INVESTIGATION OF HEAT SHOCK ON TENDON AND LIGAMENT CELLS IN VITRO

By

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ABSTRACT OF THE THESIS

Investigation of Heat Shock on Tendon and Ligament Cells in Vitro

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Thesis Director:

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Tendons and ligaments (T/L) are collagenous connective tissues that play a role in locomotion and mechanical stabilization of the joints. These tissues possess relatively low cellularity and vascularity, resulting in a long and potentially incomplete healing response following injury. For sub-failure injuries such as strains and sprains, the most common course of treatment is an implementation of rest, ice, compression, and elevation. However, this method relies mostly on the tissue's natural ability to heal, which can leave the tissue prone to re-injury and failure. As a potential aid in the T/L healing process, we investigated the effects of thermal stress on human tenocytes in vitro. Heat shock has been demonstrated to improve regeneration in other tissues of the musculoskeletal system, including bone and muscle. Exposure of cells to an increase in temperature induces the production of heat shock proteins (HSPs) which act as molecular chaperones to assist in protein assembly and transport. In this project, heat shock at 40°, 44°, and 48°C was applied for 5, 10, 15, or 20 minutes. The data shows an increase in cellular proliferation following 15 and 20 minutes of thermal conditioning at 44° and 48°C as soon as 4 hours after heat shock. Protein secretion and gene expression of collagens type I and III and TGF-β suggest that that the heat shock response of tenocytes is similar to that of natural wound healing. Lastly, an in vitro scratch wound healing

assay revealed that the use of heat may hasten the recovery time following T/L sub-failure injuries.

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Chapter 1: Introduction

1.1 Tendon and Ligament Function and Anatomy

Tendons and ligaments (T/L) are similar types of connective tissue within the musculoskeletal system. Tendons connect muscle and bone, allowing for the motion of bone by transferring the forces generated by muscle. Ligaments connect bone to bone in a addition to functioning for joint stabilization. T/L share a similar composition consisting of fibroblasts supported by an extracellular matrix (ECM). The ECM is composed of primarily collagen, elastin, proteoglycans, and water.[1-3] Collagen is the most abundant protein in T/L, accounting for approximately 65 to 80% of the dry weight of tendons[4] and about 80% of ligament dry weight.[5] Several types of collagen compose T/L, but the most common types are type I and type III collagen. Approximately 85% of the collagen content is type I,[6] which exists in a nearly 9:1 ratio alongside type III collagen.[1] The collagen is arranged in a hierarchical structure that aids in the tissue's ability to be subjected to high tensile loads along their longitudinal axes.[7,8] At the lowest architectural level, the collagen molecule is made of glycine-X-Y chains arranged in a triple helical structure, where X and Y are commonly the amino acids proline and hydroxyproline.[9] The collagen molecules are arranged to form fibrils that exhibit a crimp pattern which helps T/L to stretch without sustaining irreversible damage.[7] Fibrils are further arranged into collagen fibers. Groups of collagen fibers create bundles known as fascicles that are aligned parallel along the loading axis of T/L tissue.

1.2 Tendon and Ligament Injury and Healing

There are approximately 32 million injuries to the musculoskeletal system that occur in the United States each year; T/L injuries account for 45% of that number.[10,11]

T/L injuries can occur when the collagenous structure of the tissue becomes damaged from excessive mechanical stress or genetic disorders such as Ehlers-Danlos Syndrome, where individuals possess deficiencies in healthy collagen production.[12] Tendon overuse or trauma can result in strain injuries or inflammation and irritation known as tendonitis. Ligament injuries that occur from excessive mechanical loading are referred to as sprains. Sprains can be classified into one of three grades according to the symptoms and clinical evaluation of the injury.[13,14] Grade I sprains are considered mild stretches with little to no macroscopic tearing and retention of tissue function and joint stability. Partial ligament tears are classified as grade II and are associated with symptoms such as moderate pain, swelling, and tenderness. Grade III sprains occur when there is a complete macroscopic tear through the ligament. Swelling, hemorrhage, and tenderness are common symptoms that occur during grade III sprains.

Following injury, T/L initiate a natural healing response that occurs in three overlapping phases. Immediately after injury, during the inflammatory phase, injured blood vessels in the tissue form a hematoma. This results in the release of chemotactic factors that increase vasculature and attract inflammatory cells to the site of injury.[15] Monocytes and macrophages then work to remove the blood clot and cellular debris through phagocytosis.[4,15] A proliferative phase occurs a few days after the start of the inflammatory phase. During this second phase of healing, fibroblasts recruited to the wound proliferate rapidly and produce a high concentration of ECM proteins and water content.[4,15] Larger amounts of type III collagen are produced and secreted than type I collagen, resulting in less organization and scar-like tissue formation.[16] The final phase of healing, remodeling and maturation, occurs after about six to eight weeks after the injury.

Cellular metabolic levels remain high as type I collagen production increases, but cell number, matrix production, and type III collagen expression decrease.[4,15] Over the course of up to a year, the healing tissue regains tensile strength as cellular metabolic levels and vascularization decrease. However, the resulting scar tissue will not possess the same mechanical properties that it had prior to injury.

1.3 Current Methods of Tendon and Ligament Treatment and Regeneration

T/L are relatively poorly vascularized tissue, and thus the healing response to injury is a slow process.[17] However, with proper care and attention, minor injuries such as grade I and II sprains can be treated non-operatively with rest, ice compression, and elevation (RICE method).[13] For more severe injuries where complete tears occur through the tissue, surgical intervention becomes necessary. Surgical procedures make use of biological grafts to replace the injured or diseased tissue with a healthy alternative. Surgical grafts include autografts derived from the patient's own body or allografts taken from a donor or cadaver. For example, common anterior cruciate ligament (ACL) repairs are performed by obtaining a portion of the patient's patellar, hamstring, or quadriceps tendon with the bony attachment.[2,18] The damaged ACL is removed and replaced by the tendon graft. This procedure is advantageous because the graft will possess good mechanical and biological properties, in addition to eliminating the risk of eliciting an unwanted immune response and graft rejection. However, the drawbacks of autografts include the need for two surgical sites, potential donor site morbidity, and an increased recover time.[19] On the contrary, allografts do not require a second surgery, but the foreign tissue can be rejected by the patient's body.[20] Sterilization of an allograft to prevent disease transmission is also associated with a compromise in the graft's mechanical

strength. As a potential future tool in T/L regeneration, tissue engineering involves the utilization of engineering and biological principles to develop alternative solutions to fully repair or aid in the healing of tissue.[21] Investigations of tissue engineering methods used for T/L regeneration include, but are not limited to, the use of biomaterial scaffolds and treatment with drugs or growth factors.

1.3.1 Biomaterial Scaffolds

Various materials have been investigated for their application in tendon and ligament tissue engineering. Among natural biomaterials, collagen is an obvious option to examine because of its contribution to T/L biological and mechanical functions. However, there are no current methods that allow for collagen organization in a structure similar to native tissue.[22] Collagen scaffolds have thus been observed to be fully resorbed and fail prematurely in vivo.[23] Conversely, silk is an alternative natural, biodegradable polymer used in T/L tissue engineering because of its high tensile strength, toughness, and ability to be manipulated to make gels, films, individual nanofibers, or braided fibers.[22] Recent studies have looked to use the mechanical properties of silk in conjunction with collagen to develop a prospective scaffold for T/L reconstruction.[24] Sterilization of these natural polymers is necessary before they can be implanted, potentially compromising the scaffolds mechanical's properties. For this reason, among others, investigators have studied a number of synthetic polymers for T/L reconstruction, including poly-L-lactic acid (PLLA),[25] polyglycolic acid (PGA),[26] polylactide-*co*-glycolide (PLGA),[26] polycaprolactone (PCL),[22] polyethylene glycol (PEG) based polymers,[27] and poly desaminotyrosyl-tyrosine ethyl carbonate (poly DTE carbonate).[28]

1.3.2 Growth Factors

During the T/L healing process, fibroblast cells excrete growth factors that are believed to assist in restoring the function of the tissue. Specifically, transforming growth factor-β (TGF-β), insulin-like growth factor-I (IGF-I), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) have been well studied for their roles in T/L wound healing.[29] The effects of these growth factors on wound healing are summarized in Table 1.1. TGF-β expression is elevated during the inflammatory phase of wound healing, during which it stimulates the migration and proliferation of cells within the site of injury.[15,30] TGF-β has also been demonstrated to increase collagen production and ECM deposition, while simultaneously suppressing the activity of matrix metalloproteinases (MMPs).[31] In vivo studies have shown that supplementing injured rats with TGF-β_1 significantly increased both the stiffness and load bearing properties of healing rat Achilles tendons.[32] Similar to TGF-β, IGF-I plays a role in the migration and proliferation of fibroblasts during the inflammatory and proliferative phases.[29] During the remodeling phase of healing, the increased expression of IGF-I stimulates collagen production and ECM deposition.[33] The synthesis of IGF-I and other growth factors is increased by the presence of PDGF during the proliferative phase.[29] Later, during the remodeling phase, PDGF causes an increase in both collagenous and non-collagenous proteins, increasing the structural properties of healing T/L tissue.[34] VEGF expression increases during the proliferative and remodeling phases to stimulate angiogenesis.[29] VEGF also plays a role in cell proliferation and the remodeling of the ECM to accommodate cellular migration.[35] Like VEGF, bFGF also

promotes cellular proliferation and angiogenesis.[36] It is believed that bFGF plays a role in modulating type III collagen expression and ECM deposition.[36]

Table 1.1 Growth Factors in Tendon and Ligament Healing	
Growth Factor	Role in T/L Healing
TGF-β	• Cell proliferation and migration[15,30] • Increases collagen production and ECM deposition[31] • Decreases MMP activity[31]
IGF-I	• Cell migration and proliferation[29] • Increases collagen production and ECM deposition[33]
PDGF	• Promotes synthesis of other growth factors[29] • Increases total protein production[34]
VEGF	• Cell proliferation and migration[35] • Angiogenesis[35]
bFGF	• Cell proliferation and migration[36] • Angiogenesis[36] • Modulates ECM deposition[36]

Exogenous delivery of the growth factors discussed above could potentially improve the healing response of T/L sub-failure injuries. It is also possible to stimulate an increase in the expression and production of growth factors through other means, such as mechanical loading or thermal preconditioning.

1.4 Heat and Heat Shock Proteins

The use of heat has not been extensively investigated for therapeutic use in T/L regeneration. However, several studies have demonstrated the benefits of heat and thermal conditioning for the regeneration of muscle,[37-39] skin,[40] cardiac tissue,[41] teeth,[42] and bone.[43-46] Heat induces an increase in the expression of a family of intracellular molecules known as heat shock proteins (HSPs). HSPs are molecular chaperones that play a role in protein folding and assembly, protein transport, and DNA replication and repair.[47] Thus, they are involved in mitosis, cellular differentiation, stabilization of the

cytoskeleton, protein processing, immune system control and macrophage activation, and wound healing.[46] Basal levels of HSPs are constitutively expressed, but increases in HSP expression can be induced from a variety of stresses other than heat, such as ischemia, hypoxia, hypothermia, ultraviolet radiation, depletion of ATP, free radicals, amino acid analogs, glucose analogs, heavy metals, protein kinase C stimulators, calcium-increasing agents, nitric oxide, microbial infections, hormones, or antibiotics.[48-50] HSPs are released into the extracellular milieu after cell death, where they can interact with other surviving cells.[51-53] Cells that have been exposed to an HSP inducing stress and survive become tolerant to subsequent applications of lethal or sub-lethal stress.[49]

HSPs are classified according to their molecular weight. They can be divided into six smaller subfamilies ranging from small HSPs (18-30 kDA) to the 100 kDa HSP (HSP100) family. In particular, the 47 kDa HSP (HSP47) and the 70 kDA HSP (HSP70) are of interest for T/L healing because of their functions in collagen production and cellular proliferation.

4.1.1 Heat Shock Protein 47

The folding of newly synthesized procollagen, the precursor to collagen, within the endoplasmic reticulum (ER) is assisted by the ER-resident chaperones HSP47, the 78 kDa glucose-regulated protein (GRP78, also called BiP), GRP94, protein disulfide isomerase (PDI), and prolyl 4-hydroxylase (P4H).[54,55] HSP47 is unique among chaperones because it has been observed to bind specifically to procollagen and collagen types I-V.[56-58] It is believed that HSP47 transiently binds to the collagen triple helical conformation in order to assist in the quality control of collagen production.[9,58] This includes accelerating the collagen folding process and prevention of insoluble

aggregation of procollagen molecules.[59] Studies have shown that HSP47 expression is an important part of healthy collagen production and ECM formation. For instance, it has been reported that the disruption of HSP47 activity in fibroblast cells resulted in abnormal collagen fiber production and an accumulation of procollagen aggregates within the ER.[55] Additionally, the lack of HSP47 in cells has been demonstrated to cause improper folding and diminished secretion of type IV collagen, an important component of the basement membrane.[54]

4.1.2 Heat Shock Protein 70

The HSP70 family consists of four members: the inducible HSP70 (also referred to as HSP72), the constitutively expressed HSC70 (or HSP73), mitochondrial HSP70 (mtHSP75), and GRP78.[60,61] As a molecular chaperone, HSP70 assists in the proper folding and assembly of newly synthesized proteins, refolds denatured proteins, disassembles aggregated proteins within the cell, assists in the translocation of proteins across membranes, and regulates the activity of proteins.[62,63] In response to a stress that causes protein damage, these functions of HSP70 improve cell survivability and protect cells from apoptosis.[64] HSP70 has been reported to promote cellular proliferation, evidenced by high levels of the chaperone molecule in proliferating and transformed cells.[65,66] This claim is further supported by the apparent translocation of HSP70 into the nucleus during the synthesis phase of the cell cycle.[67] The proliferative and anti-apoptotic effects of HSP70 have been noted in cancer models, where the expression of HSP70 is highly elevated.[68] As a result of HSP70 expression, tumors can become resistant to chemotherapeutic agents.[48]

1.5 Goal of the Thesis

The overall goal of this thesis was to investigate the effects of heat shock on cells in vitro as a potential therapeutic tool for sub-failure T/L injuries. This was done by heating cells at temperatures of 40°, 44°C, and 48°C for varying amounts of time. Data was collected to examine cellular proliferation, protein production, and gene expression after heat shock. It was hypothesized that the use of heat would induce HSP expression to improve cellular proliferation and ECM production in human tenocytes.

Chapter 2: Materials and Methods

2.1 Cell Culture

Human hamstring tenocytes (hHT) were cultured as a monolayer in tissue culture flasks with growth media consisting of alpha Minimum Essential Medium (αMEM) (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) in an incubator at 5% CO_2 and 37° C. Cells were obtained from a 25 year old male donor as outlined in a previous study by Ekwueme et al.[69] Upon reaching confluency, the cells were trypsinized, counted, and seeded onto tissue culture well plates with the same media conditions used for growth. Cells between passages 3 and 8 were used for the following studies.

2.2 Heat Shock

An incubator set at a constant temperature (T = 40°, 44°, 48°C) and 5% CO_2 was employed to provide heat shock to the experimental groups. Prior to the application of heat shock, αMEM media supplemented with 10% FBS and 1% P/S was preheated to the desired heat shock temperature. To apply heat shock, media was aspirated, wells were washed with phosphate buffered saline (PBS), and warm media was added to the monolayer of cells. A non-heated group served as a control. The cells were then incubated at the desired temperature for heating durations of 5, 10, 15 or 20 minutes. Subsequently, the warm media was removed, wells were washed with PBS, and fresh media at 37°C was added to the cells. Following the heat shock protocol, the cells were incubated at 37° to allow for recovery from the thermal stress and examined at time points of 4 and 8 hours post-heating.

2.3 Cellular Metabolic Activity

The metabolic activity of the cells (n=4) was evaluated using a Presto Blue Mitochondrial Activity/Cell Viability assay according to manufacturer's instructions. Briefly, at each time point following heat shock, media was aspirated from the wells and 10% (v/v) Presto Blue (Life Technologies) solution in αMEM media supplemented with 10% FBS and 1% P/S was added. The cells were incubated with the Presto Blue solution for one hour. Absorbance values were measured at 560 nm excitation and 590 nm emission wavelengths in technical duplicate using a microplate reader (Tecan, Medford, MA).

2.4 Secreted Protein Quantification

Conditioned medium (n=4) was collected from hHT at each time point following heat shock. The total protein concentration in the media was quantified using a Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA) according to manufacturer's instructions. The colorimetric assay was run in technical duplicate and the absorbance values were read at 562 nm using a microplate reader.

2.5 Immunostaining

To obtain images of cell morphology, cells were washed with PBS and fixed with 4% paraformaldehyde solution at various time points after heat shock. The fixed cells were permeabilized with 0.1% Triton X-100/PBS and blocked with 2% BSA/PBS-Tween (0.1%). Afterwards, the cells were stained with Phalloidin fluorophore (1:200) to image the cytoskeleton. The cells were then washed with PBS and counterstained for DAPI to image nuclei. Images were taken using an EVOS FL Microscope (Life Technologies). The images were analyzed using ImageJ software (NIH).

2.6 Real-Time Quantitative Polymerase Chain Reaction (qPCR)

Gene expression of collagen (types I and III), TGF-β, HSP47, and elastin 4 hours post-heating was assessed using qPCR. Briefly, total RNA (n=3) was extracted from each group and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). The concentration of isolated RNA in each sample was measured using a NanoDrop 2000c Spectrophotometer (Life Technologies). After normalization, RNA was converted to cDNA using the Reverse Transcription System (Promega Corporation, Madison, WI). Following reverse transcription, the cDNA was mixed with SYBR Green PCR Master Mix (Life Technologies) and the primers for markers of interest. The forward and reverse primer sequences are presented in Table 2.1. The samples and reagents were subjected to qPCR using a PikoReal Real Time PCR System (Thermo Scientific). Relative fold induction of genes was calculated using the $\Delta\Delta C_T$ method and normalized with GAPDH, which served as an endogenous control.

Table 2.1 Primer Sequences and Product Sizes for qPCR

Gene		5' DNA sequence 3'	Product Size (bp)
Collagen I	Forward	5' GTCACCCACCGACCAAGAAACC 3'	121
	Reverse	5' AAGTCCAGGCTGTCCAGGGATG 3'	
Collagen III	Forward	5' GCCAACGTCCACACCAAATT 3'	88
	Reverse	5' AACACGCAAGGCTGTGAGACT 3'	
HSP47	Forward	5' TGCTGAGCCCGGAAACTC 3'	63
	Reverse	5' TTCAGGGCAGGCAGAATG 3'	
TGF-β_1	Forward	5' CCCAGCATCTGCAAAGCTC 3'	101
	Reverse	5' GTCAATGTACAGCTGCCGCA 3'	
Elastin	Forward	5' GGCCATTCCTGGTGGAGTTCC 3'	106
	Reverse	5' AACTGGCTTAAGAGGTTTGCCTCCA 3'	
GAPDH	Forward	5' CTGGGCTACACTGAGCACC 3'	101
	Reverse	5' AAGTGGTCGTTGAGGGCAATG 3'	

2.7 Scratch Wound Healing Assay

Migration of hHT was assessed using a scratch wound healing assay as previously described by Liang et al.[70] Briefly, $5x10^4$ cells/cm^2 were seeded into 24 well plates and allowed to reach confluence (n=3). A scratch to simulate a wound was applied across the cell monolayer with the use of either a 1 mL or a 200 µL pipette tip. After the scratch was induced, the culture medium was replaced with fresh medium and heat shock was applied as discussed above. Cells in the heat shock group were treated with 15 minutes of heat at 48°C. The migration of cells as the wound closed was monitored using light microscopy after various time points. The images were analyzed using ImageJ.

2.8 Statistical Analysis

All of the quantitative data is presented as mean ± standard deviation. A two-way ANOVA with a Bonferroni's multiple comparison post-hoc analysis was utilized for comparing groups in the metabolic activity and protein secretion studies. A Student's *t*-test was employed for comparing groups in the qPCR and scratch assay experiments (OriginPro Software 2016, Northampton, MA). Unless stated otherwise, differences are reported as statistically significant for *p*-values less than 0.05.

Chapter 3: Results

3.1 The Effect of Heat Shock on Cellular Metabolic Activity

Using the Presto Blue fluorometric assay, the metabolic activity of cells that underwent heat shock was measured 4 and 8 hours post-heating (Figures 3.1A and 3.1B, respectively). Following 4 hours of recovery from heat shock, there were significant increases in cellular activity when cells were conditioned at 40°C for 15 min, 44°C for 15 min ($p<0.01$) and 20 min ($p<0.001$), and 48°C for 15 and 20 min ($p<0.001$) when compared to a non-heated control. Furthermore, metabolic activity was significantly higher for cells heated at 48°C for 20 min ($p<0.01$) than all other groups except for the cells heated for 15 min at the same temperature. Heating at 48°C for 15 min increased metabolic activity compared to all 40°C groups, 44°C groups heated for durations of 10 minutes or less ($p<0.001$), and 48°C groups heated for durations of 10 min or less ($p<0.01$). Metabolic activity was significantly lower for cells heated at 44°C for 5 min when compared to groups heated for 10 min or more at 40°C or 48°C, as well as 15 or 20 min of heating at 44°C ($p<0.001$). Thermal conditioning of cells at 48°C for 5 min resulted in significantly lower metabolic activity than cells heated at 44°C for 15 min ($p<0.01$) and 20 min.

At 8 hours post-heating, there were significant decreases in metabolic activity for cells that were heated at 40°C for 10 min and significant increases in activity among cells that were heated at 48°C for 15 and 20 min compared to the non-heated control. A trend showed that heating at 44°C for less than 20 min decreased metabolic activity 8 hours after heat shock. A 20 min heat shock at 48°C resulted in significantly higher metabolic activity among cells compared to all 40°C and 44°C groups ($p<0.001$) and cells heated at

48°C for durations of 10 min or less. Cellular activity was also significantly higher for cells heated at 48°C for 15 min compared to all 40°C and 44°C groups. Heating at 48°C for 10 min caused significantly higher metabolic activity compared to heating at 40°C for 10 min or less and heating at 44°C for 15 min or less. Significantly higher metabolic activity was seen among cells heated at 48°C for 5 min compared to those heated at 44°C for 15 min or less ($p<0.01$) and 40°C for 10 min or less ($p<0.01$). Significant comparisons among heated groups are summarized in Table 3.1.

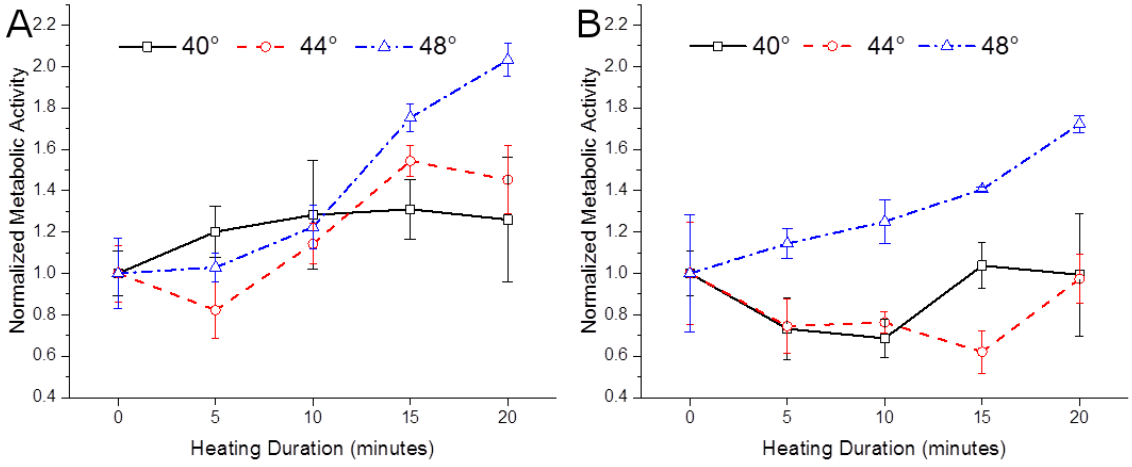

Figure 3.1: Normalized metabolic activity of hHT 4 hours (A) and 8 hours (B) post-heating.

Table 3.1 Significant Comparisons of Metabolic Activity Among Heated Groups			
Hours Post-heating	Group		Significantly higher metabolic activity than groups heated at:
	Temperature	Duration	
4 hours	40°C	10 min	44°C for 5 min
		15 min	44°C for 5 min (#)
	44°C	15 min	44°C for 5 min (%); 48°C for 5 min (#)
		20 min	44°C for 5 min (%); 48°C for 5 min
	48°C	10 min	44°C for 5 min
		15 min	40°C for 5 min (%); 40°C for 10 min (#); 40°C for 15 min; 40°C for 20 min (#); 44°C for 5 min (%); 44°C for 10 min (%); 48°C for 5 min (%); 48°C for 10 min (#)
		20 min	40°C for 5 min (%); 40°C for 10 min (%); 40°C for 15 min (%); 40°C for 20 min (%); 44°C for 5 min (%); 44°C for 10 min (%); 44°C for 15 min (#); 44°C for 20 min (%); 48°C for 5 min (%); 48°C for 10 min (%)
8 hours	40°C	15 min	40°C for 10 min; 44°C for 15 min (#)
	44°C	20 min	44°C for 15 min
	48°C	5 min	40°C for 5 min (#); 40°C for 10 min (%); 44°C for 5 min (#); 44°C for 10 min (#); 44°C for 15 min (%)
		10 min	40°C for 5 min (%); 40°C for 10 min (%); 44°C for 5 min (%); 44°C for 10 min (%); 44°C for 15 min (%)
		15 min	40°C for 5 min (%); 40°C for 10 min (%); 40°C for 15 min; 40°C for 20 min (#); 44°C for 5 min (%); 44°C for 10 min (%); 44°C for 15 min (%); 44°C for 20 min (#)
		20 min	40°C for 5 min (%); 40°C for 10 min (%); 40°C for 15 min (%); 40°C for 20 min (%); 44°C for 5 min (%); 44°C for 10 min (%); 44°C for 15 min (%); 44°C for 20 min (%); 48°C for 5 min (%); 48°C for 10 min

Mean comparisons were considered significant for $p<0.05$ (#$p<0.01$, %$p<0.001$).

3.2 Serum Protein Concentration Following Heat Shock

Conditioned medium was collected at 0 hours (immediately after heat shock), 4 hours, and 8 hours post-heating and analyzed for the total protein concentration in the serum (Figures 3.2A, 3.2B, and 3.3C, respectively). Immediately after heat shock, there

was significantly more total protein in the groups heated at 40°C for 5 and 20 min and groups heated at 48°C for 5, 15, and 20 min compared to those heated for 10 and 15 min at 40°C ($p<0.01$). Serum protein levels were higher when the cells were treated at 48°C for 5 min compared to the non-heated control ($p<0.001$), all groups heated at 44°C ($p<0.01$), and the serum from cells heated at 48°C for 10 min ($p<0.001$).

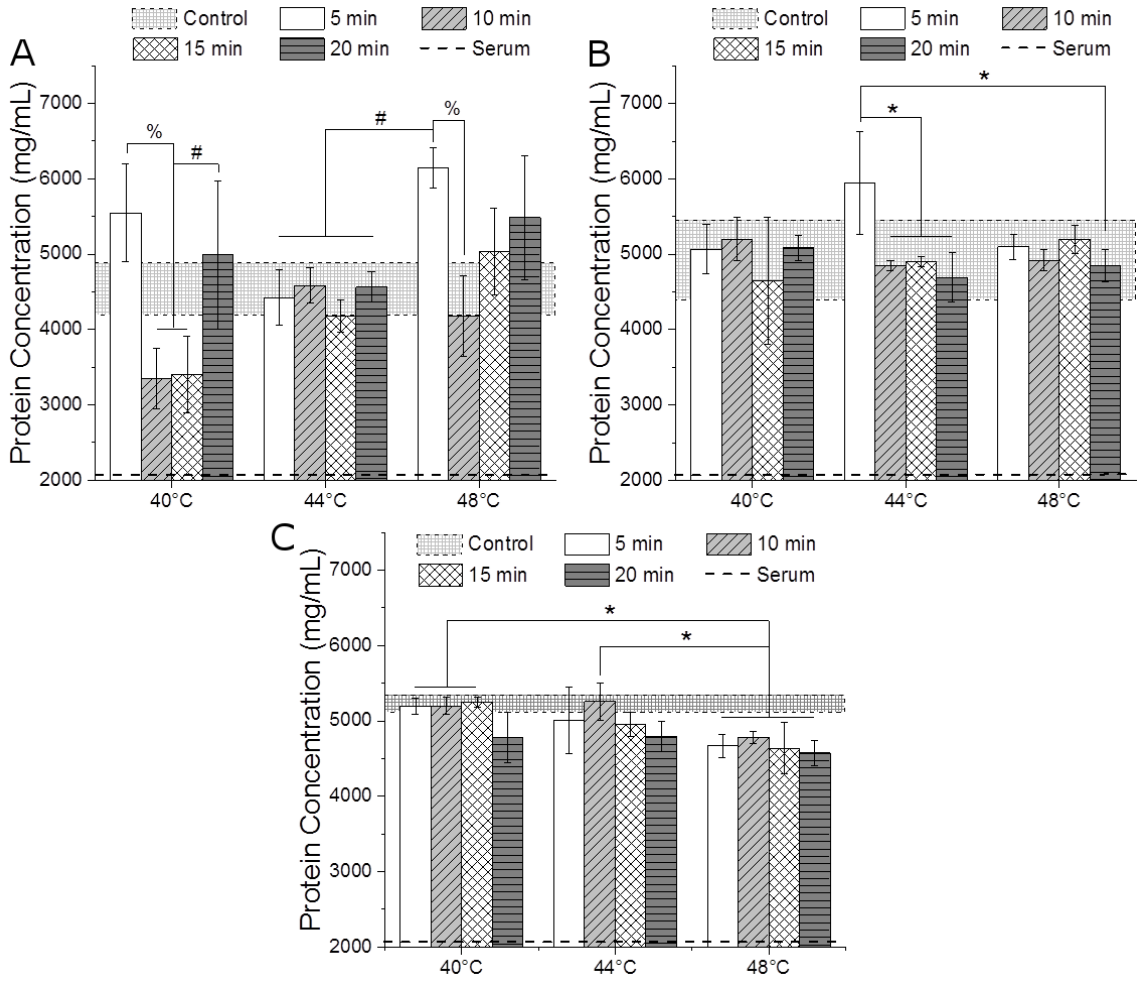

Figure 3.2: Serum protein concentration immediately (A), 4 hours (B), and 8 hours (C) following heat shock (*$p<0.05$, #$p<0.01$, %$p<0.001$). Medium collected from cells that were not exposed to heat shock served as the control. Non-treated serum had a protein concentration of 2070 mg/mL.

At 4 hours after heat shock, conditioned medium protein concentration was significantly higher among cells heated at 44°C for 5 min compared to those heated at the same temperature for longer durations. At 8 hours of recovery post-heating, the groups heated at 48°C had significantly lower protein content in serum than the non-heated control, groups heated at 40°C for 5 to 15 min, and cells heated at 44°C for 10 min.

3.3 Morphological Changes Following Heat Shock

Fluorescent images were taken of the cytoskeleton (green) and nuclei (blue) of cells 4 (Figure 3.3) and 8 hours (Figure 3.5) after heat shock. Mean fluorescence values 4 hours (Figure 3.4) and 8 hours (Figure 3.6) are provided from the cytoskeleton images.

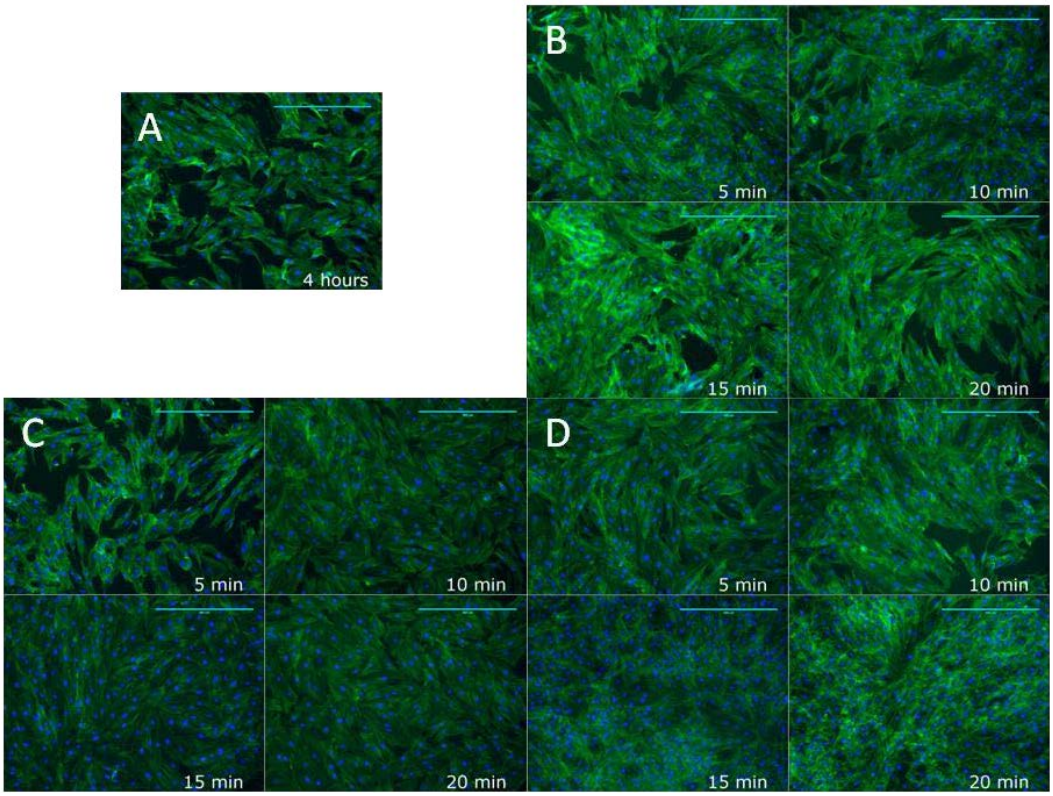

Figure 3.3: Fluorescent images of cells 4 hours post-heating. (A) Control; (B) 40°C heat shock; (C) 44°C heat shock; (D) 48°C heat shock (scale bar = 400 μm).

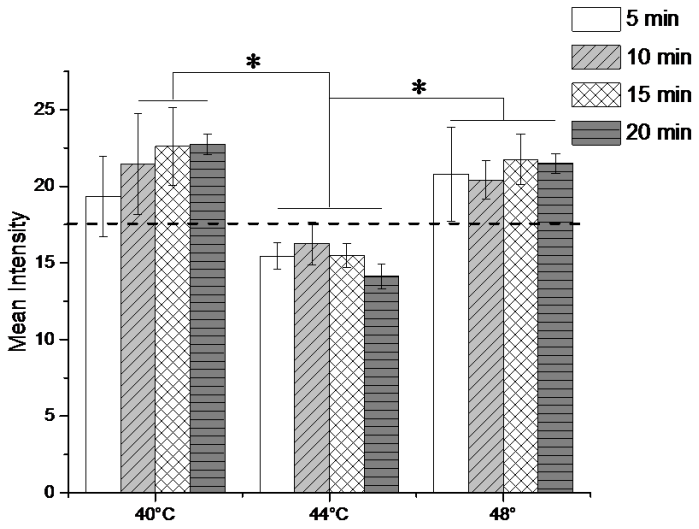

Figure 3.4: Mean intensity values of cytoskeleton fluorescence 4 hours following heat shock (*$p<0.05$). The average intensity of the non-heated cells was 17.57±1.51

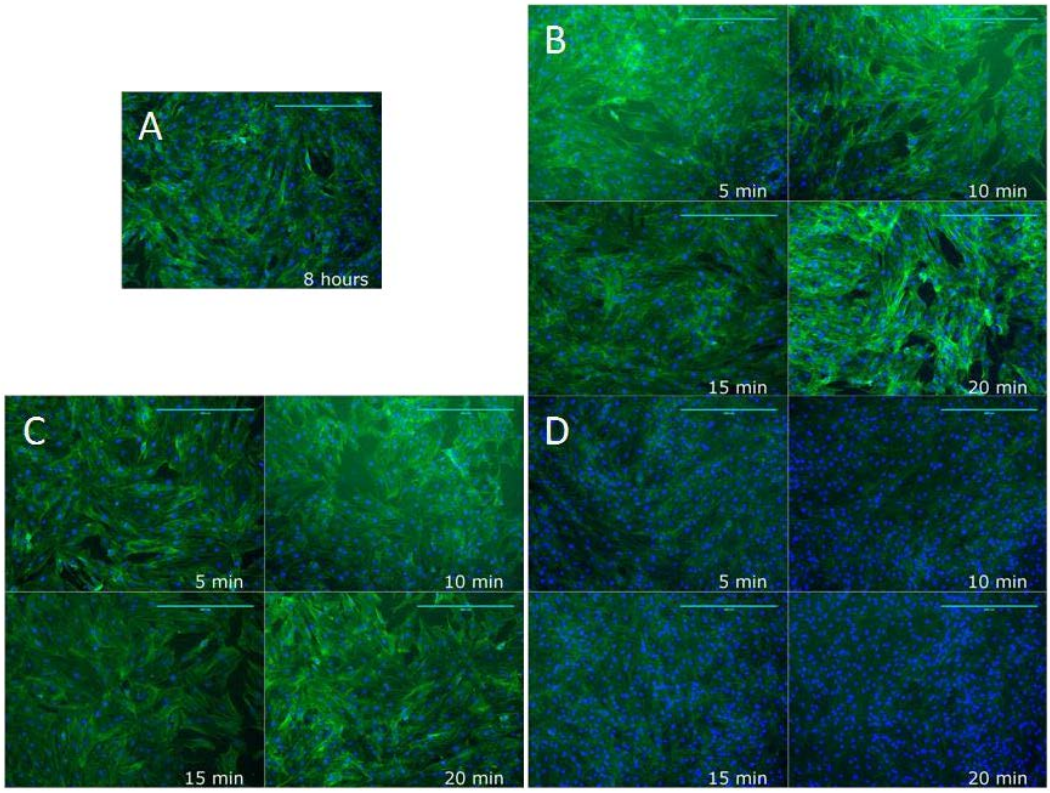

Figure 3.5: Fluorescent images of cells 8 hours post-heating. (A) Control; (B) 40°C heat shock; (C) 44°C heat shock; (D) 48°C heat shock (scale bar = 400 μm).

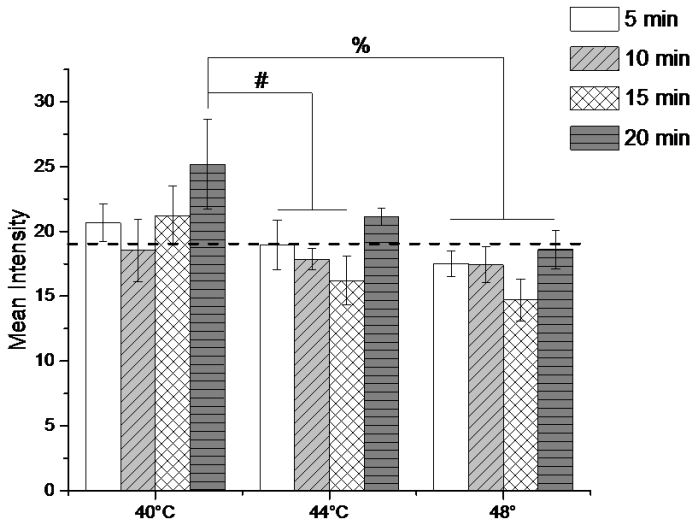

Figure 3.6: Mean intensity values of cytoskeleton fluorescence 8 hours following heat shock (#p<0.01, %p<0.001). The average intensity of the non-heated cells was 19.05±1.67.

3.4 Gene Expression Following Heat Shock

The relative expression of collagen types I and III, HSP47, TGF-β_1, and elastin was determined by running qPCR 4 hours post-heating at 44°C (Figure 3.7A) and 48°C (Figure 3.7B). Analysis of type I collagen and elastin showed a significant decrease in expression among all groups heated at 44°C. There was a significant decrease in collagen expression in the cells heated at 48°C for 5 min. Type III collagen expression was significantly higher than the non-heated control following 5 min of heat shock at 44°C. Type III collagen and TGF-β_1 expression trended to increase following 44°C heat shock compared to the control. HSP47 expression was significantly overexpressed following 15 min of thermal conditioning at 48°C. No data was obtained for the expression of HSP47 in cells heated at 48°C for 20 min.

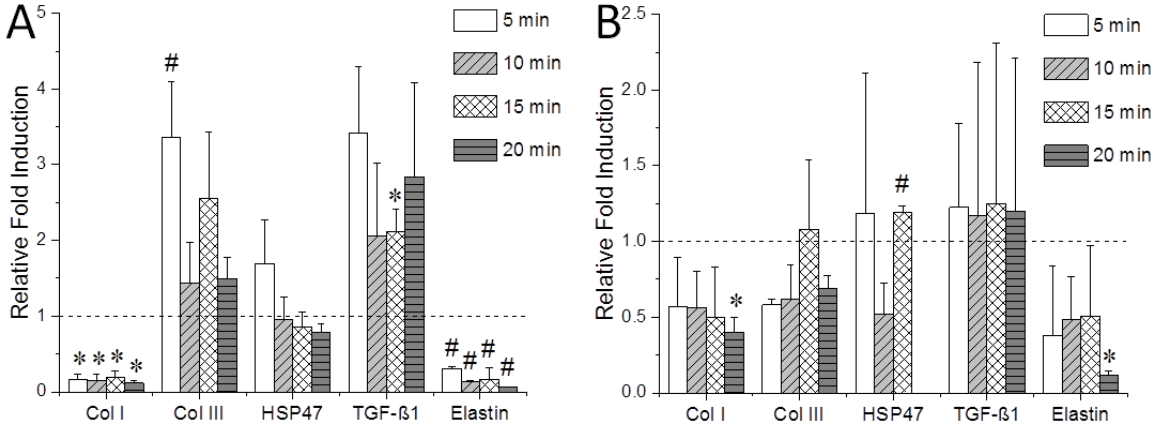

Figure 3.7: Relative gene expression 4 hours following heat shock at 44°C (A) and 48°C (B) (*$p<0.05$, #$p<0.01$).

3.5 Scratch Wound Healing Assay

A scratch was applied across a monolayer of cells to simulate a wound in vitro. The cells were then subjected to heat shock at 48°C for 15 minutes. These parameters were chosen based on the increase in proliferation seen 4 and 8 hours after heat shock and the gene expression of collagen and TGF-β_1 4 hours post-heating. Cells that were not treated with heat after the creation of the scratch served as the control. The closure of the induced wound was monitored 4, 8, 12, 16, 24, 48, and 72 hours following heat shock. The wound had closed fully by the 48 hour time point for all groups. The initial wound widths are provided in table 3.2. The closure of the scratch is shown in Figure 3.8A, and the average rate of cellular migration is displayed in Figure 3.8B. Among the scratches created using a 1 mL pipette tip, heat shock produced a significant relative closure of the wound after 8 hours ($p<0.01$). Among the groups where a 200 µL was employed to create the scratch, there was a trend which showed that heat shock caused the wound the close

relatively quicker than the control between 4 and 12 hours. Heat also produced a significantly larger cell migration rate between the 0 and 4 hour time points.

Table 3.2: Initial Scratch Assay Wound Width	
Group (Size of pipette used to make scratch)	Wound Width (μm)
Control (1 mL)	817.05 ± 138.08
Heat (1 mL)	971.37 ± 85.01
Control (200 μL)	722.29 ± 117.44
Heat (200 μL)	758.82 ± 81.30

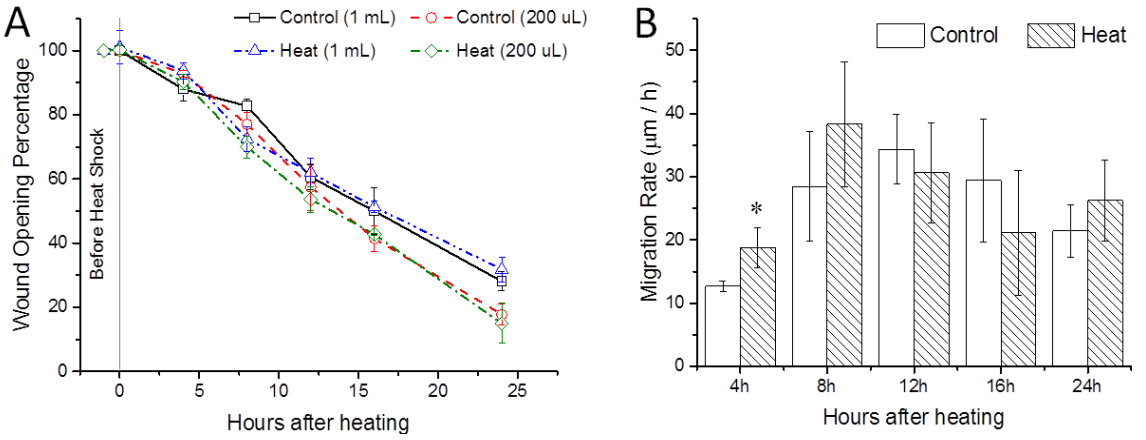

Figure 3.8: The effects of heat shock on the closure of a scratch in vitro. (A) The relative opening of the induced "wound." (B) The average cell migration rate of cells between time points across the scratch created with a 200 μL pipette tip.

Chapter 4: Discussion

This study investigated the effects of thermal conditioning on the proliferation and wound healing potential of T/L cells in vitro. To the best of our knowledge, this is the first study to assess the effects of heat shock on human tenocytes in vitro. However, thermal conditioning and HSPs have been determined to produce beneficial effects on other cells of the musculoskeletal system. Mild heat shock of 42°C has been shown to improve myogenesis and muscle development.[37-39] Osteoblasts and bone formation have also been seen to benefit from the application of thermal stress.[43-46] Based on previous studies, we hypothesized that heat shock could be used to improve the cellular proliferation and matrix production of T/L fibroblasts.

T/L contain relatively low cellularity compared to other tissues within the body, resulting in a slow healing process following injury.[17] Improving cellular growth and proliferation is a field of interest in order to improve T/L healing from sub-failure injuries. The data presented in this study shows that heat shock was able to significantly improve cellular proliferation after 4 hours, particularly following the application of thermal stress at 44° or 48°C for 15 or 20 minutes. Previous studies have shown that following heat shock, there is an accumulation of HSP70 within the nucleus.[71] Localization of HSP70 in the nucleus has also been observed during the synthesis phase of the cell cycle.[67,72] Thus, it can be inferred that heat shock stimulates an increase in cellular proliferation, which is consistent with the data obtained from the treated hamstring tenocytes. It is also interesting to see that there is a decrease in metabolic activity and cellular proliferation relative to the control when cells are heated at 44°C for 5 minutes, but an increase is observed if the cells are allowed to be heated for an

additional 10 or 15 minutes. This suggests that there may be a brief period of adaptation required in order for the cells to respond to heat shock which could be temperature and time dependent. Following 8 hours after heat shock, only the cells heated at 48°C displayed an increase in metabolic activity and cellular proliferation compared to the control. 20 minutes of heat shock at 48°C significantly increased proliferation of cells compared to all other groups, as seen by the abundance of nuclei in the fluorescent images. Longer durations of applied stress at 40°C and 44°C resulted in proliferation similar to the control, while shorter times decreased relative cellularity.

The effects of heat shock on protein secretion were determined by measuring the total protein concentration in conditioned medium. Immediately after heat shock, a trend can be seen among the 40° and 48°C heat shock groups where the media contained more protein 5 minutes after heat shock, followed by a dip in serum protein levels at 10 minutes. Total protein content in the conditioned media started to increase again following longer durations of heat shock. One possible reason for this observation could be that the initial application of heat could trigger the release of soluble factors that serve as stress signals and interact with nearby cells. The initial insult can also cause the death of several cells within the culture, releasing HSP70 into the extracellular milieu that would otherwise be localized within the cells.[51] Extracellular HSP70 can then interact with surviving cells, and the internalization of the protein can improve the new host's thermotolerance.[52] The uptake of protein after the initial heat shock may occur rapidly, as seen by the drop in serum protein content after 10 minutes of thermal conditioning. Upon adjusting to the increase in temperature, the cells may then start producing and secreting matrix proteins important for the wound healing process. However, this potential

explanation of the cellular response to heat shock is challenged by the data seen in the 44°C group. The same trend seen among the 40° and 48°C protein data is not seen at 44°C, but a decrease in cellularity after 5 minutes of heat shock at 44°C followed by an increase after longer durations of stress are noted at 4 hours.

Overall, heat shock at 40°C did not appear to produce a beneficial cellular response compared to the non-heated control. Therefore, this group was omitted from mRNA analysis. PCR was performed on mRNA collected 4 hours post-heating to determine the effect of heat shock on gene expression. Heat shock at 44°C caused a significant downregulation of type I collagen among all heating durations, but a trend in type III collagen upregulation was observed. A trend showed a decrease in type I collagen expression following 48°C heat shock as well. Type III collagen appeared to be expressed more than type I collagen when cells were heated at 48°C for 15 and 20 minutes. HSP47 plays a major role in the production and secretion of collagen molecules.[9,55] However, differences in HSP47 expression were not seen at 44°C, but a significant upregulation of the collagen-specific chaperone was seen in cells heated at 48°C for 15 minutes. The upregulation of HSP47 can lead to an increase in collagen production and matrix deposition at later time points. TGF-β expression has also been linked with collagen and ECM production in fibroblasts.[30,31,73] TGF-β_1 expression was seen to increase following heat shock at 44°C, with significant upregulation of the gene occurring after 5 and 15 minutes. Type III collagen appeared to be coregulated with TGF-β_1 following these two heating durations. Additionally, a trend showed an slight increase in TGF-β_1 expression 4 hours after a 48°C heat shock. These findings suggest that heat shock induces a response in tenocytes similar to that of wound healing.

Wound healing in T/L occurs in three phases: inflammation, proliferation, and remodeling and maturation. During the proliferative phase, fibroblasts rapidly increase in number, working to produce a large concentration of matrix proteins.[4,15] This phase is also characterized by an increase in type III collagen production and a decrease in type I collagen expression.[16] The characteristics of the proliferative phase are seen in the cellular proliferation and mRNA expression data obtained 4 hours post-heating. A decrease in cell number is observed during the remodeling and maturation phase of wound healing.[4,15] A trend displaying a decline in metabolic activity is seen 8 hours after the 44° and 48°C heat shock, when comparing normalized values. Therefore, it was hypothesized that in addition to causing a wound healing response in vitro, heat shock may also accelerate the process. To test this hypothesis, a scratch wound healing assay was performed to compare the closure of an in vitro "wound" and cellular migration after 15 minutes of thermal conditioning at 48°C with a non-heated control. When comparing wound closure of scratches of similar initial width, a trend showed that between 4 and 12 hours after thermal treatment, heat shock induced a better healing response than the control. During later time points, the control and heated groups displayed a similar healing response. Further in vivo studies are needed to determine whether heat shock does accelerate natural T/L wound healing. If this is the case, then thermal stress could be used as a therapeutic tool for sub-failure T/L injuries. A shorter healing period can also help minimize further or repeated injury that may occur before the tissue is fully healed.

Chapter 5: Conclusions and Future Work

In conclusion, this thesis investigated the effects of thermal conditioning on human tenocytes in vitro. Heat shock proteins are known to play a role in cellular proliferation and protein production. Heat shock appeared to improve cellular proliferation after the cells were stressed for 15 or 20 minutes at temperatures of 44° and 48°C. Serum protein concentrations and gene expression suggest that the heat shock response of tenocytes is similar to the natural healing response of T/L tissue. If thermal stress accelerates the healing response and results in the generation of healthy extracellular matrix, heat shock could be a potential tool in treating sub-failure T/L injuries that would otherwise take several weeks to heal. Further investigation of heat shock on T/L fibroblasts is needed to determine if it is a viable therapeutic tool. In vivo heat shock experiments can provide more insight on the effect of heat on the wound healing capacity of living tissue. Additionally, heat shock can be used in combination with other therapies, such as exogenous growth factor delivery, to develop a hybrid technique to enhance tendon and ligament repair.

Appendix A: The Effects of Heat Shock on Human Achilles Tenocytes and Mesenchymal Stem Cells In Vitro

A.1 Abstract

Due to the poor healing capabilities of ligaments and tendons (L/T), current treatments involve the use of biological or tissue engineered grafts to replace the injured tissue. However, these surgical methods are often limited by a lack of ideal physiological, biological, and mechanical properties among grafts. As a potential alternative for tissue regeneration and method to improve graft response, investigators have explored the use of thermal stress conditioning protocols to enhance tissue development in bone and cartilage. Thermal stress induces the production of heat shock proteins (HSPs) within cells, which then improve cell thermotolerance, proliferation, and matrix protein production and secretion. In this project, we investigated the effect of thermal conditioning on the cellular response of human Achilles tenocytes (hAT) and bone marrow-derived stromal cells (hMSC). Following exposure to thermal stress at 44° C, hAT and hMSC both experienced similar changes in metabolic activity. After five minutes of exposure, metabolic activity dropped compared to a non-heated control, but after 10-20 minutes of heating, the cells appeared to be able to recover and displayed comparable activity to the control. After exposure to thermal stress, hAT displayed an increase in total protein secretion in media compared to the control. Among these proteins, a trend showed that the mRNA expression of HSP47 and collagen I was increased after 5 minutes of heating. On the contrary, hMSC secreted less protein with an increase in heating time. Thermal conditioning applied for 10-20 minutes appeared to

improve the response of protein secretion among tenocytes while maintaining their metabolic activity. The opposite appeared true for the stromal cells.

A.2 Introduction

Ligaments and tendons (L/T) are connective tissue in the musculoskeletal system that share similar characteristics. Ligaments hold bones together and stabilize the joints, while tendons lie between muscle and bone to transfer the forces generated by muscles into the movement of limbs. L/T are composed of mostly water; collagen (types I, III, V, VI, XI, XIV) accounts for most of the dry weight of the tissue, with the rest of the solid components consisting of proteoglycans, elastin, glycoproteins, and other proteins.[6,22] L/T exhibit a hierarchical structure of collagen arrangement.[8,11] Bundled fascicles arranged in a parallel formation are constructed of collagen fibers, which are subdivided into fibrils.[7] The fibrils are further divided into microfibrils which are composed of individual collagen molecules. An insult to the L/T structure can result in varying degrees of injury. Often, L/T injuries involve the tearing of the tissue, with complete tears through the tissue requiring surgical reconstruction.[15] For less severe injuries, treatment can be administered via rest, ice, compression, and elevation (RICE).[13] This method makes use of the natural healing process of L/T, which is divided into inflammation, proliferation, and maturation phases.[4]

Growth factors such as transforming growth factor-β (TGF-β) play a prominent role during L/T wound healing. TGF-β is expressed during all phases of L/T healing, during which it is involved in cellular migration, cellular proliferation, and types I and III collagen production.[15,31] TGF-β has also been suggested to regulate the expression of heat shock protein 47 (HSP47), a chaperone protein that assists in the synthesis of collagen

molecules.[53] Heat shock proteins (HSPs) are a family of chaperone molecules, classified by their molecular weight, which serve a variety of functions within the cell, including protein folding, prevention of protein aggregation, and protein transport.[47] HSPs are constitutively expressed under homeostatic conditions, but in response to thermal, mechanical, or oxidative stress, their expression is upregulated to develop the cell's tolerance to subsequent stresses.[49] In particular, the 70 kDa HSP (HSP70) has been thoroughly investigated to promote thermotolerance of cells after the application of a sub-lethal heat stress.[64,74] HSP70 has also been observed to induce cellular proliferation and prevention of apoptosis.[50,66]

Through the use of heat shock, the induction of HSP production could potentially present a method of L/T treatment and repair following sub-failure injuries. To test this hypothesis, thermal stress was applied to cells in vitro, and the effects of heat shock on cell viability, protein secretion, and gene expression were investigated. In the field of L/T tissue engineering, there is a debate on the cell source that would produce the best results in tissue regeneration. Investigators have indicated that mesenchymal stem cells (MSC) have more potential for L/T regeneration than ligament fibroblasts or tenocytes.[5,75-78] Previous research has shown greater matrix deposition and faster proliferation rates in MSC than L/T cells.[79-81] Furthermore, MSC have the potential to differentiate into L/T phenotypes.[80,82] In the present study, human Achilles tenocytes and MSC were subjected to a temperature of 44°C for varying periods of time. The effects of heat shock on both cell types were monitored 12 hours post-heating.

A.2 Materials and Methods

A.2.1 Cell Culture

Immortalized human Achilles tenocytes (hAT) transfected to express green fluorescent protein (GFP) were cultured as a monolayer with growth media composed of alpha Minimum Essential Medium (αMEM; Life Technologies, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) in an incubator at 5% CO_2 and 37° C. Human mesenchymal stem cells (hMSC) were cultured with growth media composed of αMEM supplemented with 20% FBS and 1% P/S in an incubator at 5% CO_2 and 37° C. Cells were grown in 175-cm^2 T-flasks until they reached confluency. Then, they were trypsinized, counted, and seeded onto well plates with a density of $2.5x10^4$ cells/cm^2. Plated cells were cultured with αMEM supplemented with 10% FBS and 1% P/S and allowed to attach overnight.

A.2.2 Heat Shock

An incubator at 5% CO_2 and 44° C was utilized to provide thermal conditioning to experimental specimens. Prior to applying heat shock to the cells, 10% FBS media was pre-warmed to 44° C. To apply the heat shock, media was aspirated, wells were washed with phosphate buffered saline (PBS), and fresh media warmed to 44° C was added to the monolayer of cells. The cells were then incubated at 44° C for the desired amount of time (5, 10, 15, 20 minutes). A separate group in which 10% FBS media warmed to 37° C was added to the cells and then incubated at 37° C served as a control. After the desired amount of time, media was aspirated from the wells, wells were washed with PBS, and fresh 10% FBS media at 37° C was added to the cells. The cells were then incubated at 37° C for a pre-determined amount of time (12 or 24 hours).

A.2.3 Viability Study

Following heat shock, a Presto Blue Mitochondrial Activity/Cell Viability assay was performed at the set time point (n=3). 10% Presto Blue (Life Technologies, CA) solution was prepared by adding Presto Blue to media composed of αMEM supplemented with 10% FBS and 1% P/S. The Presto Blue solution was then added to the monolayer of cells. Following one hour of incubation at 37° C, the conditioned Presto Blue media was transferred to a new 96 well plate. The absorbance values of the solution were measured at 560 nm excitation and 590 nm emission wavelengths in technical triplicate by a microplate reader (Tecan, Medford, MA). Absorbance values correlated to the relative metabolic activity of the cells, which served as an indirect measure of cell count.

A.2.4 Imaging

The GFP expression of the hAT was utilized to examine the effects of heat shock on cellular morphology. The cells were allowed 24 hours to recover from heat shock. They were then imaged for GFP using an EVOS® FL Microscope (Life Technologies). Intensity analysis of the images was performed using ImageJ software (NIH).

A.2.5 Total Protein Assay

A total protein assay was performed using the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA) according to manufacturer's instructions. Pooled media was collected from treated cells 12 hours after heat shock. Samples were run in triplicate and the absorbance values were read at 562 nm using a microplate reader.

A.2.6 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

The gene expression of select genes 12 hours after heat shock was evaluated with the use of qPCR. Briefly, RNA was extracted from the samples (n=3) and purified with

the use of the RNeasy Mini Kit (Qiagen, Valencia, CA). A NanoDrop 2000c Spectrophotometer (Life Technologies) was employed to determine the concentration of isolated RNA from each sample. RNA concentrations were normalized prior to cDNA conversion, which was performed using the Reverse Transcription System (Promega Corporation, Madison, WI). After reverse transcription, cDNA was mixed with SYBR Green PCR Master Mix (Life Technologies) and primers for the target gene. The forward and reverse primer sequences used in this study are provided in Table A.1. PCR was run through a PikoReal Real Time PCR System (Thermo Scientific). Relative gene expression was calculated by employing the $\Delta\Delta C_T$ method and normalization to glyceraldehyde 3-phosphate dehydrogenase (GADPH) as an endogenous control.

Table A.1: Primer Sequences and Product Sizes for qPCR

Gene		5' DNA sequence 3'	Product Size (bp)
Collagen I	Forward	5' GTCACCCACCGACCAAGAAACC 3'	121
	Reverse	5' AAGTCCAGGCTGTCCAGGGATG 3'	
Collagen III	Forward	5' GCCAACGTCCACACCAAATT 3'	88
	Reverse	5' AACACGCAAGGCTGTGAGACT 3'	
HSP47	Forward	5' TGCTGAGCCCGGAAACTC 3'	63
	Reverse	5' TTCAGGGCAGGCAGAATG 3'	
TGF-β_1	Forward	5' CCCAGCATCTGCAAAGCTC 3'	101
	Reverse	5' GTCAATGTACAGCTGCCGCA 3'	
GAPDH	Forward	5' ACAACTTTGGTATCGTGGAA '3	458
	Reverse	5' AAATTCGTTGTCATACCAGG '3	

A.2.7 Statistical Analysis

Quantitative data is presented as mean ± standard deviation. A one-way ANOVA with a Bonferroni's multiple comparison post-hoc analysis was used to compare groups in the viability study. A pairwise Student's t-test was utilized to compare groups in the PCR studies (OriginPro Software 2016, Northampton, MA). A p-value less than 0.05 was considered to be statistically significant, unless stated otherwise.

A.3 Results

A.3.1 The Effect of Heat Shock on Cell Viability

The metabolic activity of cells (hAT and hMSC) conditioned with thermal stress of 44° C for 0, 5, 10, 15, and 20 minutes was measured 12 and 24 hours post-heating (Figure A.1). Both hAT and hMSC showed a significant decline in metabolic activity following 5 minutes of heating compared to the control ($p<0.001$). It appeared that following 10 and 15 minutes of heating, both cell types were able to recover to their normal levels of activity. However, following 20 minutes of heating, the metabolic activity of hAT was significantly lower than the control cells. At 24 hours post-heating, there was a significant decrease in the metabolic activity of hAT that were heated for 10 minutes at 44°C ($p<0.01$). The other groups displayed similar levels of metabolic activity. Among the hMSC, there was a trend that showed an increase in the metabolic activity of cells subjected to heat shock compared to the control.

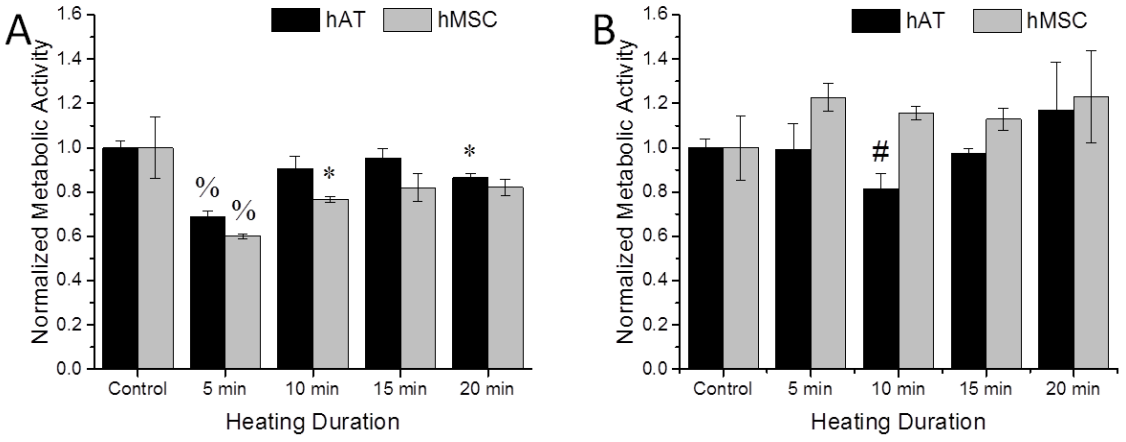

Figure A.1: Normalized metabolic activity of hAT and hMSC 12 hours (A) and 24 hours (B) post-heating at 44°C (*$p<0.05$, #$p<0.01$, %$p<0.001$).

A.3.2 The Effect of Heat Shock on Total Protein Content

The concentration of proteins in serum was measured from the conditioned media collected from the wells containing either hAT or hMSC 12 hours after they had been heated at 44° C for 0 minutes (control), 5 minutes, 10 minutes, 15 minutes, or 20 minutes (Figure A.2). Because pooled media was used to quantify serum protein levels, no error bars are present in the figure. For the media collected from the hAT groups, there was a trend that showed an increase in the total protein concentration of serum following heat shock. On the contrary, in the media collected from the hMSC groups, there was a trend that showed a decrease in the total protein concentration following 15 and 20 minutes of heating when compared to the control.

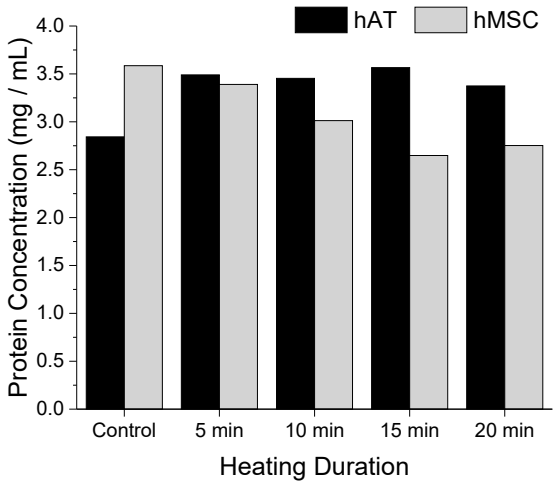

Figure A.2: Protein concentration 12 hours post-heating in conditioned media collected from hAT and hMSC samples.

A.3.3 Gene Expression After Heat Shock

Quantitative real-time polymerase chain reaction (qPCR) was run to determine the relative gene expression of hAT and hMSC 12 hours after heat shock (Figure A.3). The genes investigated were type I collagen (Col I), type III collagen (Col III), HSP47, and

TGF-β_1. There was a significant upregulation of type I collagen in the hAT heated for 15 minutes ($p<0.001$). The expression of TGF-β_1 in hAT was significantly lower than the control when the cells were heated for 10 or 15 minutes. There was little change among the expression of the investigated genes when thermally treated hMSC were compared to the control.

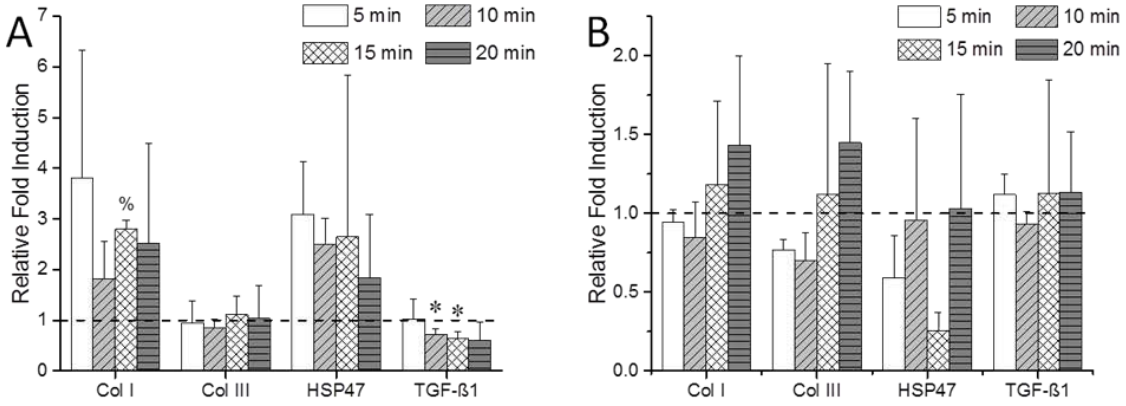

Figure A.3: Relative gene expression 12 hours after heat shock in hAT (A) and hMSC (B) (*$p<0.05$, %$p<0.001$).

A.3.4 Images of Cells After Heat Shock

Fluorescent images of hAT were taken 24 hours after heat shock (Figure A.5). From the images, it appeared that the cellular response improved among the cells heated for 5, 10, and 15 minutes in comparison to the control. The cells heated for 5 and 15 minutes also appeared to demonstrate more elongation than the other groups, which is suggestive of increased collagen production.

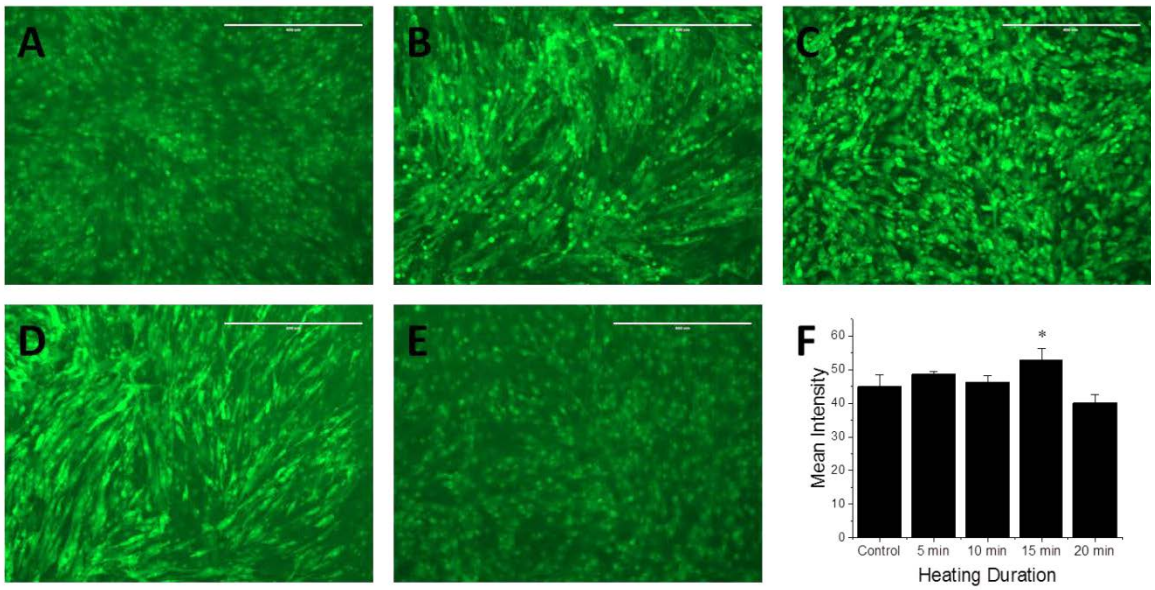

Figure A.4: Fluorescent images of hAT 24 hours following heat shock at 44°C for durations of 0 min (A), 5 min (B), 10 min (C), 15 min (D), and 20 min (E); mean intensity of the images (F). The scale bar represents a length of 400 μm (*$p<0.05$).

A.4 Discussion

In the present study, we examined the effects of heat shock at 44°C on human Achilles tenocytes (hAT) and human mesenchymal stem cells (hMSC) in vitro. It was hypothesized that the thermal stress would improve the cellular response of both cell types compared to a non-heated control. At 12 hours post-heating, there was a significant decline in metabolic activity in the hAT heated for 5 and 20 minutes compared to the control. The hAT heated for 10 and 15 minutes displayed metabolic levels similar to the control. At 24 hours post-heating, the metabolic activity of hAT in heated groups was comparable to that of the control, except for the cells heated for 10 minutes which showed significantly lower metabolic levels. However, the images of GFP expression taken 24 hours after heat shock suggest that heat shock at 44°C for 15 minutes improves

tenocyte elongation and cytoskeleton development, which aids in matrix deposition. While the metabolic data did not reveal if heat shock has much of an effect on hAT, the concentration of total protein in the serum displayed a trend of increased protein secretion 12 hours following heat shock compared to the control. This suggests that the hAT do elicit a response to the thermal treatment.

Genetic analysis 12 hours post-heating shows trends of upregulation of HSP47 and type I collagen in heated hAT. Type I collagen was significantly upregulated following 15 minutes of thermal stress applied at 44°C. Type III collagen expression appeared to remain constant among all groups. The genetic data suggests that heat shock produces a positive effect on hAT as type I collagen is the most abundant and important protein in healthy L/T tissue, playing a large role in providing the tissue with its tensile properties.[1] Moreover, type III collagen is associated with the formation of scar tissue during the L/T wound healing process.[15] Interestingly, TGF-β_1 expression is significantly lower than the control when hAT are heated for 10 or 15 minutes. TGF-β has been shown to increase the expression of both HSP47 and type I collagen.[31,45,53] However, the qPCR data suggests that there is no co-regulation of TGF-β_1 with HSP47 or type I collagen. This does not necessarily mean that TGF-β_1 plays no role in the expression of these proteins. It is possible that TGF-β_1 was expressed at an earlier time point following heat shock, leading to an accumulation of cellular and secreted TGF-β_1. The increased amount of TGF-β_1 in the samples would then influence an upregulation of type I collagen and HSP47 expression.

In response to heat shock, the hMSC displayed a significant decline in metabolic activity 12 hours following thermal stress applied for 5 and 10 minutes. Examination of

the serum protein levels 12 hours post-heating displayed a trending decline as the heating duration increased. Furthermore, no significant changes in the expression of any of the investigated gene were observed among any of the groups. The metabolic levels of hMSC 24 hours after heat shock were consistent among all heated groups and the control. Therefore, it appears as though heat shock does not have much of a beneficial effect, if any at all, on cultured hMSC.

In conclusion, heat shock at 44°C appeared to improve the cellular response of human Achilles tenocytes in vitro. Thermal conditioning applied for 15 or 20 minutes maintained cellular metabolic activity and viability while elevating type I collagen mRNA expression in hAT. On the contrary, mesenchymal stem cells appeared to be unaffected by the thermal stress. More studies are needed to fully understand the effect of heat shock on these two cell types. An examination of the cellular response at different time points could provide insight on the cellular mechanisms invoked by heat shock. Exposing the cells to other temperatures, such as 40° or 48°C, may produce an increase in proliferation and matrix protein expression compared to 44°C heat shock. Based on the findings of this study, there is potential for heat shock to be employed as a method of treating L/T sub-failure injuries.

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