass transport on hBMSC-seeded scaffolds, noting that ECM mineralization was inhibited with increasing mass transport [30].

In this study, minimal response was observed with a single application of stress, but multiple applications of stress enhanced gene expression for OCN and OPN. OPN was significantly upregulated for all groups, with combined stress having the greatest induction. For OCN gene expression, although all stress treatments increased mRNA levels compared to the control, there was no significant difference among stress treatments. For protein concentrations, repeated stress treatments were able to increase OPN secretion. However, no stress treatments stimulated a significant change in OCN protein secretion. In agreement with our results, prior studies have shown that OPN gene expression can be regulated by stress. One study observed increased OPN gene expression on Day 19 following weekly thermal stress applications for 1 hr at 41°C, compared to non-stressed cell cultures [15]. Gonzales et al also described significant upregulation of OPN using higher magnitudes of shear stress (>24 dynes/cm^2) and primary osteoblasts. Another study using the same preosteoblast cell line as the current study, observed that fluid flow enhanced OPN gene expression in response to oscillatory fluid flow [13]. One of

the few prior studies investigating combined stress was performed by our group and demonstrated the greatest induction of OPN mRNA levels following combined thermal stress (44°C) and cyclic tension [73], which is consistent with our results. In comparison to these studies, the current study used a less sensitive cell line, 2D substrate, and lower magnitudes of stress and was still able to to generate a significant upregulation of OPN. Contrary to our results, some studies observed increased OCN synthesis and deposition following repeated shear stress at 1.6 dynes/cm^2 [36]. However, different cell types (MSCs, SAOS-2 cells) were used, different shear stresses, and different method for OCN analysis were used in these previous studies compared to the current study, which may contribute to the discrepancies observed in the stress response. Despite its large involvement in bone mineralization, few studies have reported stress induced changes in OCN expression, possibly due to a limited ability to stimulate a strong response from this protein. In addition, analyzing OCN and OPN accumulation in the media may not be the best way to assess their synthesis and secretion. Since these proteins are involved in ECM development and maturation, their accumulation in the media may be minimal or not an accurate representation of their true concentrations. An alternative assessment could involve immunocytochemical staining of the ECM for these proteins, supported by western blot analysis of protein synthesis.

Although osteoblasts have been extensively studied for their high sensitivity to mechanical stimulation, hyperthermic responsiveness can also play an important role in modifying osteogenic differentiation as demonstrated in previous studies by increased HSP expression, ALP activity, calcium deposition, OPN, and BMP2 expression following mild thermal stress of MSCs [15, 16]. The results from our study showed significant HSP induction following thermal stress. In addition, repeated exposure to thermal stress stimulated significant upregulation of OPN and enhanced calcium deposition, which was not observed with a single application of thermal stress. These results may be explained by the notion that repeated exposure to thermal stress can overcome the temporary decrease in metabolic activity typically associated with heat shock, while still inducing HSP expression and the associated beneficial effects [61].

As expected, early response markers related to mechanotransduction, such as VEGF, COX-2, and PGE accumulation, were significantly upregulated with shear stress, while thermal stress had little influence on these factors. Likewise, thermal stress induced changes in temperature-responsive proteins like HSPs, yet shear stress did not stimulate any differences. More importantly, the ability of combined stress to simulate a response in all the factors above as well as late stage differentiation markers, speaks to the potential of combined stress protocol to capitalize on the stimulatory effects of shear stress sensitivity and thermal stress-induced changes Furthermore, the simultaneous application of shear and thermal stress reveals new possibilities for stimulating the osteogenic potential of cells. In this study, the combination of

shear and thermal stress induced some interesting changes in gene and protein expression. In some cases, combined stress produced strong responses that had a more potent effect than individual shear or thermal stress, which was observed in enhanced HSP expression, COX-2 induction, and VEGF upregulation. Alternatively, combined stress seemed to show some inhibitory effects as seen with PGE_2 accumulation and mineralization. Although hyperthermic temperatures may have hindered the full potential of combined stress, PGE_2 accumulation was still significantly increased in combined stress compared to no stress. A better understanding of the complex signaling involved with combined shear and thermal stress will be necessary to provide insight into the underlying mechanism(s) directing cellular response.

2.5 Conclusion

To our knowledge, this is the first study investigating the effect of combined shear and thermal stress on preosteoblasts. Prior work by our group has explored thermal stress in combination with cyclic tension and observed an enhanced osteogenic response [73]. The results of this study demonstrate that combined stress has shown positive stimulatory effects in most of the factors measured in this study, whereas individual stress has responded differentially by inducing a response in some factors, but not others. Only mineralization was significantly decreased as a result of combined stress, but this may be due to a number of factors. Overall, combined stress can provide the appropriate cues to activate multiple signaling pathways essential in osteogenesis, essentially capitalizing on the beneficial effects associated with the thermal stress response and shear-induced mechanotransduction.

Bone tissue engineering strategies to improve healing and regeneration within critical-sized defects are necessary to solve a pervasive issue affecting many orthopedic patients. Evidence provided in this study demonstrates that repeated applications of combined shear and thermal stress can effectively stimulate cytoprotective proteins, early signaling factors integral to bone development, and late-stage markers of osteoblast differentiation. As a result, this protocol can be an effective technique providing the appropriate cues to activate multiple signaling pathways in osteogenesis, essentially capitalizing on the beneficial effects associated with the thermal stress response and shear-induced mechanotransduction.

Chapter 3: Future Work

The results of this study demonstrated that combined shear and thermal stress has a broad impact on osteoblast differentiation and has an enhanced effect compared to individual stress treatments. Although this study was able to show the potential of combined stress as an effective stress conditioning technique, there remain many unexplored issues. For example, the mechanism regulating the stimulatory effects of combined stress is unknown, yet this information is valuable in better understanding the cellular response to combined stress. Also, conservative stress parameters and simple protocols were chosen in this study to minimize the complexity in evaluating the effects of combined stress. However, additional parameters for combined stress should be explored to determine if an improved cellular response can be achieved. In addition, it is not clear whether the successful results observed in this study using *in vitro* experiments with a cell monolayer will translate within a 3D environment that is necessary for developing bone. Therefore, future studies related to this work should focus on 1) determining the mechanism involved with combined shear and thermal stress, 2) developing an optimized combined stress protocol, and 3) applying combined stress regimes to cell-seeded 3D scaffolds to develop a bone tissue engineered constructs.

In order to gain knowledge of the ability of combined stress to regulate osteoblast differentiation and induce significant changes in gene and protein expression, it is important to define the underlying mechanism(s) involved. This can be achieved by identifying which signaling pathways and regulatory factors are involved with combined stress. Results from this study have shown that COX-2 is mainly upregulated with shear stress and HSPs are predominately induced by thermal stress, yet both of these proteins are greatly increased by combined stress. Based on this, if future studies evaluate the effects of inhibiting COX-2 activity with NS-398 as well as blocking HSP70 activity using VER-155008, it can be helpful in determining the role of these important factors as well as determine how each individual stress contributes to the overall combined effect. In addition, the inhibition of MAPK signaling pathways involved with external stress, such as p38 MAPK using SB203580 and JNK using SP600125, may be helpful in determining if combined shear and thermal stress act along similar or distinct pathways. These future studies will provide insight into why certain factors are upregulated, downregulated, or have no response. By comparing the results of combined stress to individual thermal or shear stress, it will help determine whether the combined response has a synergistic, additive, or inhibitory effect.

To explore the full potential of combined shear and thermal stress treatments, systematic variation of parameters can determine which conditions will be optimal for promoting bone development. Future studies on combined stress should vary the magnitude of shear stress (1 dynes/cm^2 to 20 dynes/cm^2) and temperature (41 to 44°C) to evaluate which combination

produces the most beneficial cellular response and upregulation of bone-related proteins. Also, varying the frequency of repeated stress treatments from every 2 days to once a week will determine the appropriate times to stimulate cells and the necessary recovery period between stress applications. Furthermore, future work should investigate applying combined stress simultaneously (as in this study) or sequentially to determine if the effects produced from one stress can promote an enhanced response with the subsequent stress. This sequential application of combined stress treatments should be investigated with thermal stress followed by shear stress and vice versa, with the time in between stress treatments ranging from 1 hr to 12 hrs apart. Information gathered from these studies can be used to develop and optimize a combined shear and thermal stress protocol to rapidly induce bone related proteins, enhance ECM development, and increase mineralization

Future studies would also include translating this work from 2D cell monolayers to 3D constructs in order to assess the capability of combined shear and thermal stress protocols to produce mineralized, functional bone substitutes. MC3T3-E1 cells will be seeded within a 3D biodegradable, porous matrix made of a biocompatible polymer. Although there are a number of different techniques to produce these scaffolds, it is important to consider methods where scaffold properties can be easily controlled and modified, such as electrospinning, 3D bioprinting, photolithography, and molding [74-79]. Due to the fact that scaffold properties can have a significant impact on cellular behavior [80], future *in vivo* studies will consist of determining the appropriate geometry, porosity, and mechanical properties of scaffolds that will facilitate bone development following combined stress treatments. Lastly, implanting these stress conditioned bone substitutes into a rat calvarial bone defect model, are essential in evaluating the functionality of these constructs by measuring the progression of bone healing and regeneration. Ultimately, with the additional knowledge gained from future studies, combined shear and thermal stress has the potential to be a useful technique in bone tissue engineering.

References

[1] A. S. Brydone, D. Meek, and S. Maclaine, "Bone grafting, orthopaedic biomaterials, and the clinical need for bone engineering," *Proc Inst Mech Eng H,* vol. 224, pp. 1329-43, Dec 2010.

[2] R. Dimitriou, E. Jones, D. McGonagle, and P. V. Giannoudis, "Bone regeneration: current concepts and future directions," *BMC Med,* vol. 9, p. 66, 2011.

[3] R. Quarto, M. Mastrogiacomo, R. Cancedda, S. M. Kutepov, V. Mukhachev, A. Lavroukov*, et al.*, "Repair of large bone defects with the use of autologous bone marrow stromal cells," *N Engl J Med,* vol. 344, pp. 385-6, Feb 1 2001.

[4] S. Weinbaum, S. C. Cowin, and Y. Zeng, "A model for the excitation of osteocytes by mechanical loading-induced bone fluid shear stresses," *J Biomech,* vol. 27, pp. 339-60, Mar 1994.

[5] M. R. Kreke and A. S. Goldstein, "Hydrodynamic shear stimulates osteocalcin expression but not proliferation of bone marrow stromal cells," *Tissue Eng,* vol. 10, pp. 780-8, May-Jun 2004.

[6] L. A. Sharp, Y. W. Lee, and A. S. Goldstein, "Effect of low-frequency pulsatile flow on expression of osteoblastic genes by bone marrow stromal cells," *Ann Biomed Eng,* vol. 37, pp. 445-53, Mar 2009.

[7] S. Kapur, D. J. Baylink, and K. H. Lau, "Fluid flow shear stress stimulates human osteoblast proliferation and differentiation through multiple interacting and competing signal transduction pathways," *Bone,* vol. 32, pp. 241-51, Mar 2003.

[8] T. L. Donahue, T. R. Haut, C. E. Yellowley, H. J. Donahue, and C. R. Jacobs, "Mechanosensitivity of bone cells to oscillating fluid flow induced shear stress may be modulated by chemotransport," *J Biomech,* vol. 36, pp. 1363-71, Sep 2003.

[9] H. Kaneki, I. Takasugi, M. Fujieda, M. Kiriu, S. Mizuochi, and H. Ide, "Prostaglandin E2 stimulates the formation of mineralized bone nodules by a cAMP-independent mechanism in the culture of adult rat calvarial osteoblasts," *J Cell Biochem,* vol. 73, pp. 36-48, Apr 1 1999.

[10] H. Mayer, H. Bertram, W. Lindenmaier, T. Korff, H. Weber, and H. Weich, "Vascular endothelial growth factor (VEGF-A) expression in human mesenchymal stem cells: autocrine and paracrine role on osteoblastic and endothelial differentiation," *J Cell Biochem,* vol. 95, pp. 827-39, Jul 1 2005.

[11] I. Owan, D. B. Burr, C. H. Turner, J. Qiu, Y. Tu, J. E. Onyia*, et al.*, "Mechanotransduction in bone: osteoblasts are more responsive to fluid forces than mechanical strain," *Am J Physiol,* vol. 273, pp. C810-5, Sep 1997.

[12] J. T. Triffitt, "Initiation and enhancement of bone formation. A review," *Acta Orthop Scand,* vol. 58, pp. 673-84, Dec 1987.

[13] J. You, G. C. Reilly, X. Zhen, C. E. Yellowley, Q. Chen, H. J. Donahue*, et al.*, "Osteopontin gene regulation by oscillatory fluid flow via intracellular calcium mobilization and activation of mitogen-activated protein kinase in MC3T3-E1 osteoblasts," *J Biol Chem,* vol. 276, pp. 13365-71, Apr 20 2001.

[14] G. N. Bancroft, V. I. Sikavitsas, J. van den Dolder, T. L. Sheffield, C. G. Ambrose, J. A. Jansen*, et al.*, "Fluid flow increases mineralized matrix deposition in 3D perfusion culture of marrow stromal osteoblasts in a dose-dependent manner," *Proc Natl Acad Sci U S A,* vol. 99, pp. 12600-5, Oct 1 2002.

[15] J. Chen, Z. D. Shi, X. Ji, J. Morales, J. Zhang, N. Kaur*, et al.*, "Enhanced osteogenesis of human mesenchymal stem cells by periodic heat shock in self-assembling peptide hydrogel," *Tissue Eng Part A,* vol. 19, pp. 716-28, Mar 2013.

[16] C. X. Shui and A. Scutt, "Mild heat shock induces proliferation, alkaline phosphatase activity, and mineralization in human bone marrow stromal cells and Mg-63 cells in vitro," *J Bone Miner Res,* vol. 16, pp. 731-741, Apr 2001.
[17] E. Chung and M. N. Rylander, "Response of preosteoblasts to thermal stress conditioning and osteoinductive growth factors," *Cell Stress Chaperones,* vol. 17, pp. 203-14, Mar 2012.
[18] I. Riederer, E. Negroni, A. Bigot, M. Bencze, J. Di Santo, A. Aamiri*, et al.*, "Heat shock treatment increases engraftment of transplanted human myoblasts into immunodeficient mice," *Transplant Proc,* vol. 40, pp. 624-30, Mar 2008.
[19] T. Hojo, M. Fujioka, G. Otsuka, S. Inoue, U. Kim, and T. Kubo, "Effect of heat stimulation on viability and proteoglycan metabolism of cultured chondrocytes: preliminary report," *J Orthop Sci,* vol. 8, pp. 396-9, 2003.
[20] K. Yoshida, K. Uoshima, K. Oda, and T. Maeda, "Influence of heat stress to matrix on bone formation," *Clin Oral Implants Res,* vol. 20, pp. 782-90, Aug 2009.
[21] K. R. Diller, "Stress protein expression kinetics," *Annu Rev Biomed Eng,* vol. 8, pp. 403-24, 2006.
[22] U. Fiege and R. I. Morimoto, "Stress-Inducible Cellular Responses," 1996.
[23] T. R. Dafforn, M. Della, and A. D. Miller, "The molecular interactions of heat shock protein 47 (Hsp47) and their implications for collagen biosynthesis," *J Biol Chem,* vol. 276, pp. 49310-9, Dec 28 2001.
[24] M. N. Rylander, Y. Feng, K. Zimmermann, and K. R. Diller, "Measurement and mathematical modeling of thermally induced injury and heat shock protein expression kinetics in normal and cancerous prostate cells," *Int J Hyperthermia,* vol. 26, pp. 748-64, 2010.
[25] H. K. Dinh, B. Zhao, S. T. Schuschereba, G. Merrill, and P. D. Bowman, "Gene expression profiling of the response to thermal injury in human cells," *Physiol Genomics,* vol. 7, pp. 3-13, Oct 10 2001.
[26] J. A. Frangos, L. V. McIntire, and S. G. Eskin, "Shear stress induced stimulation of mammalian cell metabolism," *Biotechnol Bioeng,* vol. 32, pp. 1053-60, Oct 5 1988.
[27] D. Brindley, K. Moorthy, J. H. Lee, C. Mason, H. W. Kim, and I. Wall, "Bioprocess forces and their impact on cell behavior: implications for bone regeneration therapy," *J Tissue Eng,* vol. 2011, p. 620247, 2011.
[28] H. Zhang and S. A. Fisher, "Conditioning effect of blood flow on resistance artery smooth muscle myosin phosphatase," *Circ Res,* vol. 100, pp. 730-7, Mar 16 2007.
[29] J. T. Selsby, S. Rother, S. Tsuda, O. Pracash, J. Quindry, and S. L. Dodd, "Intermittent hyperthermia enhances skeletal muscle regrowth and attenuates oxidative damage following reloading," *J Appl Physiol (1985),* vol. 102, pp. 1702-7, Apr 2007.
[30] D. Li, T. Tang, J. Lu, and K. Dai, "Effects of flow shear stress and mass transport on the construction of a large-scale tissue-engineered bone in a perfusion bioreactor," *Tissue Eng Part A,* vol. 15, pp. 2773-83, Oct 2009.
[31] J. B. Lian and G. S. Stein, "Concepts of osteoblast growth and differentiation: basis for modulation of bone cell development and tissue formation," *Crit Rev Oral Biol Med,* vol. 3, pp. 269-305, 1992.
[32] T. D. Brown, "Techniques for mechanical stimulation of cells in vitro: a review," *J Biomech,* vol. 33, pp. 3-14, Jan 2000.
[33] Y. J. Li, N. N. Batra, L. You, S. C. Meier, I. A. Coe, C. E. Yellowley*, et al.*, "Oscillatory fluid flow affects human marrow stromal cell proliferation and differentiation," *J Orthop Res,* vol. 22, pp. 1283-9, Nov 2004.
[34] H. L. Holtorf, T. L. Sheffield, C. G. Ambrose, J. A. Jansen, and A. G. Mikos, "Flow perfusion culture of marrow stromal cells seeded on porous biphasic calcium phosphate ceramics," *Ann Biomed Eng,* vol. 33, pp. 1238-48, Sep 2005.

[35] C. R. Jacobs, C. E. Yellowley, B. R. Davis, Z. Zhou, J. M. Cimbala, and H. J. Donahue, "Differential effect of steady versus oscillating flow on bone cells," *J Biomech,* vol. 31, pp. 969-76, Nov 1998.

[36] M. R. Kreke, W. R. Huckle, and A. S. Goldstein, "Fluid flow stimulates expression of osteopontin and bone sialoprotein by bone marrow stromal cells in a temporally dependent manner," *Bone,* vol. 36, pp. 1047-55, Jun 2005.

[37] L. Fassina, L. Visai, L. Asti, F. Benazzo, P. Speziale, M. C. Tanzi*, et al.*, "Calcified matrix production by SAOS-2 cells inside a polyurethane porous scaffold, using a perfusion bioreactor," *Tissue Eng,* vol. 11, pp. 685-700, May-Jun 2005.

[38] V. I. Sikavitsas, J. S. Temenoff, and A. G. Mikos, "Biomaterials and bone mechanotransduction," *Biomaterials,* vol. 22, pp. 2581-93, Oct 2001.

[39] A. D. Bakker, K. Soejima, J. Klein-Nulend, and E. H. Burger, "The production of nitric oxide and prostaglandin E2 by primary bone cells is shear stress dependent," *J Biomech,* vol. 34, pp. 671-677, 2001.

[40] F. M. Pavalko, N. X. Chen, C. H. Turner, D. B. Burr, S. Atkinson, Y. F. Hsieh*, et al.*, "Fluid shear-induced mechanical signaling in MC3T3-E1 osteoblasts requires cytoskeleton-integrin interactions," *Am J Physiol,* vol. 275, pp. C1591-601, Dec 1998.

[41] K. M. Reich, C. V. Gay, and J. A. Frangos, "Fluid shear stress as a mediator of osteoblast cyclic adenosine monophosphate production," *J Cell Physiol,* vol. 143, pp. 100-4, Apr 1990.

[42] R. G. Bacabac, T. H. Smit, S. C. Cowin, J. J. Van Loon, F. T. Nieuwstadt, R. Heethaar*, et al.*, "Dynamic shear stress in parallel-plate flow chambers," *J Biomech,* vol. 38, pp. 159-67, Jan 2005.

[43] J. Klein-Nulend, C. M. Semeins, N. E. Ajubi, P. J. Nijweide, and E. H. Burger, "Pulsating fluid flow increases nitric oxide (NO) synthesis by osteocytes but not periosteal fibroblasts--correlation with prostaglandin upregulation," *Biochem Biophys Res Commun,* vol. 217, pp. 640-8, Dec 14 1995.

[44] T. N. McAllister and J. A. Frangos, "Steady and transient fluid shear stress stimulate NO release in osteoblasts through distinct biochemical pathways," *J Bone Miner Res,* vol. 14, pp. 930-6, Jun 1999.

[45] J. G. McGarry, J. Klein-Nulend, and P. J. Prendergast, "The effect of cytoskeletal disruption on pulsatile fluid flow-induced nitric oxide and prostaglandin E2 release in osteocytes and osteoblasts," *Biochem Biophys Res Commun,* vol. 330, pp. 341-8, Apr 29 2005.

[46] M. M. Thi, D. A. Iacobas, S. Iacobas, and D. C. Spray, "Fluid shear stress upregulates vascular endothelial growth factor gene expression in osteoblasts," *Ann N Y Acad Sci,* vol. 1117, pp. 73-81, Nov 2007.

[47] D. E. Brooks, J. W. Goodwin, and G. V. Seaman, "Interactions among erythrocytes under shear," *J Appl Physiol,* vol. 28, pp. 172-7, Feb 1970.

[48] T. Takahashi, "A mathematical model for the distribution of hemodynamic parameters in the human retinal microvascular network," *Journal of Biorheology,* vol. 23, pp. 77-86, 2009.

[49] I. J. Benjamin and D. R. McMillan, "Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease," *Circ Res,* vol. 83, pp. 117-32, Jul 27 1998.

[50] N. D. Kirkpatrick, S. Andreou, J. B. Hoying, and U. Utzinger, "Live imaging of collagen remodeling during angiogenesis," *Am J Physiol Heart Circ Physiol,* vol. 292, pp. H3198-206, Jun 2007.

[51] C. Ye, L. Bai, Z. Q. Yan, Y. H. Wang, and Z. L. Jiang, "Shear stress and vascular smooth muscle cells promote endothelial differentiation of endothelial progenitor cells via activation of Akt," *Clin Biomech (Bristol, Avon),* vol. 23 Suppl 1, pp. S118-24, 2008.

[52] S. Biosciences, "L-glutamine Stability," 2005.

[53] K. Arii, H. Kobayashi, T. Kai, and Y. Kokuba, "Degradation kinetics of L-glutamine in aqueous solution," *Eur J Pharm Sci,* vol. 9, pp. 75-8, Oct 1999.
[54] E. A. Nauman, R. L. Satcher, T. M. Keaveny, B. P. Halloran, and D. D. Bikle, "Osteoblasts respond to pulsatile fluid flow with short-term increases in PGE(2) but no change in mineralization," *J Appl Physiol,* vol. 90, pp. 1849-54, May 2001.
[55] E. Tzima, M. Irani-Tehrani, W. B. Kiosses, E. Dejana, D. A. Schultz, B. Engelhardt*, et al.*, "A mechanosensory complex that mediates the endothelial cell response to fluid shear stress," *Nature,* vol. 437, pp. 426-31, Sep 15 2005.
[56] M. N. Rylander, Y. Feng, J. Bass, and K. R. Diller, "Thermally induced injury and heat-shock protein expression in cells and tissues," *Ann N Y Acad Sci,* vol. 1066, pp. 222-42, Dec 2005.
[57] S. Wang, K. R. Diller, and S. J. Aggarwal, "Kinetics study of endogenous heat shock protein 70 expression," *J Biomech Eng,* vol. 125, pp. 794-7, Dec 2003.
[58] M. R. Kreke, L. A. Sharp, Y. W. Lee, and A. S. Goldstein, "Effect of intermittent shear stress on mechanotransductive signaling and osteoblastic differentiation of bone marrow stromal cells," *Tissue Eng Part A,* vol. 14, pp. 529-37, Apr 2008.
[59] S. Scaglione, D. Wendt, S. Miggino, A. Papadimitropoulos, M. Fato, R. Quarto*, et al.*, "Effects of fluid flow and calcium phosphate coating on human bone marrow stromal cells cultured in a defined 2D model system," *J Biomed Mater Res A,* vol. 86, pp. 411-9, Aug 2008.
[60] R. C. Riddle, A. F. Taylor, D. C. Genetos, and H. J. Donahue, "MAP kinase and calcium signaling mediate fluid flow-induced human mesenchymal stem cell proliferation," *Am J Physiol Cell Physiol,* vol. 290, pp. C776-84, Mar 2006.
[61] L. A. Mizzen and W. J. Welch, "Characterization of the thermotolerant cell. I. Effects on protein synthesis activity and the regulation of heat-shock protein 70 expression," *J Cell Biol,* vol. 106, pp. 1105-16, Apr 1988.
[62] L. D. Kaplan, C. R. Chu, J. P. Bradley, F. H. Fu, and R. K. Studer, "Recovery of chondrocyte metabolic activity after thermal exposure," *Am J Sports Med,* vol. 31, pp. 392-8, May-Jun 2003.
[63] M. F. Cellier, M. Taimi, M. T. Chateau, A. Cannat, and J. Marti, "Thermal stress as an inducer of differentiation of U937 cells," *Leuk Res,* vol. 17, pp. 649-56, Aug 1993.
[64] D. S. Latchman, "Protective effect of heat shock proteins: potential for gene therapy," *Gene Ther Mol Biol,* vol. 7, pp. 245-254, 2003.
[65] S. Wang, W. Xie, M. N. Rylander, P. W. Tucker, S. Aggarwal, and K. R. Diller, "HSP70 kinetics study by continuous observation of HSP-GFP fusion protein expression on a perfusion heating stage," *Biotechnol Bioeng,* vol. 99, pp. 146-54, Jan 1 2008.
[66] H. Itoh, A. Komatsuda, H. Ohtani, H. Wakui, H. Imai, K. Sawada*, et al.*, "Mammalian HSP60 is quickly sorted into the mitochondria under conditions of dehydration," *Eur J Biochem,* vol. 269, pp. 5931-8, Dec 2002.
[67] F. S. Wang, R. W. Wu, J. Y. Ko, M. H. Tai, H. C. Ke, D. W. Yeh*, et al.*, "Heat shock protein 60 protects skeletal tissue against glucocorticoid-induced bone mass loss by regulating osteoblast survival," *Bone,* vol. 49, pp. 1080-9, Nov 2011.
[68] N. E. Ajubi, J. Klein-Nulend, M. J. Alblas, E. H. Burger, and P. J. Nijweide, "Signal transduction pathways involved in fluid flow-induced PGE2 production by cultured osteocytes," *Am J Physiol,* vol. 276, pp. E171-8, Jan 1999.
[69] P. P. Cherian, A. J. Siller-Jackson, S. Gu, X. Wang, L. F. Bonewald, E. Sprague*, et al.*, "Mechanical strain opens connexin 43 hemichannels in osteocytes: a novel mechanism for the release of prostaglandin," *Mol Biol Cell,* vol. 16, pp. 3100-6, Jul 2005.
[70] S. Harada, J. A. Nagy, K. A. Sullivan, K. A. Thomas, N. Endo, G. A. Rodan*, et al.*, "Induction of vascular endothelial growth factor expression by prostaglandin E2 and E1 in osteoblasts," *J Clin Invest,* vol. 93, pp. 2490-6, Jun 1994.

[71] P. J. Bouletreau, S. M. Warren, J. A. Spector, D. S. Steinbrech, B. J. Mehrara, and M. T. Longaker, "Factors in the fracture microenvironment induce primary osteoblast angiogenic cytokine production," *Plast Reconstr Surg,* vol. 110, pp. 139-48, Jul 2002.

[72] C. Maniatopoulos, J. Sodek, and A. H. Melcher, "Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats," *Cell Tissue Res,* vol. 254, pp. 317-30, Nov 1988.

[73] E. Chung, A. C. Sampson, and M. N. Rylander, "Influence of heating and cyclic tension on the induction of heat shock proteins and bone-related proteins by MC3T3-E1 cells," *Biomed Res Int,* vol. 2014, Article ID 354260, 2014.

[74] K. Wei, Y. Li, K. O. Kim, Y. Nakagawa, B. S. Kim, K. Abe*, et al.*, "Fabrication of nano-hydroxyapatite on electrospun silk fibroin nanofiber and their effects in osteoblastic behavior," *J Biomed Mater Res A,* vol. 97, pp. 272-280, Jun 1 2011.

[75] S. H. Oh, I. K. Park, J. M. Kim, and J. H. Lee, "In vitro and in vivo characteristics of PCL scaffolds with pore size gradient fabricated by a centrifugation method," *Biomaterials,* vol. 28, pp. 1664-1671, Mar 2007.

[76] T. D. Roy, J. L. Simon, J. L. Ricci, E. D. Rekow, V. P. Thompson, and J. R. Parsons, "Performance of degradable composite bone repair products made via three-dimensional fabrication techniques," *J Biomed Mater Res A,* vol. 66, pp. 283-291, Aug 1 2003.

[77] M. P. Cuchiara, A. C. Allen, T. M. Chen, J. S. Miller, and J. L. West, "Multilayer microfluidic PEGDA hydrogels," *Biomaterials,* vol. 31, pp. 5491-5497, Jul 2010.

[78] A. P. Golden and J. Tien, "Fabrication of microfluidic hydrogels using molded gelatin as a sacrificial element," *Lab Chip,* vol. 7, pp. 720-725, Jun 2007.

[79] G. Huang, X. Zhang, Z. Xiao, Q. Zhang, J. Zhou, F. Xu*, et al.*, "Cell-encapsulating microfluidic hydrogels with enhanced mechanical stability," *Soft Matter,* vol. 8, pp. 10687-10694, 2012.

[80] N. Annabi, N.E. Vrana, P. Zorlutuna, F. Dehghani, and A. Khademhosseini, "Engineering Biomimetic Scaffolds (Chapter 7)," in *Scaffolds for Tissue Engineering: Biological Design, Materials, and Fabrication*, C. Migliaresi and A. Motta, Eds., ed: Pan Stanford Publishing Pte. Ltd., 2014, pp. 199-238.