

AN ABSTRACT OF THE THESIS OF

Kirsty A. Husby for the degree of Master of Science in Veterinary Science presented on June 15, 2016.

Title: In Vitro Evaluation of Therapeutic Laser Treatment on Equine Tendon Fibroblasts.

Abstract approved:

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Abstract:

Previous investigations have failed to show benefits of low level laser therapy (LLLT) for tendon healing in horses, contrary to reports in other species. We hypothesized that delivery of LLLT at manufacturer-recommended energy fluence through equine cadaver skin to equine tendon-derived fibroblasts in vitro would increase cell proliferation, metabolic activity and migration. Fibroblasts from grossly normal front superficial digital flexor tendons of four horses euthanatized for reasons unrelated to the study were cultured in monolayer. Cells were treated through one of three equine cadaver skin samples (light, medium, or dark colored) at manufacturer-recommended energy fluence. Untreated cells served as controls. At 24 hours after treatment, cell proliferation was determined via manual cell counts and cellular metabolic rate was assessed via resazurin sodium salt reduction assay. Cell migration was determined by scratch assay and by transwell migration assay. Data was analyzed by repeated measures ANOVA with $p < 0.05$ considered significant. There was no

difference between groups in cell numbers ($p=0.2497$), cell viability ($p=0.4326$), in fraction of reduced resazurin salt ($p=0.2613$), in number of cells migrating along a chemotactical gradient ($p=0.4914$), or for scratch width ($p=0.6685$) and scratch scores ($p=0.5129$). No beneficial effects of LLLT were found for tendon healing in horses under the study conditions.

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In Vitro Evaluation of Therapeutic Laser Treatment on Equine Tendon Fibroblasts

by
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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented June 15, 2016
Commencement June 2017

Master of Science thesis of Kirsty A. Husby presented on June 15, 2016

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ACKNOWLEDGEMENTS

The author expresses sincere appreciation to my graduate advisor, Dr. Katja Duesterdieck-Zellmer, for outstanding mentorship and encouragement through the duration of my laboratory endeavors as well as sample collection, assistance in the laboratory, data interpretation, and manuscript preparation. I would also like to thank the other members of my graduate committee, Dr. Stacy Semevolos, Dr. Oleh Taratula, and Dr. Adam Alani for their time and help during my Master's program.

I would also like to thank Maureen Larson for her excellent technical assistance and guidance. Her encouragement and contagious positive attitude kept me going during long hours in the laboratory.

Financial support was provided by internal funding through Oregon State University and by a grant from the American Holistic Veterinary Medical Foundation.

TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	1
2. Literature Review	3
2.1 Tendinopathies	3
2.1.1 Tendon Anatomy and Histology	3
2.1.2 Tendon Function	6
2.1.3 Effect of Aging and Exercise on Tendons	7
2.1.4 Tendon Injury	10
2.1.5 Tendon Healing	13
2.1.6 Diagnosis of Tendon Injury	14
2.1.7 Medical Treatment of Tendon Injuries	18
2.2 Low Level Laser Therapy	27
3. Objectives and Hypotheses	34
4. Materials and Methods	35
4.1 Cell Culture	35
4.2 Laser Protocol	37
4.3 Cell Proliferation and Viability Determination	38
4.4 Resazurin Salt Assay	40
4.5 Transwell Migration Assay	41
4.6 In Vitro Wound Healing	42
4.7 Statistical Analysis	43
5. Results	48

TABLE OF CONTENTS (Continued)

	<u>Page</u>
5.1 Laser Fluence	48
5.2 Cell Proliferation and Viability	49
5.3 Resazurin Salt Assay	50
5.4 Transwell Migration	50
5.5 In Vitro Wound Healing	51
6. Discussion	59
7. Conclusion	65
8. Bibliography	66
9. Appendices.....	84
Appendix A: ANOVA tables	84
Appendix B: Assessment of efficacy of small interfering RNA (siRNA) on equine articular chondrocytes in vitro	86

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1. Diagram of tendon structure	31
2. Stress-Strain Curve	32
3. Hysteresis Figure	33
4. Custom steel stand	45
5. Cadaver skin samples	46
6. Lasering procedure	47
7. Cell numbers at 24 hours	53
8. Cell viability at 24 hours	54
9. Reduction of resazurin salt at 24 hours	55
10. Number of cells migrated through transwell membrane at 24 hours	56
11. Scratch width remaining at 24 hours	57
12. Scratch score at 24 hours	58

LIST OF TABLES

<u>Table</u>	<u>Page</u>
1. Cell count at 24 hours	49
2. Percent viability at 24 hours	49
3. Percent resazurin reduction at 24 hours	50
4. Number of cells migrated through transwell membrane at 24 hours	51
5. Percent scratch width remaining at 24 hours	52
6. Scratch scores at 24 hours	52

Introduction

Low level laser therapy (LLLT), also known as cold or soft laser therapy, is a therapeutic modality that uses low powered laser light to stimulate a biological response. The general principle behind this therapeutic measure is exposure of the tissues to red and near infrared light which is absorbed, resulting in increased adenosine triphosphate (ATP) production, modulation of reactive oxygen species, and stimulation of various transcription factors (Chung et al., 2012). Over many years this nontraditional modality has been used both as a primary and adjunct treatment for a variety of ailments. It has been utilized to promote wound healing and tissue repair (Ghamsari et al., 1997; Pinto et al., 2010), reduce inflammation and edema (Piller and Thelander, 1998; Gautam et al., 2015), and provide analgesia for a multitude of acute and chronic conditions (Chow et al., 2009; Konstantinovic et al., 2010a; Fabre et al., 2015). Despite its widespread use in both human and veterinary medicine, there are inconsistent published results and scientific evidence supporting its efficacy in equids is lacking.

Tendinopathies are common amongst both human and equine athletes, and are considered a multifactorial condition affected by age and exercise or training volume (Birch et al., 1998; Kujala et al., 2005; Avella et al., 2009; Thorpe et al., 2010; Reardon et al., 2012; Dakin et al., 2012). In the horse, superficial digital flexor tendinopathy is one of the most common musculoskeletal injuries, and affects up to 30% of Thoroughbred racehorses (Marr et al., 1993; Williams et al., 2001; Kasashima et al., 2004; Takahashi et al., 2004; Ely et al., 2010). The mainstay of treatment typically involves a rest and rehabilitation program whereby exercise is increased

incrementally over time (Gillis, 1997; Avella and Smith, 2012). This is often combined with adjunctive therapies such as nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, extracorporeal shockwave therapy (ESWT), platelet rich plasma (PRP), mesenchymal stem cells, and surgical intervention in some cases (Avella and Smith, 2012; Smith et al., 2014). Treatment of tendonitis in the horse may be costly, and it can take up to a year or longer for full recovery (Avella and Smith, 2012). Once recovered, recurrence is common with many horses being retired or euthanized due to continued lameness (Genovese et al., 1996; Dyson, 2004; Dowling et al., 2010; O'Meara et al., 2010). LLLT has been examined in several species, including the horse, for tendon healing with variable success (McKibbin and Paraschak, 1983; Ryan and Smith, 2007), and effective energy doses for equine tenocytes are unknown.

Literature Review

Tendon Anatomy and Histology

Tendons are composed of a hierarchy of subunits that form strong, fibrous structures connecting muscles to bone (Figure 1). Most muscles have two tendons that connect to bone; one proximal and one distal. Through these attachments to bone the tendons allow transmission of force created in the muscle to bone (Avella and Smith, 2012). Tendons are surrounded by connective tissue called epitenon. In extrathecal locations epitenon is surrounded on its outer surface by paratenon which allows reduction of friction and free gliding movement of tendons against surrounding tissues (Hess et al., 1989). The paratenon contains numerous blood vessels which may be important in supplying cellular elements required for tendon repair (Avella and Smith, 2012). The epitenon is continuous on its inner surface with the endotenon which surrounds the tendon subunits, known as fascicles. The endotenon serves to bind the fascicles together, carries blood vessels and nerves to the deeper structure of the tendon, and is believed to be a source of mesenchymal stem cells within the tendon (Avella and Smith, 2012).

The internal structure of the tendon is composed of approximately 70% water with almost 30% collagen (Kannus, 2000). The high water content is thought to be important in maintaining tendon elasticity (Birch, 2007). The collagen content is predominantly type I collagen with elastin, embedded in a proteoglycan-water matrix. Other minor collagens, present in small amounts, include types III, V, XI, XII, and XIV (Birch et al., 2014). Collagen fibrils are comprised of a triple helix of collagen molecules which are produced by tenoblasts and tenocytes. Collagen fibrils are cross-

linked by the enzyme lysyl oxidase to form collagen fibers that are grouped together in fascicles (Bailey et al., 1998). Fascicles have a characteristic wavy formation, known as crimp, which allows elongation of collagen fibers and thus imparts elasticity to the tendon (Smith, 2011).

Collagen fibrils are separated by the cellular component of tendons, called tenocytes, which synthesize and degrade the proteoglycan and collagenous matrix components of the tendon (Birch et al., 2014). Three types of tenocytes are described based on their morphology. Type I have thin, spindle shaped nuclei and are thought to be inactive forms of type II cells which have more rounded nuclei (Goodrich, 2011). It is thought that type II cells are more metabolically active as they are found more frequently in young horses while type I cells are typically present in mature horses (Smith, 2011). Type III cells, which resemble chondrocytes, are found commonly in areas of mature tendon that are sustaining high compressive loads and are thought to be associated with aging change (Smith, 2011). Cellular numbers within tendons vary, and are highest in young horses. Although tenocyte numbers may remain stable once maturity is reached, certain areas such as the center of the superficial digital flexor or deep digital flexor tendons may become acellular (Stanley et al., 2008).

Non-collagenous components that make up the proteoglycan matrix include cartilage oligomeric matrix protein (COMP), elastin, fibronectin, thrombospondin, decorin, fibromodulin, lumican, aggrecan, and versican (Smith, 2011). COMP, which is the most abundant non-collagenous glycoprotein, is thought to be vital in collagen fibril organization and cross-linking during tendon development. It is proposed to function by bringing together collagen molecules to form collagen fibrils in the

characteristic helix or “quarter stagger” arrangement, however once the fibrils are formed COMP detaches and is no longer functional (Smith et al., 1997; Smith et al., 2002). COMP levels in equine flexor tendons are noted to be highest during growth where they reach their peak around 2 years of age, and are lower in skeletally mature animals (Birch et al., 2014). It has been suggested that higher COMP levels during growth may be correlated to greater ultimate tensile strength of the tendon at skeletal maturity (Smith et al., 2002). COMP has recently been shown to be involved in tendon remodeling and repair along with type III collagen (Södersten et al., 2013). The non-collagenous composition may vary along the length of the tendon reflecting different functional and metabolic environments. Small proteoglycans such as decorin, fibromodulin, and lumican tend to predominate in areas of tension, while the larger proteoglycans such as aggrecan and versican tend to be found in areas where there is compression such as where tendons change direction over a bony prominence (Avella and Smith, 2012). The greater water holding capacity of the larger proteoglycans will allow the tendon to resist compression in needed areas, while the smaller proteoglycans are thought to play a role in tenocyte function, collagen fibrillogenesis, and spatial organization of collagen fibers (Hedbom and Heinegård, 1993; Svensson et al., 1995; Gu and Wada, 1996).

Nutrient supply to tendons is obtained via both perfusion and diffusion. Diffusion occurs where tendons are within a sheath via synovial fluid. Perfusion by way of blood delivery to the tendon occurs via vessels that originate from muscular attachments and osseous insertions, as well as intratendinous vasculature (Kraus-Hansen et al., 1992; Kraus et al., 1995). Intratendinous vasculature has been

identified to be most abundant on the periphery of the tendon, as opposed to the center of the tendon (Strömberg and Tufvesson, 1969).

Tendon Function

Tendons are elastic structures that transmit forces generated by muscle to bones to allow movement of the skeleton. They can be classified into two functional groups; positional tendons, such as the common digital extensor tendon in the horse, which serve primarily to maintain alignment of the limb, and energy storing tendons which are weight bearing tendons that store elastic energy necessary for locomotion, such as the superficial and deep digital flexor tendons (Goodrich, 2011). These energy storing tendons act as springs with associated muscle bodies serving to dampen vibrations (Wilson et al., 2001). The biomechanical properties of tendons are optimized for their function, and are usually described by way of the stress-strain curve (Figure 2) (Avella and Smith, 2012). Initially the tendon is very compliant and stretches under very little load which is associated with elimination of the crimp that is present within the fascicles; this is known as the elastic phase or toe region, and is the portion of the stress-strain curve which is nonlinear. Beyond the elastic phase the linear region represents where the tendon is elongating in an elastic fashion due to elongation of collagen fibrils and sliding of fibrils/fibers. As the load increases, the stiffness increases and ability of the tendon to extend decreases until the yield point is reached. At the yield point, irreparable damage including rupture of the collagen crosslinks and slippage of collagen fibrils leads to irreversible lengthening that can ultimately result in tendon rupture. Normal strains recorded in an *in vivo* study for the

digital flexor tendons in the horse were 3-8% at the walk, 7-10% at the trot, and 12-16% at the gallop (Stephens et al., 1989; Riemersma et al., 1996). While it is difficult to simulate natural conditions in the laboratory, equine tendons have been reported to rupture in vitro at strain values of 10-12%, although values as high as 20% have been recorded (Goodship et al., 1994).

Another important biomechanical property of tendon is that there is a loss of energy between the loading and unloading cycle, which is known as hysteresis (Figure 3) and is estimated to be about 5% in equine tendons (Birch et al., 1997; Goodrich, 2011). This energy is lost in the form of heat which is transferred to the tendon itself resulting in an increased core temperature during repeated loading and unloading cycles. Core temperatures during high speed exercise can reach 45° C within the equine superficial digital flexor tendon (Birch et al., 1997). In vitro studies have found that tenocytes isolated from the center of equine superficial digital flexor tendons remain viable at increasing temperatures typical of the exercising horse, while those from the outer surface of the tendon do not (Birch et al., 1997). The exact effect of hysteresis on tendon function and contribution to pathology is unknown, although it seems like cells have some adaptation to the increased temperatures.

Effect of Aging and Exercise on Tendons

The mechanical loading environment of developing tendons can influence their ultimate composition and structural properties. Immature tendons have a higher proportion of type III collagen than adult tendons, and it is arranged primarily in small collagen fibrils (Parry et al., 1978; Birk and Mayne, 1997). As the animal

matures the proportion of type III collagen decreases, the proportion of type I collagen increases, and collagen fibril size diameters become bimodal with larger fibrils present within the center of the tendon and smaller fibrils at the tendon periphery (Parry et al., 1978; Birk and Mayne, 1997; Lin et al., 2005; Patterson-Kane et al., 2009). Changes are rapid initially, and then decrease in rate with the equine superficial digital flexor tendon considered to be mature at approximately 2 years of age (Kasashima et al., 2002). Interestingly, the deep digital flexor tendon is thought to require longer to reach full maturation (Kasashima et al., 2002).

A higher proportion of small collagen fibrils have been noted in the central portion of the superficial digital flexor tendon in mature horses that have been undergoing long term exercise on a high speed treadmill compared to horses that were only subject to walking (Patterson-Kane et al., 1997b). There was no noted change in total collagen content, and thus this could be the result of a degenerative process whereby larger collagen fibril crosslinks are becoming detached within the fascicles and potentially resulting in increased strain or stiffness to the tendons. In addition, treadmill exercise and increasing age resulted in alteration to crimp angles of tendon fascicles, especially at the center of the superficial digital flexor tendon which may predispose to injury in this area. Crimp angles in a population of wild horses were found to be decreased in the superficial digital flexor tendon core in older horses (> 5 years) compared to young horses (< 5 years) (Patterson-Kane et al., 1997a). In addition, a group of young Thoroughbreds (2 years old) that underwent a controlled high speed exercise program on the treadmill over an 18-month period had a decreased crimp angle compared to a control group that received only walking

exercise (Patterson-Kane et al., 1998). Normal crimp angles in the midmetacarpal region of the superficial digital flexor tendon in the young horse is approximately 19-20° with a crimp length of 17-19 μm , however in the adult horse these values are reduced to 12-17° and 11-15 μm (Wilmink et al., 1992). With decreased crimp angles in the core of the tendon this region will be loaded first and will have to withstand a greater percentage of the total overall load in comparison to the periphery of the tendon. Superficial digital flexor tendons harvested from mature horses (> 2 years) have been shown to be stiffer with a higher average elastic modulus compared to tendons harvested from 2 year old horses (Gillis et al., 1995). These age-related changes may predispose tendons to degeneration and subsequent injury.

As previously mentioned, levels of the non-collagenous glycoprotein, COMP, decrease after tendon maturity is reached around 2 years of age (Birch et al., 2014), and this may be influenced by exercise as loading of a tendon appears to be an important stimulus for the production of COMP (Smith et al., 1997; Smith and Heinegård, 2000; Smith et al., 2002; Kasashima et al., 2008; Smith et al. 2010). Early reports indicated that early exercise could accelerate age-related decreases in COMP levels (Smith and Birch, 1998; Smith et al., 2010), however more recently foals enrolled in a long term treadmill exercise program were found to have no difference in COMP levels in the superficial digital flexor tendon compared to foals allowed 4 hours of pasture turnout daily (Kasashima et al., 2008). As such, early exercise may not be as deleterious to developing tendons as initially thought. Tendons may undergo adaptations in response to cumulative fatigue and degenerative changes. As COMP levels in tendon decrease beyond the age of 2, there may be a decreased ability for

effective tendon remodeling in response to the biomechanical stressors (Södersten et al., 2013).

Tendon Injury

Tendons may be injured through excessive strain or acute percutaneous trauma, such as lacerations or kicks. Overstrain injuries may occur as a result of a sudden overload of the tendon which exceeds its capacity for resistance, or as a result of degenerative changes within the tendon that weaken it. Lack of fitness, fatigue, and poor conformation may promote excessive biomechanical forces on a tendon in the equine athlete. Risk factors for development of superficial digital flexor tendinopathies have been evaluated in racehorses and include older age at time of first race, male gender, heavy bodyweight, fatigue, lack of fitness, uncoordinated action, conformation, firm ground, long race distance, and frequent high speed work (Mohammed et al., 1992; Estberg et al., 1995; Kasashima et al., 2004; Perkins et al., 2005; Lam et al., 2007; Butcher et al., 2007; Reardon et al., 2012).

Tendon degeneration is likely multifactorial, although exact etiologies have not yet been determined. Mechanical forces through overextension and repetitive loading may result in microdamage, which are likely cumulative over time due to mature tendons' reduced ability to adapt or coordinate a reparative response. It has been suggested that quantity of loading cycles or frequent exercise at speeds with higher loading rates may be a factor in tendon degeneration (Avella and Smith, 2012). The physical force placed upon a tendon during high speed exercise may damage the tendon matrix by disrupting collagen fibrils and matrix proteins (Lavagnino et al.,

2006; Arnoczky et al., 2007; Fortier and Smith, 2008). In addition, the heat produced within the core region of the tendon during high speed exercise may induce damage, although tenocytes themselves have been shown to remain viable at these temperatures (Wilson and Goodship, 1994; Birch et al., 1997). Post-mortem studies of normal adult horses have identified gross and microscopic tendon lesions, which corroborate age-related degenerative changes (Webbon, 1977; Birch et al., 1998). In addition, epidemiologic studies have found a strong association between injury rates and age in both human and equine athletes (Pickersgill, 2000; Williams et al., 2001; Kasashima et al., 2004). An in vitro experiment evaluating the effect of cyclical loading on an equine tendon explant model found that older specimens were more likely to weaken under normal strain rates of 5%, and had increased degradation of the extracellular matrix (Dudhia et al., 2007). More recent work has identified less sliding between fascicles at the endotendon, or interfascicular matrix, of the superficial digital flexor tendon in horses aged 17-20 years compared to horses aged 3-7 years which may predispose the tendon to injury (Thorpe et al., 2013; Thorpe et al., 2015).

Overstrain injuries may be preceded by an alteration in the composition of the tendon matrix that occurs during aging, however clinical injury occurs when the physical load overwhelms the elastic capacity of the tendon. During movement of the horse the greatest loads on the superficial digital flexor tendon and suspensory ligament occur as the heel strikes the ground (early weight bearing phase) which is related to the degree of fetlock extension, while the deep digital flexor tendon is loaded more slowly with the greatest load occurring during late stance phase

(Goodship et al., 1994). When peak load of the tendon reaches and exceeds the yield point, physical disruption to the tendon matrix occurs with breaking of collagen fibril crosslinks, fibrillar slippage, and finally rupture with separation of tendon tissue. Higher speeds and harder surfaces will increase the peak load on the tendons which explains the high incidence of tendinopathies in racehorses (Goodship et al., 1994; Kasashima et al., 2004; O'Meara et al., 2010).

The importance of inflammation in development of tendon injuries is unknown. Analysis of 5 injured equine superficial digital flexor tendons identified positive immunostaining for proinflammatory cytokines IL-1 α , IL-1 β , TNF- α , and IFN- γ which were not recognized in 4 normal superficial digital flexor tendons (Hosaka et al., 2002). Rodent models of tendon injuries have found upregulation of genes involved in control of inflammation (Millar et al., 2009). Prostaglandin E₂ is an arachidonic acid derivative that is involved in both normal physiologic processes as well as during inflammation. Its effects during the inflammatory process may be both beneficial and deleterious to the tendon based on in vitro and murine models (Khan et al., 2005; Ferry et al., 2012), however it is not well investigated in the horse. Another product of the arachidonic acid pathway, lipoxin A₄, functions in an anti-inflammatory capacity by regulating neutrophil responses and thereby promoting resolution of inflammation (Serhan et al., 2000). A single in vitro study exists that investigated the role of arachidonic acid metabolites in equine tendon disease. During acute injury, levels of prostaglandin E₂ were decreased while lipoxin A₄ levels were increased compared to normal and chronically injured tendons (Dakin et al., 2012). Interestingly, the investigators also identified an age-associated decline in the lipoxin

A₄ receptor (FPR2/ALX) expression with concurrently increased levels of Prostaglandin E₂, and increased expression of the receptor in tendon explants from younger horses (< 10 years) that were treated with IL-1 β (Dakin et al., 2012). Since lipoxin A₄ is thought to be beneficial in restoring tissue homeostasis following injury, decreased expression of its receptor could indicate reduced capacity for resolution of inflammation in older horses.

Tendon Healing

Tendons go through the same three phases of healing as do other tissues; inflammation, reparation, and remodeling. The inflammatory phase is typically short and involves intratendinous hemorrhage and edema which bring an influx of neutrophils, macrophages, and proteolytic enzymes to induce removal of damaged tissue. While these processes are important in tendon healing, the response may become excessive resulting in further tendon damage (Goodrich, 2011). Reduction of the initial inflammatory reaction may be accomplished with administration of nonsteroidal anti-inflammatories (NSAIDs). The reparative phase can last for several months and includes angiogenesis with infiltration of fibroblasts and synthesis of scar tissue which has a higher ratio of type III collagen content that is more haphazardly arranged compared to normal tendon tissue (Smith, 2011). The presence of type III collagen confers early stability to the site of injury (Williams et al., 1980). During the remodeling stage there is slow transformation of a large portion of type III collagen into type I collagen as the scar tissue matures. During this process the collagen fibrils become larger and alignment is improved, however the original tendon strength is

never restored due to the abnormal composition and arrangement of the tendon matrix (Pool, 1996). Healed tendon remains stiffer than prior to injury due to scar tissue; which in combination with the slow rate of healing may predispose to re-injury.

Diagnosis of Tendon Injury

Horses are typically lame with pain, heat, and swelling over the affected area, however once the inflammatory phase has passed, the lameness is often markedly improved. In cases of severe injury, such as tendon rupture, there may be an alteration to limb appearance or biomechanics. With severe damage or rupture of the superficial digital flexor tendon the affected limb may have greater than normal extension of the fetlock joint, especially when loading on that limb increases (Avella and Smith, 2012). Rupture of the deep digital flexor tendon results in the characteristic toe-up appearance to the hoof (Avella and Smith, 2012).

Ultrasonographic evaluation is the most commonly used tool for diagnosis and follow-up evaluation of tendon pathology, although injuries associated with lacerations or wounds may be difficult to image because of air artifact. It is minimally invasive and allows identification and localization of the lesion, measurement of lesion size and tendon cross-sectional area, and evaluation of fiber alignment (Rantanen et al., 1985). Although it has been investigated, ultrasound has not been found to be beneficial in detecting subclinical tendinopathies or predicting likelihood of acute injury (Avella et al., 2009). Optimal time for evaluation of tendon injury is 7-10 days after the onset of the injury as lesions often expand during the first week, and may be subtle enough to be missed early on (Goodrich, 2011). It is important to

evaluate both limbs as bilateral tendinopathy has been reported to occur in 35% of horses (Avella et al., 2009). Ultrasound of tendons and ligaments of the distal limb is typically performed with a 7.5 to 14 MHz linear transducer after preparing the limb by clipping and cleaning to enhance image quality. Evaluation should be performed in a systematic manner in both transverse and longitudinal planes from proximal to distal, and may include both nonweightbearing and dynamic imaging (Avella and Smith, 2012; Smith and Cauvin, 2014). Assessment of echogenicity, fiber alignment, and cross-sectional area should be performed.

A normal tendon should be viewed ultrasonographically as hyperechogenic in nature compared to the surrounding soft tissues. In long axis, hyperechoic parallel striations should be visible along the length of the tendon which are representative of the fascicular structure, and in transverse section these often appear as hyperechoic granular dots (Coudry and Denoix, 2012; Smith and Cauvin, 2014). The appearance of the tendon should be consistent across the visible image and throughout its length. Decreased echogenicity is often seen in injured tendons as a result of increased water content in the tendon due to edema or infiltration of granulation tissue which is also more hypoechogenic to normal tendon tissue (Coudry and Denoix, 2012; Smith and Cauvin, 2014). Tendons will lack the clear hyperechogenic striations or granular appearance at the location of the lesion, and instead have poorly marginated areas of decreased echogenicity. There may also be increased peritendinous swelling or edema, although this is not specific to tendinopathies.

Cross-sectional area can be measured objectively, however there is considerable variation among breeds and types of horses, as well as the location

where it is measured. One study of multiple breeds identified the upper limits of normal cross-sectional area for the superficial digital flexor tendon and deep digital flexor tendon in the mid-metacarpal region at 22.67 mm² and 29.22 mm² respectively, although ponies were noted to have smaller cross-sectional area (Smith et al., 1994). Other studies in Thoroughbreds have identified a range for cross-sectional area of superficial digital flexor tendon at 7.2-19.3 mm², and Arabians' and ponies' cross-sectional areas are even smaller at 6-8 mm² (Reef, 1998; Avella et al., 2009). There is one published report of normal cross-sectional area of several tendon structures in the tarsal region at multiple levels proximodistally (Vilar et al., 2011). Despite variation among horses, increased tendon cross-sectional area is a sensitive indicator of tendon fiber damage (Smith et al., 1994; Genovese et al., 1996), and an increase in cross-sectional area of more than 10% during rehabilitation for a tendon injury may be indicative of re-injury (Reef, 1998).

Elastography is an ultrasonographic technique that is used to evaluate mechanical properties of the tissue by evaluating tissue strain. This is performed by measuring ultrasound echos before and after a compression force is applied, and gives information about the stiffness of the tissue (Lustgarten et al., 2014). It has been used to evaluate multiple organs (Ophir et al., 1991; Sousaris and Barr, 2016; Huang et al., 2016; Agladioglu et al., 2016), and more recently has been investigated for evaluation of tendon injuries in humans and horses (Lustgarten et al., 2014; Aubry et al., 2015; Cortes et al., 2015; Suydam et al., 2015; Lustgarten et al., 2015). Normal flexor tendons were classified to be predominantly hard with no difference in stiffness between weightbearing and nonweightbearing (Lustgarten et al., 2014). Injured

tendons were noted to be softer with more acute lesions being significantly softer than more chronic lesions, and stiffness was found to increase with progression of healing (Lustgarten et al., 2015). Furthermore, a correlation between degree of hypoechogenicity on greyscale ultrasound and increased softness on elastography was identified (Lustgarten et al., 2015). These findings are promising for future use of this technique for evaluation of tendon pathology.

Other potential diagnostic imaging tools include magnetic resonance imaging (MRI) and contrast enhanced computed tomography (CT). MRI is an excellent diagnostic for identifying soft-tissue lesions, however this is typically expensive in the horse and superior high-field magnet imaging requires general anesthesia. As such, MRI tends to be reserved for identifying those lesions contained within the hoof capsule that are more difficult to image by other means. Contrast enhanced CT has recently been validated for identifying deep digital flexor tendon lesions in the foot with 93% sensitivity (Hamel et al., 2014).

Research has also focused on identifying biomarkers for tendon disease, however at the current time these tests are not routinely performed and are not recommended as a sole diagnostic. Serum biomarkers for type I collagen synthesis (procollagen type I C-terminal propeptide; PICP) and degradation (type I collagen degradation product; ICTP) were evaluated in a group of horses with tendon pathology and compared to normal horses (Jackson et al., 2003). Findings indicated that PICP serum concentrations were elevated in injured compared to normal horses, although PICP is not tendon specific (Jackson et al., 2003). As mentioned earlier, COMP has been evaluated as a biomarker in tendon injury. Elevated COMP levels in

synovial fluid from the digital flexor tendon sheath appear to be indicative of intrathecal tendon or ligament tearing (Smith and Heinegård, 2000; Smith et al., 2011), while serum COMP levels were not significantly elevated in tendinopathy (Smith and Heinegård, 2000).

Medical Treatment of Tendon Injuries

Considering treatment options for tendon injuries can produce an exhaustive list, however the most important aspects include rest and remedies to decrease inflammation and pain. Cold therapy through either cold hosing or application of ice packs to a limb can act as both an anti-inflammatory and analgesic. These effects are mediated through increased vasoconstriction, decreased enzymatic activity, reduced formation of inflammatory mediators, and diminished nerve conduction (Avella and Smith, 2012). Typically cold hosing is recommended for 20 minutes at least twice daily, although research has shown cold treatment for 1 hour with mean core temperatures of 10° C in the superficial digital flexor tendon does not appear to be harmful to tendon cells (Petrov et al., 2003). An elaborate hypertonic cold water spa bath has been investigated and found to be a beneficial cold therapy option (Hunt, 2001).

Support for acute tendon injuries can take the form of compression or coaptation depending on the severity of the injury. Pressure in the form of a bandage applied to the affected area will increase hydrostatic pressure and thus reduce inflammation and edema, although no studies have evaluated these effects. Tendon injuries that result in fetlock hyperextension or those that result in instability of the

limb should be stabilized with external coaptation to reduce likelihood of further injury and increase comfort level for the horse. Surgically and collagenase induced superficial digital flexor tendon lesions in horses treated with cast immobilization showed a reduction in lesion propagation (Bosch et al., 2010b; David et al., 2012). One of these studies was performed on cadaver limbs which is unlikely to accurately reflect in vivo conditions, however limbs were loaded and unloaded 5000 times over an 8 hour period (Bosch et al., 2010b). Another study evaluated short term cast immobilization compared to bandaging alone for 10 days post bilateral surgically induced superficial digital flexor tendon core lesions (David et al., 2012). Computerized ultrasonographic tissue characterization performed at various time points and post-mortem findings revealed that lesion length and width was smaller in casted limbs compared to bandaged limbs indicating that short term immobilization may be beneficial in the early inflammatory period (David et al., 2012).

Rest and controlled return to exercise is a vital component of any therapeutic program for tendon or ligament injury. This allows for healing to occur while maintaining gliding function and promoting optimal collagen remodeling (Gillis, 1997). Due to the slow healing capacity of tendons, a controlled rehabilitation program of 9-18 months is required with frequent ultrasound evaluations before full athletic activity is resumed (Avella and Smith, 2012). Usually stall rest is prescribed for the initial inflammatory period followed by a slow incremental increase in exercise that is thought to maximize the elasticity of scar tissue formation without placing the horse at risk for further injury. Since strains are lowest at the walk, exercise programs start with minimal duration of walking and progress up to higher

speed gaits which are known to have higher strains (Stephens et al., 1989; Goodship et al., 1994; Riemersma et al., 1996). Frequent ultrasonographic evaluation is recommended, especially around times when incorporation of a faster gait may be considered, allowing tailoring of the rehabilitation program to the individual horse. An increase in tendon cross-sectional area of 10% during this period may indicate re-injury and exercise should be reduced (Reef, 1998). Although widely accepted, the benefits of the controlled exercise program have been proven only in one small clinical study where 71% of Thoroughbred racehorses with superficial digital flexor tendinopathy that were placed in a rehabilitation program returned to racing compared to 25% that were managed with uncontrolled turnout (Gillis, 1997).

Extracorporeal shock wave therapy has been used for several years in an attempt to enhance the healing of tendons and ligaments and shorten convalescence periods, although the exact mechanism of action is unknown. High energy pressure pulses are transmitted to a focal point within the tissue to induce positive changes at the cellular level. Analgesic effects are thought to be induced via action of extracorporeal shockwave therapy on sensory nerves (Bolt et al., 2004; McClure et al., 2005), increases in TGF- β 1 and IGF-1 have been identified which upregulate extracellular matrix production by tenocytes (Abrahamsson, 1997), mitotic activity is stimulated, and cytokine production is enhanced (Kusnierczak et al., 1999; Wang et al., 2002). A rodent model found that shockwave therapy increased lubricin expression in tendons which may provide beneficial effects for healing by decreasing erosive wear (Zhang et al., 2011). In a pony model, shockwave therapy was identified to increase protein synthesis within 3 hours of treatment, although these effects were

no longer present at 6 weeks (Bosch et al., 2007). It has been used with satisfactory outcomes for treatment of multiple tendinopathies in human patients including shoulder tendonitis, plantar fasciitis, Achilles tendinopathy, and lateral elbow epicondylitis (Saxena et al., 2012; Carulli et al., 2015). Extracorporeal shockwave therapy has been used extensively in horses for treatment of tendon and ligament injuries, and one study identified increased neovascularization of induced lesions within the superficial digital flexor tendon after 3 treatments with shockwave compared with controls (Schramme et al., 2006). Extracorporeal shockwave therapy has also been identified to produce disorganization of the collagen network in normal equine flexor tendons and ligaments, although whether this is deleterious or indicative of changes signifying repair is unknown (Bosch et al., 2010a). Collagenase-induced lesions of the accessory ligament of the deep digital flexor tendon treated with a single shockwave treatment showed no difference in lesion size, microstructure, or composition compared to non-treated controls, although mRNA levels for type I collagen were higher in the shockwave treated tendons (Waguespack et al., 2011). There are no reports to date in the literature that have shown positive long term outcomes of tendon lesions treated with extracorporeal shockwave therapy compared to controls.

Nonsteroidal anti-inflammatory drugs may be beneficial in the early stages after injury to control the acute inflammatory reaction. Systemic corticosteroids should be avoided due to potential for inhibiting fibroplasia (Dowling et al., 2010), and intralesional use has been shown to cause collagen necrosis and dystrophic mineralization (Pool et al., 1980).

Polysulfated glycosaminoglycans (PSGAGs) may be administered via intramuscular or intralesional routes, and are believed to enhance tendon healing by inhibiting collagenase and metalloproteinase activity, as well as macrophage activation (Dowling et al., 2010). These effects would be beneficial in the acute stage after tendon injury only, and they have not been identified to have any significant effects on production of proteoglycan by in vitro tendon fibroblasts (Dahlgren et al., 1998). Horses treated with PSGAGs via an intramuscular route in a collagenase induced superficial digital flexor tendonitis study were found to have improved ultrasonographic appearance of the tendon earlier than the control group (Redding et al., 1999), however no difference was noted in healing when administered intralesionally (Marxen et al., 2004). Intralesional injection did result in more organized collagen bundles in another study (Moraes et al., 2009), which fits with an improved ultrasound appearance. No difference in reinjury rate has been reported for horses treated with PSGAGs (Dyson, 2004).

Despite early studies suggesting improved ultrasonographic appearance of tendons treated with hyaluronic acid (Spurlock et al., 1989; Spurlock et al., 1999; Gaughan et al., 1991), more recent work has failed to show any significant benefit for tendon healing (Foland et al., 1992), or any decrease in recurrence (Dyson, 2004). Hyaluronic acid has been shown to have benefit in reduction of adhesion formation for intrathecally located tendons (Thomas et al., 1986; Gaughan et al., 1991), and could thus be recommended post-injury in these cases.

Beta-aminopropionitrile fumarate (BAPN) is a toxic agent isolated from the seeds of the *Lathyrus* plants (Dowling et al., 2010). This toxin is a lysyl oxidase

inhibitor, and in tendon healing it will inhibit excessive crosslinking of collagen fibers during early repair which, in combination with a controlled exercise program can encourage appropriate tendon fiber alignment (Genovese, 1993). A collagenase induced tendonitis model confirmed this via intralesional injections of BAPN at 20 days with improved collagen alignment as assessed by ultrasound and histopathologic examination (Alves et al., 2001). In a retrospective study, 80% of cases treated with intralesional BAPN had improvements in ultrasonographic evaluation and measurements (Genovese, 1993). Reinjury rate was also significantly reduced over treatment with HA, PSGAGs, or controlled exercise alone in another large scale prospective study (Dyson, 2004). Clinical use of BAPN resulted in frequent inflammatory reactions which were manageable, however its popularity decreased and it is no longer commercially available. In addition, further in-vitro evaluation determined that BAPN may weaken and suppress tendon healing by decreased collagen synthesis and crosslinking of collagen fibers (Dahlgren et al., 2001).

Autologous blood derived products such as autologous conditioned serum (ACS) or platelet rich plasma (PRP) have become an increasingly popular biologic regenerative therapy option for tendinopathies. These products have high concentrations of growth factors including platelet derived growth factor (PDGF), transforming growth factor beta (TGF- β), insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor (VEGF) which stimulate cellular proliferation and matrix synthesis (Geburek et al., 2015). Surgically created core lesions within the superficial digital flexor tendon treated with a single intralesional injection of PRP had a higher collagen, glycosaminoglycan, and DNA content with better organization

of the collagen network than control tendons, as well as a higher strength at failure and elastic modulus during biomechanical testing (Bosch et al., 2010c). All horses in a group of seven with naturally occurring tendinopathies that were treated with ACS returned to their previous level of performance within 10-13 months post injury (Georg et al., 2010). A recent clinical study also found that forelimb superficial digital flexor tendinopathies responded positively to a single intralesional ACS injection with significantly improved ultrasonographic echogenicity, reduction in ultrasonographic lesion size, and greater collagen type I expression than untreated controls (Geburek et al., 2015). These findings are encouraging, however long term outcomes have not been investigated.

Several growth factors have been investigated for their effects on tendon healing. Intralesional insulin-like growth factor-1 (IGF-1) resulted in decreased lesion size, increased collagen content, and improved mechanical characteristics compared to saline treated controls in a collagenase-induced model of SDF tendonitis (Dahlgren et al., 2002). A retrospective study on 40 Thoroughbred racehorses found that intralesional IGF-1 administered every other day for 4 or 5 treatments resulted in ultrasonographic improvement of the lesion, however there was only a moderate prognosis for return to racing with 62% returning to race at least once (Witte et al., 2011). In addition, 46% of horses had recurrence of tendonitis or developed tendonitis in a different location subsequent to treatment (Witte et al., 2011). Recombinant equine growth hormone (rEGH) administered intramuscularly was found to have a negative effect on early tendon healing with larger tendon cross-sectional areas and lower tensile strength compared to a control group in a collagenase induced

superficial digital flexor tendonitis model (Dowling et al., 2002). Additional investigation with regard to growth factors is necessary before they may be incorporated into treatment plans.

There have been an abundance of studies looking at regenerative effects of mesenchymal stem cells (MSCs) for tendon lesions in recent years. Injection of MSCs introduces the potential for regeneration of tendon matrix by newly differentiated tenocytes. Adipose derived nucleated cell fractions injected into collagenase induced superficial digital flexor tendon core lesions resulted in improved tendon fiber organization and increased expression of COMP compared to controls (Nixon et al., 2008). MSCs used alone or IGF-1 enhanced MSCs significantly improved tendon histological scores 8 weeks after injection in a collagenase induced tendonitis model (Schnabel et al., 2009). Naturally occurring superficial digital flexor lesions treated with intralesional MSCs found improved histologic organization and reduced stiffness compared to saline treated controls (Smith et al., 2013). A surgical model of superficial digital flexor tendonitis found no difference in collagen fibril size between limbs treated with intralesional MSCs and saline control limbs indicating that it is unlikely that MSCs impact collagen fibril size (Caniglia et al., 2012). A retrospective study on 141 racehorses treated with intralesional MSCs in combination with a controlled exercise program for superficial digital flexor tendon overstrain injuries found that intralesional injection of autologous MSCs was safe without any adverse effects (Godwin et al., 2012). Long term follow-up was available for 113 horses from this study, and 98% returned to racing with a significantly lower

reinjury rate of 27.4% compared to other comparable published data with various other treatment modalities (Godwin et al., 2012).

Low Level Laser Therapy

Laser is an acronym that stands for light amplification by the stimulated emission of radiation. This is essentially the delivery of monochromatic, collimated, coherent beams of light. Low level laser therapy (LLLT), also called “cold laser therapy” or photobiomodulation, is meant to not induce heating of tissues primarily like surgical lasers, but is instead supposed to stimulate photochemical reactions within cells as a result of emission of certain wavelengths of light (Chung et al., 2012), although heating is probably unavoidable. Typically, these lasers emit < 15 watts (W) of power within the red or near infrared spectrum (600 nm – 1000 nm) (Huang et al., 2009). These lasers can be further subdivided by their power output. Class 3a emit a maximum of 5 mW of power, Class 3b emit a maximum of 500 mW of power, and Class 4 lasers cover all lasers that emit > 500 mW of power. Laser tissue effects are determined as a result of the wavelength, power, irradiation time, beam area, continuous or pulsed modes, number of treatments, interval between treatments, and tissue characteristics (Jenkins and Carroll, 2011). Unfortunately, there is no agreed upon method among the scientific community for measuring or reporting LLLT dosage. The most commonly reported variables describing LLLT are wavelength, power, and energy fluence. Power is calculated as follows:

$$\text{Power (W)} = \frac{\text{Laser energy (J)}}{\text{Pulse duration (seconds)}}$$

Energy fluence, also commonly referred to as energy dose, is calculated as follows:

$$\text{Fluence (J/cm}^2\text{)} = \frac{\text{Laser energy (J)}}{\text{Focal spot area (cm}^2\text{)}}$$

The precise mechanisms resulting in therapeutic effects of LLLT are not well established and investigations are ongoing, however mitochondria are thought to play

an important role. It is hypothesized that chromophores within the mitochondria absorb the photons emitted by LLLT which results in excitement of electrons and an increase in stored potential energy (Chung et al., 2012). Evidence suggests that cytochrome C oxidase, a large transmembrane protein involved in the electron transport chain, is the chromophore that acts as photoacceptor and electron excitement in this molecule results in increased ATP production and a production of a small amount of reactive oxygen species (Karu, 1999; Eells et al., 2004). Extra energy in the form of ATP can be used by the cell for protein and nucleic acid synthesis as well as provide energy for various cellular metabolic activities. Reactive oxygen species activate transcription factors which can upregulate genes related to cellular proliferation (Moore et al., 2005; AlGhamdi et al., 2012; Ejiri et al., 2014), migration (Ejiri et al., 2014; Farivar et al., 2014), and production of cytokines and growth factors (Zhang et al., 2003; de Souza da Fonseca et al., 2013; Fernandes et al., 2013; Martignago et al., 2015).

LLLT has been evaluated as a therapeutic for various ailments to promote healing (Mester et al., 1971; Medrado et al., 2003; Hawkins et al., 2005; Dawood and Salman, 2012), reduce inflammation (Marcos et al., 2011; Farivar et al., 2014; Jang et al., 2015; Fabre et al., 2015), and provide relief from pain (Konstantinovic et al., 2010b; Ruaro et al., 2014; Paschoal et al., 2014; Fabre et al., 2015). In animal models of tendonitis, LLLT has been shown to decrease gene expression of the proinflammatory biomarkers IL-1 β , IL-6, TNF- α , and Prostaglandin E₂ (PGE₂) (Marcos et al., 2012; Torres-Silva et al., 2015), as well as increase expression of type I and type III collagen (Xavier et al., 2014). It has also been found to stimulate tenocyte

migration by upregulation of dynamin 2 expression (Tsai et al., 2012), and enhance tenocyte proliferation (Tsai et al., 2014). Inhibition of COX-2 may also occur as a result of LLLT (Marcos et al., 2011; Marcos et al., 2012) which could provide a safer alternative to NSAIDs. A collagenase induced deep digital flexor tendinopathy study in sheep found that laser therapy decreased the number of fibroblasts present in the tendons and improved collagen fiber organization after 10 treatments (Iacopetti et al., 2015). However, findings in human patients have been inconsistent and inconclusive (Royal Pharmaceutical Society of Great Britain, 2010; Tumilty et al., 2010a; Tumilty et al., 2010b; Steffens and Maher, 2011; Tumilty et al., 2012; Nogueira and Júnior, 2015; Haslerud et al., 2015; Doyle et al., 2016;), which could be related to variations in treatment variables between studies, such as power, laser energy dose or fluence, wavelength, number of treatments, and time intervals between treatments.

Effective doses of LLLT are currently unknown, however doses of 1-5 J/cm² have investigated in vitro for a variety of conditions (Hawkins et al., 2005; Chen et al., 2009; AlGhamdi et al., 2012; Jann et al., 2012; Marcos et al., 2012; Tsai et al., 2012; Tsai et al., 2014; Fabre et al., 2015; Kurach et al., 2015; Martignago et al., 2015). A dose of 2-2.5 J/cm² resulted in increased cell migration and proliferation of rat Achilles tenocytes in culture (Tsai et al., 2012; Tsai et al., 2014), while doses of 2-2.16 J/cm² enhanced cell proliferation in porcine Achilles tendon fibroblasts (Chen et al., 2009). In addition, delivery of 1-3 J/cm² was noted to increase gene expression of decorin and collagen type I in porcine Achilles tendon fibroblasts (Chen et al., 2009). These findings indicate that energy doses of approximately 2 J/cm² may be ideal for maximal effects on tenocytes in culture, however effective doses remain speculative.

Investigation into the effects of LLLT in veterinary medicine are limited. There were no differences found between laser treated tendons and control tendons after experimentally induced superficial digital flexor tendon lacerations in horses (Kaneps et al., 1984), and there did not appear to be any benefit to laser treatment over the use of a rehabilitation program alone in a retrospective study of 73 racehorses with superficial digital flexor tendon injuries (Marr et al., 1993). Another study evaluated 116 Standardbreds with bowed tendons that received a single treatment of low energy infrared laser (904 nm) pulsed at 146 Hz, or 292 Hz for more chronic cases, over an average of 30 minutes (McKibbin and Paraschak, 1983). 83.4% of the horses returned to racing at similar or faster final race time speeds, but no control group was included and no ultrasonographic or histopathologic data was obtained for this study population (McKibbin and Paraschak, 1983). Transmittance of laser light through equine skin may affect outcomes with this treatment modality, and it has been shown that clipping the hair and cleaning the skin with alcohol prior to laser treatment resulted in increased transmission of laser light (Ryan and Smith, 2007). Skin color and thickness play a determining role in penetrance of laser light in the horse with light colored and thinner skin allowing the greatest amount of light penetration, with lighter skin being penetrated best by laser light at a wavelength of 800 nm and darker skin being best penetrated at a wavelength of 970 nm (Duesterdieck-Zellmer et al., 2016). In addition, a certain amount of light is scattered, and thus laser light doses may need to be adjusted for skin color and scatter so that the appropriate energy dose can be absorbed. Light colored skin has been previously identified to allow approximately 22% penetration of laser energy at 800 or 970 nm,

while medium and dark colored skin allowed penetration of 8% and 2% respectively (Duesterdieck-Zellmer et al., 2016).

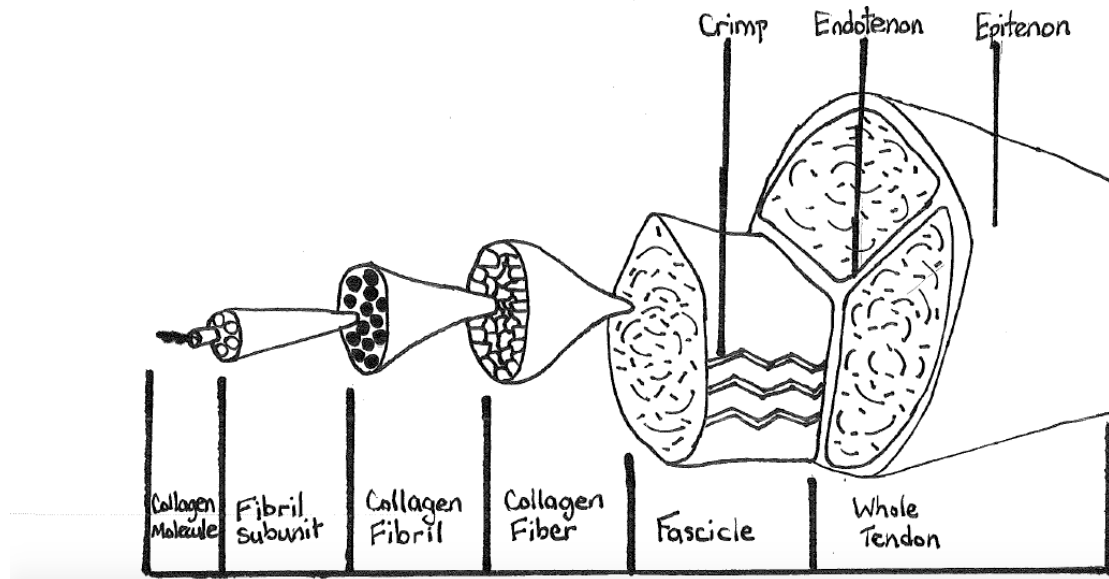


Figure 1: Diagram of normal tendon histology (adapted from: Thorpe, 2010 by Hayley Husby)

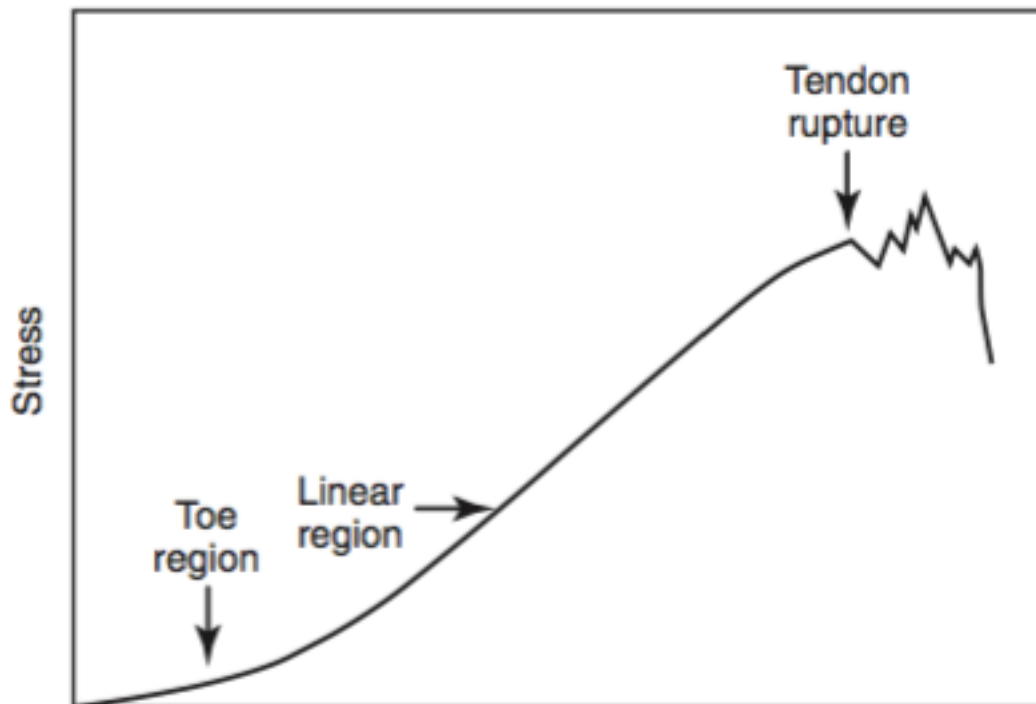


Figure 2: Stress-strain curve for tendon (from: Avella CS, Smith RKW: Diagnosis and Management of Tendon and Ligament Disorders, p 1159. In Auer JA, Stick JA (eds): Equine Surgery, Elsevier Saunders, St. Louis, 2012)

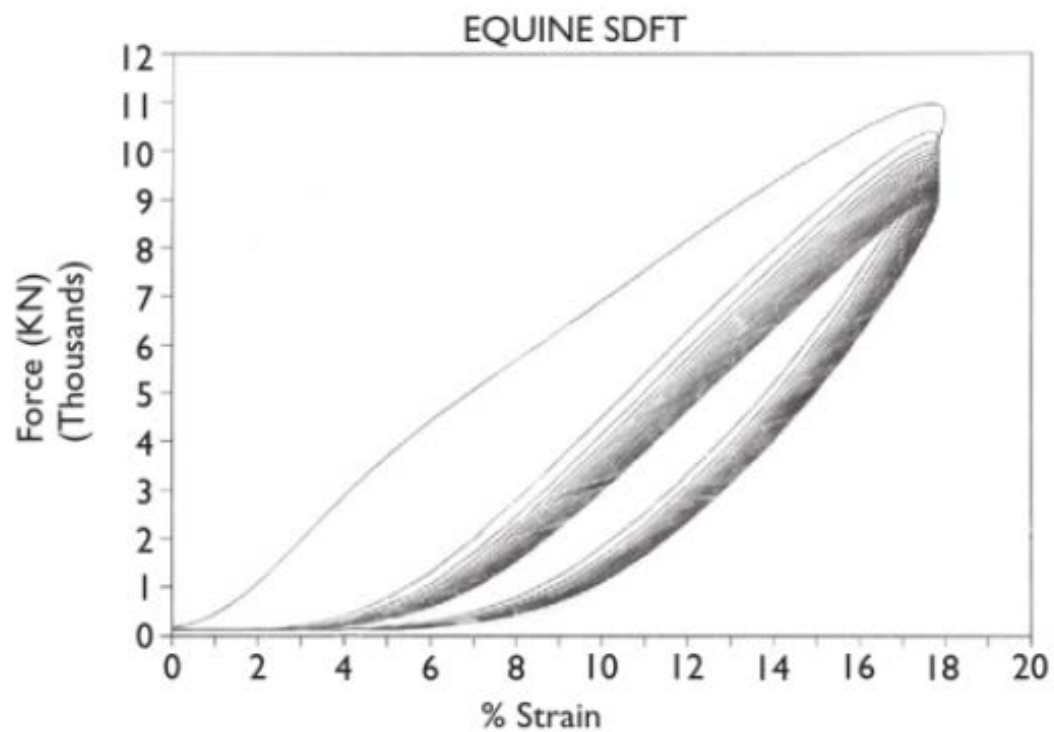


Figure 3: Hysteresis figure (from: Birch HL, Sinclair S, Goodship AE: Tendon and Ligament Physiology, p 174, In Hinchcliff KW, Kaneps AJ, Geor RJ (eds): Equine Sports Medicine and Surgery, Elsevier, Philadelphia, 2013).

Objective and Hypotheses

The objectives of this study were to evaluate the effects of manufacturer recommended therapeutic laser energy doses on equine tendon fibroblasts in vitro, and to determine if these effects were modulated by the color of skin through which the laser light was delivered. The working hypothesis was that manufacturer recommended laser energy doses would increase cell proliferation, metabolic activity and cell migration in equine tendon fibroblasts in vitro.

Materials and Methods

Cell Culture

Forelimb superficial digital flexor tendons were harvested aseptically from 4 horses (aged 3-11 years) euthanized for reasons unrelated to this study or tendinopathy within 6 hours of euthanasia. No Animal Care and Use Committee Protocol was required as tendons were harvested from fresh cadavers. The excised tendons were placed immediately into cooled phosphate buffered saline (PBS) with 100 U/ml penicillin, 100 ug/ml streptomycin, and 250 ng/ml amphotericin B for up to 30 minutes. Tendons were then transferred into tenocyte media and cut into small pieces of approximately 1-3 mm using aseptic technique. Tenocyte media consisted of high glucose Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fischer Scientific) supplemented with 10% fetal bovine serum (FBS), 100 µg/ml ascorbic acid, 300 µg/ml L-glutamine, 30 µg/ml α-ketoglutaric acid, 25 mM HEPES buffer, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B. The tendon pieces were put into 250 ml flasks with tenocyte media and collagenase (Worthington Collagenase Type 2) at 1.5 mg/ml and placed on a covered rotator that was set at 37° C and 220 rpm to digest for 16-18 hours. After the digestion period the cell suspension was filtered through sterile cheese cloth, 70 µm cell strainers, and finally 40 µm cell strainers into 50 ml conical tubes. The suspension was centrifuged at 500 *xg* for 10 minutes. The supernatant was removed and cells were resuspended in tenocyte media.

Cell number and viability were determined using 0.4 %Trypan Blue (Ameresco, VWR) and a hemocytometer. 50 µl of cell suspension was removed and

diluted with 950 μ l PBS in a 2 ml Eppendorf tube. 50 μ l of diluted cell suspension was combined with 400 μ l PBS and 50 μ l 0.4% Trypan Blue (Amersco, VWR) and allowed to sit for 5 minutes. After 5 minutes the suspension was gently mixed before 10 μ l was loaded onto each side of a hemocytometer. Using a standard microscope at 40X, all cells in each of the 4 outer squares were counted on both sides of the hemocytometer. Viable cells were unstained, and nonviable cells were stained blue. Cell count and viability were determined using the following equations:

$$\text{Cell count (cells/ml)} = \left(\frac{\text{viable cells}}{\text{number of squares}} \right) (\text{dilution factor}) (10,000)$$

$$\text{Cell viability (\%)} = \frac{\text{cell count (viable)}}{\text{total cell count (viable+nonviable)}}$$

Cells were frozen in cryovials at 10×10^6 cells/ml with 1 ml of freeze media (95% FBS + 5% DMSO) and placed in a freezing container (Nalgene® Mr. Frosty, Sigma Aldrich) which contained isopropyl alcohol and maintained at -80° F for up to 5-24 hours before transfer to liquid nitrogen.

Cells used in laser exposures were cultured from the frozen stocks. Cryovials were partially thawed in 37°C water and cells were quickly transferred to a 15 ml centrifuge tube. 10 ml of 37°C tenocyte media was slowly added to warm the cells and dilute the freeze media. The cells were centrifuged at 500 xg for 10 minutes and the cell pellet was resuspended in fresh media and plated in T-25 Flasks (Falcon) for 1 passage. Cells were grown at 37°C with 5% CO₂. On the second day of culturing, a partial media exchange was performed to remove dead cells. When cells were close to confluence, the media was removed and any remaining media was washed away with warm PBS. 1 ml of TryPLE™ Express (Gibco, Life Technologies) was added to dissociate cells from the flask surface. The flasks were incubated for 4 minutes with

gentle tapping on the sides of the flask to loosen the cells. Fresh media was added and cells were spun at 500 *xg* for 10 minutes. The supernatant was removed and fresh media was added. Cells were then transferred to T-75 Flasks (Falcon) and maintained until sufficient quantities of cells were obtained. All cells were used within 3 passages.

Laser Protocol

Laser irradiation was carried out using a commercially available multiwavelength (660 nm, 800 nm, 970 nm) Class IV laser (K-Laser Cube 3, K-Laser USA) using manufacturer recommended settings for tendon treatment in horses. The laser delivered equal amounts of all 3 wavelengths over a total treatment time of 5 minutes and 37 seconds. The treatment was divided into 6 phases of 56 seconds each that all delivered 112 J, however the pulse frequency varied during each phase. The laser settings were as follows:

Treatment phase	Time (sec)	Power (W)	Laser pulse frequency (Hz)	Duty cycle (%)	Energy delivered (J)
1	56	4	2	50	112
2	56	4	10	50	112
3	56	4	500	50	112
4	56	4	1000	50	112
5	56	4	5000	50	112
6	56	4	500	50	112

A custom steel stand (Figure 4) was designed to similar in size as the palmar metacarpal area of an average-sized horse, and to house three 3.5 cm diameter petri dishes (Falcon 353001, VWR) in darkness with circular openings that corresponded to the size of the petri dishes. The stand measured 126.5 cm², however the total area treated was slightly smaller at 105 cm². Petri dishes were placed under the custom

steel stand and the appropriate colored skin was placed over top of the stand which allowed usage of skin without potential for contamination of cell cultures. Laser attenuation had been assessed in multiple types of petri dishes in previous experiments, and the dishes used were the ones that attenuated the laser light the least of all dishes tested (5.9% attenuation). Laser treatment was carried out through shaved and cleaned light, medium, and dark colored equine cadaver skin (Figure 5) in a continuous back and forth motion ensuring that the same amount of time during each phase of the laser treatment was spent over each petri dish (Figure 6). The skin samples were all obtained from the palmar metacarpal region of cadaver horses and thickness was similar in all samples. The light colored skin sample had pink skin with light colored hair, the medium colored skin sample had dark colored skin with light brown/chestnut hair, and the dark colored skin sample had dark colored skin with dark brown/black hair. All skin samples used were evaluated to determine amount of laser energy transmittance. The light skin allowed 26% laser penetration, medium skin allowed 15.4% laser light penetration, and dark skin allowed 2.5% of laser light to be transmitted. Petri dishes used as controls were placed in the custom steel stand and black cardboard was used to cover the openings. They were not exposed to laser light.

Determination of fluence/energy dose administered to cells was calculated as follows:

$$\text{Energy (J)} = \text{Power (W)} \times \text{Time (sec)} \times \text{Duty Cycle}$$

$$\text{Fluence (J/cm}^2\text{)} = \frac{\text{Energy (J)}}{\text{Surface area (cm}^2\text{)}}$$

Cell Proliferation and Viability Determination

Tendon fibroblasts were plated in 3.5 cm diameter petri dishes (Falcon, VWR) at a density of 100,000 cells/dish and allowed 24 hours for equilibration prior to laser treatment. Treatments were performed in duplicate. Immediately following treatment, petri dishes were returned to the incubator and maintained under previously described culture conditions for 24 hours. At 24 hours the tenocyte media was removed from the dish via suction and the cells were rinsed with PBS. Cells were dissociated from the culture surface with 800 μ l TrypLETM Express cell dissociation reagent (Gibco, Life Technologies) which was placed directly into the petri dish and allowed a total of 5 minutes for maximal cell detachment. After 5 minutes 500 μ l tenocyte media was added to the suspension, and the suspension was placed in a 2 ml Eppendorf tube. To ensure all cells were removed from the petri dish, an additional 500 μ l of tenocyte media was used to rinse the dish and also added to the Eppendorf tube. The 2 ml tube was centrifuged at 500 xg for 7 minutes. The supernatant was removed from the tube and PBS was added to the remaining cell pellet to achieve a total volume of 500 μ l. The cells were gently resuspended in PBS. 500 μ l of a 0.08% Trypan Blue solution (Amresco, VWR) in PBS was added to the cell suspension and allowed to sit for 5 minutes. After 5 minutes the suspension was gently mixed before 10 μ l was loaded onto each side of a hemocytometer. Using a standard microscope at 40X, all cells in each of the 4 outer squares were counted on both sides of the hemocytometer. Viable cells were unstained, and nonviable cells were stained blue. Cell count and viability were determined using the following equations:

$$\text{Cell count (cells/ml)} = \left(\frac{\text{viable cells}}{\text{number of squares}} \right) (\text{dilution factor}) (10,000)$$

$$\text{Cell viability (\%)} = \frac{\text{cell count (viable)}}{\text{total cell count (viable+nonviable)}}$$

Resazurin Salt Assay

Tendon fibroblasts were plated in 3.5 cm diameter petri dishes (Falcon, VWR) at a density of 100,000 cells/dish and allowed 24 hours for equilibration prior to treatment. Treatment was performed in duplicate, and the petri dishes were returned to the incubator and maintained under previously described culture conditions for 24 hours. At 24 hours the tenocyte media was removed from the dish via suction, replaced with 800 μ l 0.004% Resazurin salt solution, and incubated for 2 hours. 100 μ l were removed in duplicate from each dish and placed in a 96 well plate. This was read on an absorbance based plate reader at 570 nm and 600 nm wavelengths. Percent reduction of the resazurin salt solution reagent was calculated from the absorbance data via the following equation:

$$\text{Percent reduction} = \left(\frac{(O2 \times A1) - (O1 \times A2)}{(R1 \times N2) - (R2 \times N1)} \right) \times 100$$

Where: *O1* = molar extinction coefficient of oxidized reagent at 570 nm

O2 = molar extinction coefficient of oxidized reagent at 600 nm

R1 = molar extinction coefficient of reduced reagent at 570 nm

R2 = molar extinction coefficient of reduced reagent at 600 nm

A1 = absorbance of test wells at 570 nm

A2 = absorbance of test wells at 600 nm

N1 = absorbance of media only wells at 570 nm

N2 = absorbance of media only wells at 600 nm

Transwell Migration Assay

Transwell Permeable Supports (Corning Costar, Sigma Aldrich) with 8.0 μm pores were used for the migration assay. Tendon fibroblasts were prepared as a suspension just prior to treatment. Cells were dissociated from the T75 flask (Falcon) culture surface with 2 ml TrypLETM Express cell dissociation reagent (Gibco, Life Technologies) for 4 minutes with gentle tapping. To stop the enzyme activity, 2 ml of tenocyte media was added to the suspension, and the suspension was placed in a 15 ml conical tube. An additional 2 ml of tenocyte media was used to rinse the dish and also added to the conical tube. The tube was centrifuged at 500 xg for 5 minutes. The supernatant was removed from the tube and cells were resuspended in 2 ml tenocyte media containing 0.5% bovine serum albumin (BSA) instead of 10% FBS (BSA tenocyte media). A cell count was performed using 0.4% Trypan Blue (Ameresco, VWR) and a hemocytometer as previously described. A cell suspension of 750,000 cells/ml was prepared with BSA tenocyte media and 2 ml was placed in 3.5 cm diameter petri dishes for laser or control treatment. The transwell upper and lower chambers were equilibrated with media as described below for 24 hours prior to cell addition. The upper chambers received only BSA tenocyte media as this was the media the cells were prepared in. The lower chambers were divided into two groups consisting of 3 experimental wells with tenocyte media containing 10% FBS (the chemotactic agent) and 3 control wells with BSA tenocyte media (non-chemotactic control) per laser treatment. Before addition of the cells, the lower chamber media was exchanged for fresh media of the same type, and the media was removed from the upper chambers. Following laser or control treatment, 100 μl cell suspension of

75,000 cells was added to the upper chambers. Triplicate wells were prepared for each treatment. The cells were either placed in a chamber above a well with the same media (BSA tenocyte media; non-chemotactic control) or above a well with media containing 10% FBS (chemotactic stimulus). Plates were incubated under previously described culture conditions for 6 hours. Then, cells on the upper surface which did not migrate through the pores of the membrane were removed using a cotton swab. Membranes were gently rinsed with PBS, fixed with cold methanol for 5 minutes, and allowed to air dry. This was followed by Wright-Giemsa staining for 4 minutes, double rinsing in distilled water, and air drying. Finally the membranes were removed with a #15 blade and mounted to a slide and coverslipped using Permount mounting medium (Fisher Chemical). Cells in five random fields of the membrane were counted under 400X magnification and the total for all five fields determined for each membrane. The cell numbers were then averaged with numbers from two similarly treated membranes.

In Vitro Wound Healing

Tendon fibroblasts were plated in 3.5 cm diameter gridded petri dishes (μ -Dish, ibidi USA) and grown to confluence in tenocyte media as described above. The confluent cell layer was scraped with the back of a 10 μ l sterile pipette to consistently produce a cell free line along the plate's diameter. Photographs were taken at 100X in four locations of the scraped area using the grids as reference points. Plates were subjected to laser treatment or control, and assessed at 24 hours. In vitro wound healing was photographed at 24 hours at 100X magnification in the exact same grid

locations. The photographs at 24 hours were compared to photographs prior to treatment, and distance between the cell fronts was measured in pixels (Adobe Photoshop) at two different spots in each of the four locations for each petri dish. Using these measurements, the percent of the original scratch area covered by cells at 24 hours was calculated. In addition, the number of cells between the two cell fronts over the distance of the scratch are at 24 hours was graded on a scale of 1-4, as follows:

1 = zero cells in gap

2 = 1-3 cells in gap

3 = 4-10 cells in gap

4 = >10 cells in gap

Statistical Analysis

Tendon fibroblasts from four different horses (n=4) were assigned to four different treatment groups (control – no laser treatment; light skin – laser treatment of cells through unpigmented skin; medium skin - laser treatment of cells through brown pigmented skin; dark skin - laser treatment of cells through black pigmented skin). In each treatment group, a total of four dishes with cells in media were treated, to provide one dish for determination of cell numbers and viability, one dish for the resazurin assay, one dish with cells for the transwell migration assay and one dish for the in-vitro scratch healing assay. This resulted in a total of 64 dishes used (4 horses x 4 treatment groups/horse x 4 dishes/treatment groups = 64 dishes).

Data analysis was performed using a 2-way repeated measures ANOVA (Graph Pad Prism v.6.05, GraphPad Software, Inc.) with horse as repeated measure to reflect that treatment was nested within horse and treatment as independent variable. Results are presented in means, medians and maximum and minimum values, as means may be affected by outliers especially with a small sample size as used in this study. Cell count data to assess cell proliferation was log-transformed before analysis. ANOVA tables can be reviewed in Appendix 1. Significance was set at $p < 0.05$.

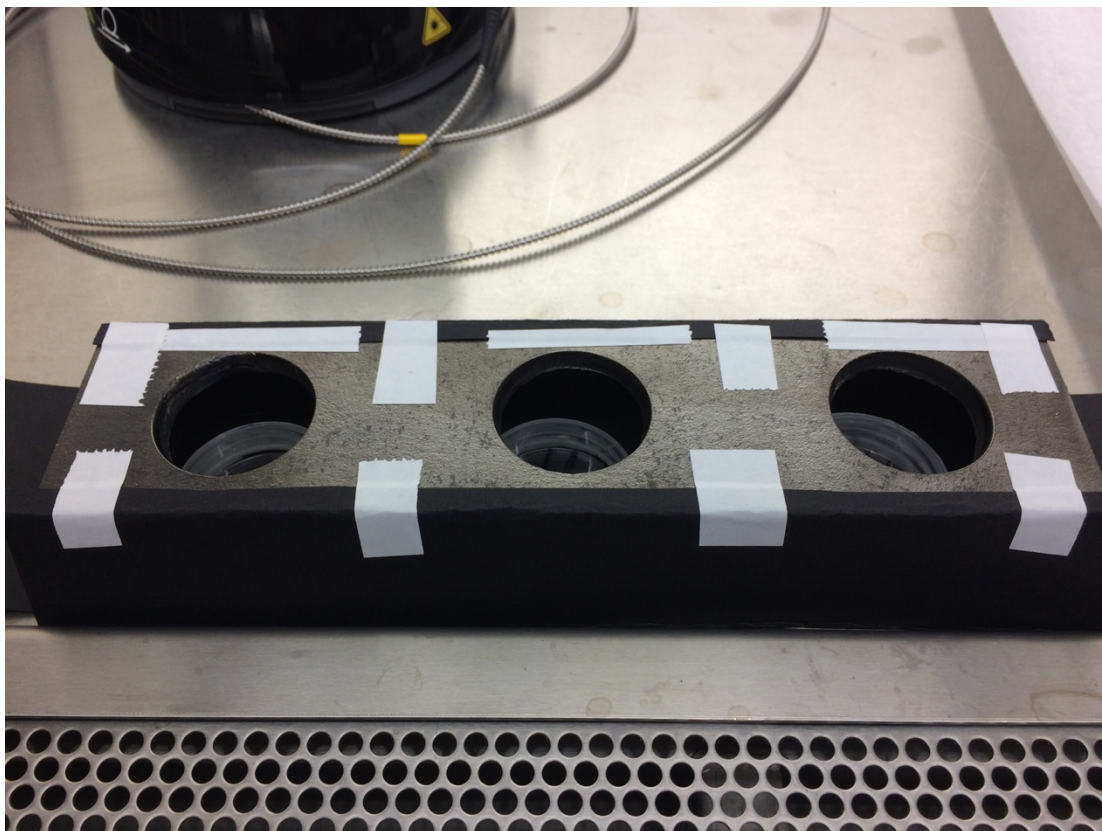


Figure 4: Custom steel stand designed to be the same size as the palmar metacarpal area of an average-sized horse with three 3.5 cm diameter circular openings

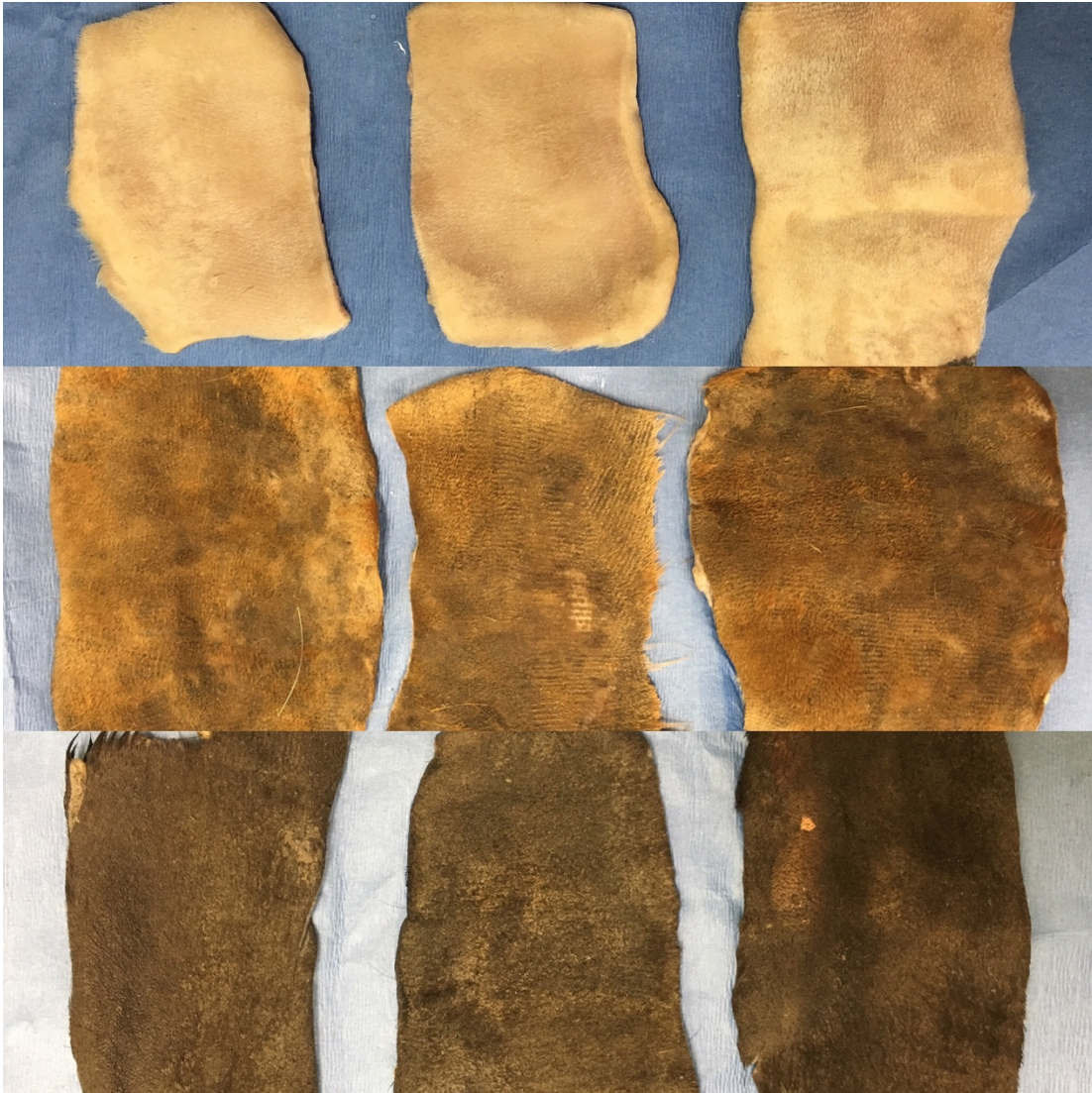


Figure 5: Cadaver skin samples used in the laser study; top = light, middle = medium, bottom = dark

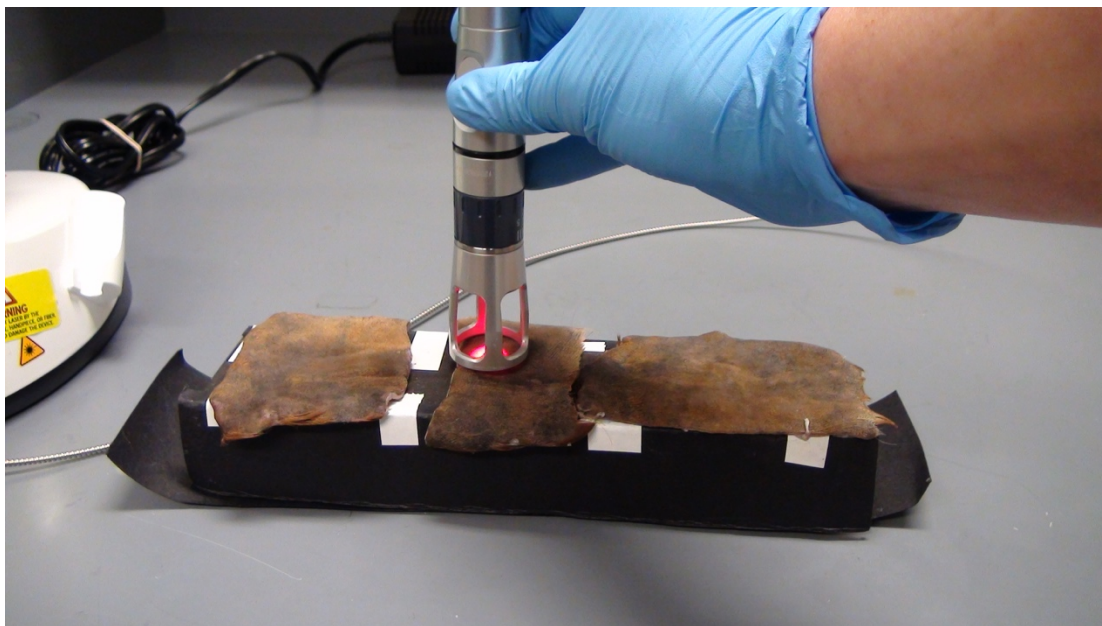


Figure 6: Lasering protocol through medium colored skin placed over top of custom steel stand.

Results

Laser Fluence

The laser fluence/total energy dose delivered to the equine tendon fibroblasts was calculated as follows based on the previously measured laser transmittance through the skin samples used in this project:

$$\text{Total energy delivered} = 6 \text{ phases} \times 112 \text{ J} = 672 \text{ J}$$

$$\text{Fluence} = \frac{672 \text{ J}}{105 \text{ cm}^2} = 6.4 \text{ J/cm}^2$$

The total fluence delivered to the surface of the skin was 6.4 J/cm^2 , however the skin and the lids of the petri dishes attenuate the laser energy. Calculation of the laser energy transmitted through the skin was calculated as follows:

$$\text{Light skin} = 6.4 \text{ J/cm}^2 \times 0.26 = 1.7 \text{ J/cm}^2$$

$$\text{Medium skin} = 6.4 \text{ J/cm}^2 \times 0.154 = 1.0 \text{ J/cm}^2$$

$$\text{Dark skin} = 6.4 \text{ J/cm}^2 \times 0.025 = 0.2 \text{ J/cm}^2$$

The laser energy dose was attenuated an additional 5.9% by the petri dish lid, and the calculation of the actual laser energy that was transmitted through the skin and petri dishes to reach the skin cells was as follows:

$$\text{Light skin} = 1.7 \text{ J/cm}^2 \times 0.059 = 1.60 \text{ J/cm}^2$$

$$\text{Medium skin} = 1.0 \text{ J/cm}^2 \times 0.059 = 0.94 \text{ J/cm}^2$$

$$\text{Dark skin} = 0.2 \text{ J/cm}^2 \times 0.059 = 0.19 \text{ J/cm}^2$$

These laser energy doses were all below the 2 J/cm^2 which appears to be ideal for having an effect on tenocytes.

Cell Proliferation and Viability

Laser treatment did not appear to influence cell proliferation as there was no significant difference in cell number at 24 hours between treatment and control groups ($p=0.2654$) (Figure 7). Mean cell number \pm standard error of mean, median cell number, minimum cell number, and maximum cell number for treatment and control groups are listed in Table 1. There was no significant difference between treatment groups with respect to cell viability at 24 hours ($p=0.4326$) (Figure 8). Based on the ANOVA model, there was a significant difference between horses for cell viability ($p=0.0416$). Mean percent viability \pm standard error of mean, median percent viability, minimum percent viability, and maximum percent viability are listed in Table 2.

Table 1: Mean \pm SEM, median, minimum, and maximum cell counts at 24 hours

Treatment Group	Mean \pm SEM	Median	Minimum Value	Maximum Value
Control	109,844 \pm 14,870 cells	96,250 cells	92,500 cells	154,375 cells
Light skin	149,063 \pm 18,685 cells	166,563 cells	93,125 cells	170,000 cells
Medium skin	139,844 \pm 7,178 cells	133,750 cells	130,625 cells	161,250 cells
Dark skin	110,000 \pm 10,517 cells	110,938 cells	89,375 cells	128,750 cells

Table 2: Mean \pm SEM, median, minimum, and maximum percent cell viability at 24 hours

Treatment Group	Mean \pm SEM	Median	Minimum Value	Maximum Value
Control	97.6% \pm 0.4	97.7%	96.5%	98.4%
Light skin	98.1% \pm 0.4	98.3%	97.1%	98.9%
Medium skin	97.7% \pm 0.2	97.8%	97.2%	98.1%
Dark skin	98.0% \pm 0.4	98.0%	97.2%	98.7%

Resazurin Salt Assay

There was no significant difference in percent reduction of resazurin salt between treatment groups ($p=0.2613$) (Figure 9). However, the factor “horse” was significant in the ANOVA model ($p=0.0004$). Mean percent reduction \pm standard error of mean, median percent reduction, minimum percent reduction, and maximum percent reduction are listed in Table 3.

Table 3: Mean \pm SEM, median, minimum, and maximum percent resazurin reduction at 24 hours

Treatment Group	Mean \pm SEM	Median	Minimum Value	Maximum Value
Control	25.66% \pm 2.50	25.23%	20.12%	32.04%
Light skin	27.65% \pm 2.52	28.18%	21.18%	33.05%
Medium skin	24.68% \pm 1.68	24.84%	20.82%	28.22%
Dark skin	26.35% \pm 1.61	26.5%	23.13%	29.25%

Transwell Migration

There was no significant difference in the number of cells that migrated through the transwell membrane from BSA tenocyte media to FBS media between treatments ($p=0.4914$) (Figure 10). There was a significant effect of horse ($p=0.0006$). Mean cell number \pm standard error of mean, median cell number, minimum cell number, and maximum cell number of migrated cells from BSA tenocyte media to media containing FBS are listed in Table 4a. There was a significant difference in the migration of cells to wells containing BSA tenocyte media compared to wells containing tenocyte media containing FBS for all groups ($p<0.0001$); more cells migrated from BSA tenocyte media to well containing FBS for all treatment groups and controls. Mean difference in cell number \pm standard error of mean difference,

median cell number, minimum cell number, and maximum cell number of migrated cells from BSA tenocyte media to BSA tenocyte media are listed in Table 4b.

Table 4a: Mean \pm SEM, median, minimum, and maximum number of cells that migrated through transwell membrane at 24 hours from BSA tenocyte media to FBS tenocyte media

Treatment Group	Mean \pm SEM	Median	Minimum Value	Maximum Value
Control	363 cells \pm 58	336 cells	258 cells	523 cells
Light skin	343 cells \pm 40	334 cells	256 cells	447 cells
Medium skin	375 cells \pm 65	352 cells	244 cells	551 cells
Dark skin	399 cells \pm 73	381 cells	243 cells	590 cells

Table 4b: Mean difference \pm SEM, median, minimum, and maximum number of cells that migrated through transwell membrane at 24 hours from BSA tenocyte media to BSA tenocyte media

Treatment Group	Mean difference \pm SEM	Median	Minimum Value	Maximum Value
Control	349 cells \pm 33	14 cells	10 cells	19 cells
Light skin	307 cells \pm 33	33 cells	10 cells	69 cells
Medium skin	334 cells \pm 33	31 cells	5 cells	97 cells
Dark skin	348 cells \pm 33	41 cells	9 cells	109 cells

In Vitro Wound Healing

There were no significant differences in width of the scratch area covered by cells ($p=0.6685$) or scratch scores ($p=0.5129$) between treatment groups compared to the control group (Figures 11 and 12). There were significant differences in scratch scores between horses ($p=0.0274$). Mean percent scratch width remaining \pm standard error of mean, median percent scratch width remaining, minimum percent scratch width remaining, and maximum percent scratch width remaining are listed in Table 5.

Mean scratch scores \pm standard error of mean, median scratch scores, minimum scratch scores, and maximum scratch scores are listed in Table 6.

Table 5: Mean \pm SEM, median, minimum, and maximum percent scratch width remaining at 24 hours

Treatment Group	Mean \pm SEM	Median	Minimum Value	Maximum Value
Control	44.1% \pm 4.7	47.8%	34.8%	49.6%
Light skin	44.7% \pm 6.1	43%	35.1%	56%
Medium skin	45.8% \pm 5.5	48.6%	35.1%	53.6%
Dark skin	51.2% \pm 3.1	54%	45%	54.6%

Table 6: Median scratch score, minimum scratch score, and maximum scratch score at 24 hours

Treatment Group	Mean \pm SEM	Median	Minimum Value	Maximum Value
Control	2.35 \pm 0.30	2.35	1.7	3
Light skin	2.4 \pm 0.40	2.65	1.3	3
Medium skin	1.93 \pm 0.37	1.7	1.3	3
Dark skin	2.33 \pm 0.42	2.5	1.3	3

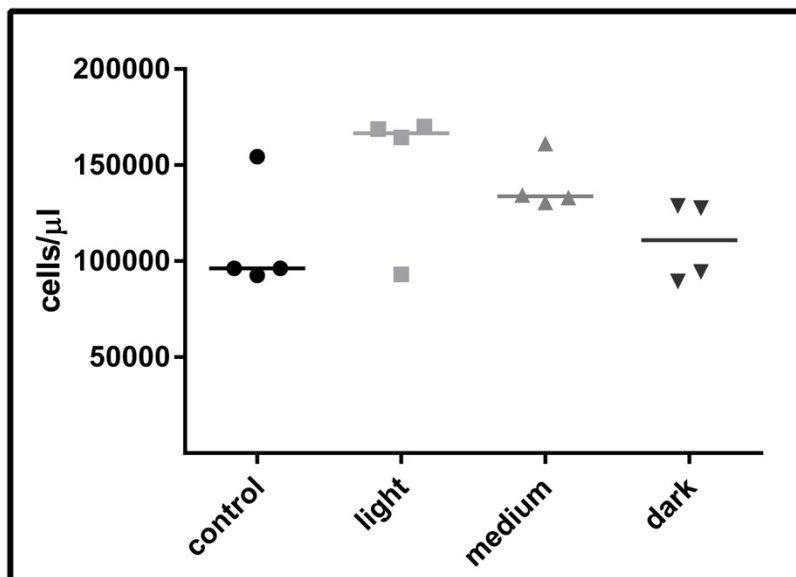


Figure 7: Cell numbers (cells/μl) 24 hours after treatment with therapeutic laser light through unpigmented (“light”), brown pigmented (“medium”) or black pigmented (“dark”) equine cadaver skin according to manufacturer recommendations. Control samples were not exposed to therapeutic laser light, but removed from the incubator for a similar amount of time as treated samples. Bars represent the median and each datapoint represents measurements from cells of individual horses (n=4).

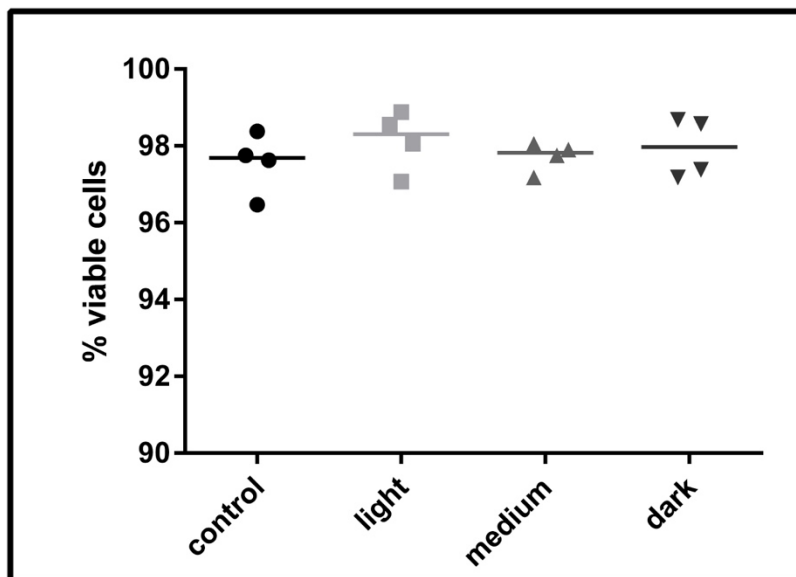


Figure 8: Percent viable cells 24 hours after treatment with therapeutic laser light through unpigmented (“light”), brown pigmented (“medium”) or black pigmented (“dark”) equine cadaver skin according to manufacturer recommendations. Control samples were not exposed to therapeutic laser light, but removed from the incubator for a similar amount of time as treated samples. Bars represent the median and each datapoint represents measurements from cells of individual horses (n=4).

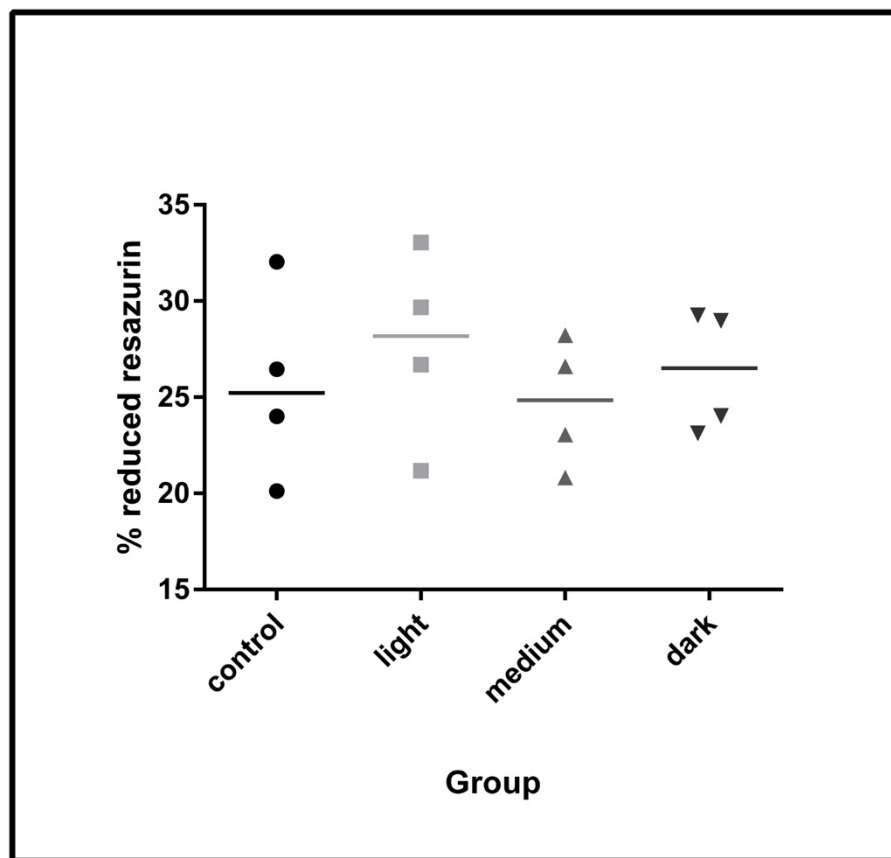


Figure 9: Percent reduced resazurin 24 hours after treatment with therapeutic laser light through unpigmented (“light”), brown pigmented (“medium”) or black pigmented (“dark”) equine cadaver skin according to manufacturer recommendations. Control samples were not exposed to therapeutic laser light, but removed from the incubator for a similar amount of time as treated samples. Bars represent the median and each datapoint represents measurements from cells of individual horses (n=4).

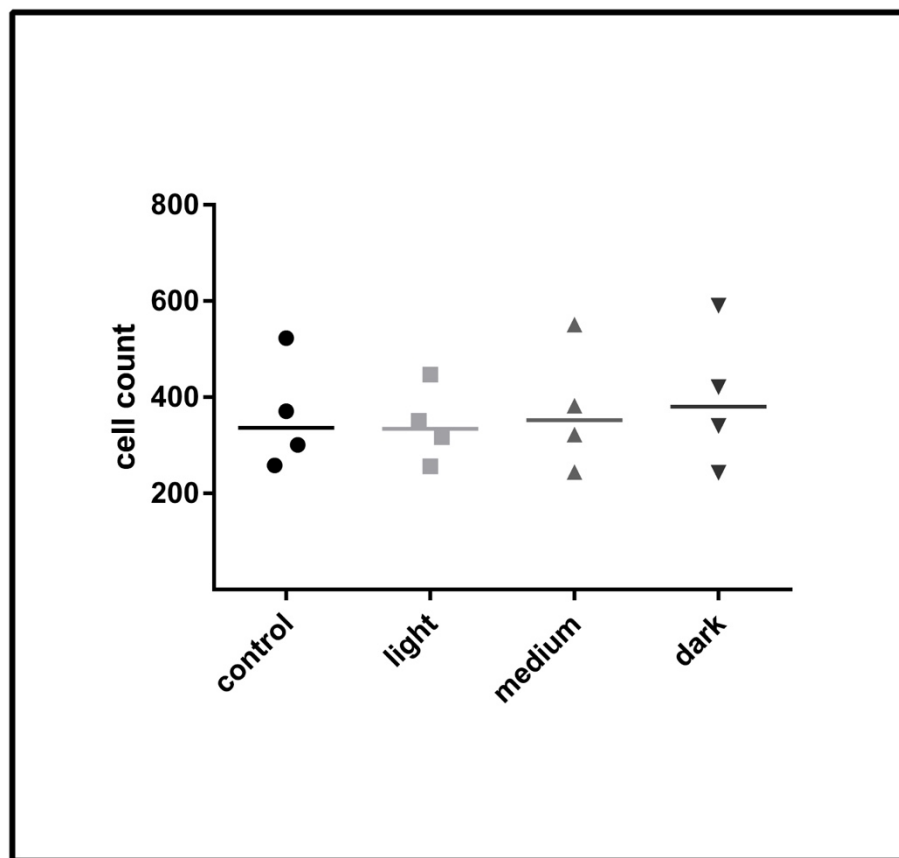


Figure 10: Number of cells that migrated through a transwell membrane driven by a chemotactic gradient for 6 hours following treatment with therapeutic laser light through unpigmented (“light”), brown pigmented (“medium”) or black pigmented (“dark”) equine cadaver skin according to manufacturer recommendations. Control samples were not exposed to therapeutic laser light, but removed from the incubator for a similar amount of time as treated samples. Bars represent the median and each datapoint represents measurements from cells of individual horses (n=4).

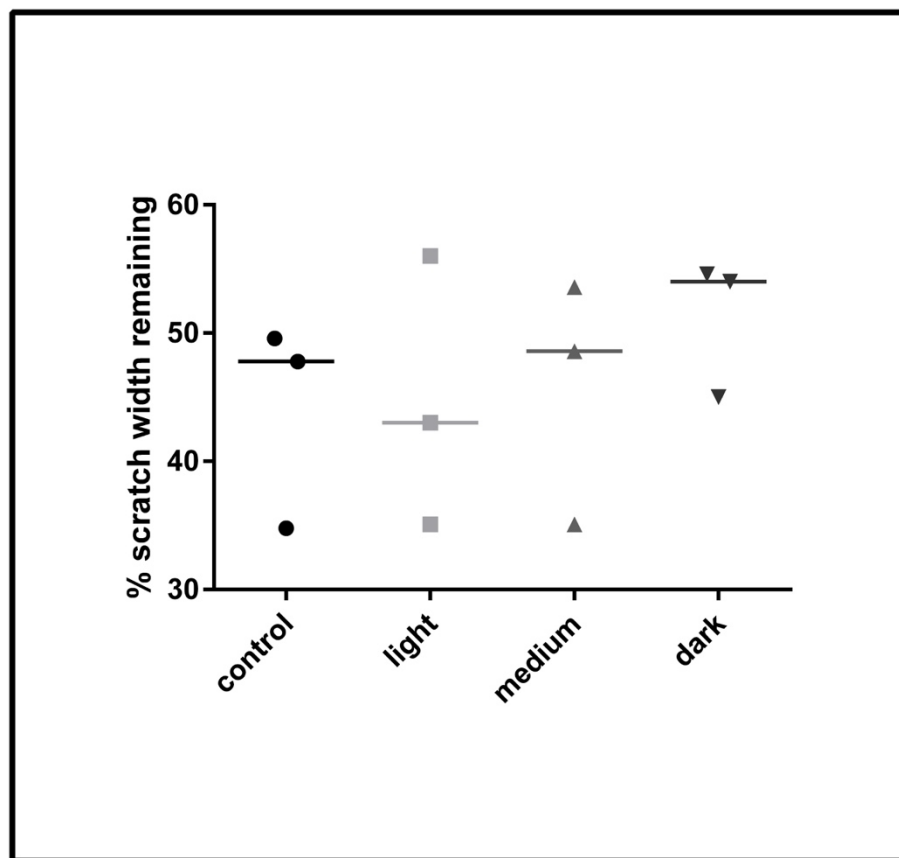


Figure 11: Percent scratch width remaining 24 hours after treatment with therapeutic laser light through unpigmented (“light”), brown pigmented (“medium”) or black pigmented (“dark”) equine cadaver skin according to manufacturer recommendations. Control samples were not exposed to therapeutic laser light, but removed from the incubator for a similar amount of time as treated samples. Bars represent the median and each datapoint represents measurements from cells of individual horses (n=3 due to missing data points for one horse).

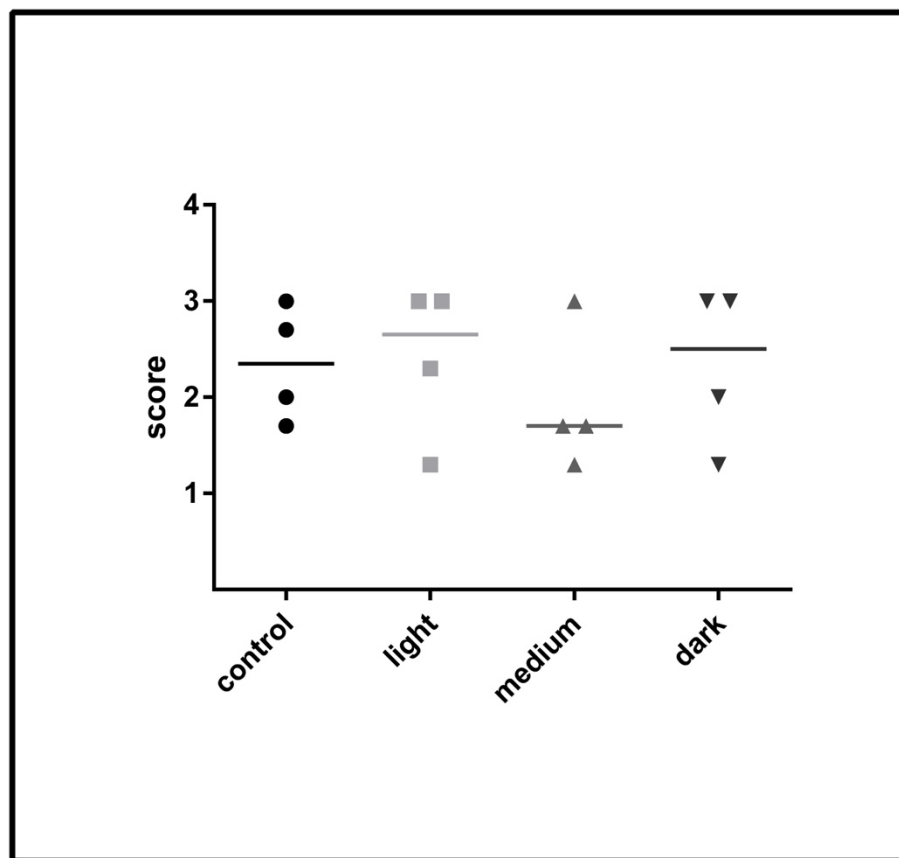


Figure 12: Scratch scores 24 hours after treatment with therapeutic laser light through unpigmented (“light”), brown pigmented (“medium”) or black pigmented (“dark”) equine cadaver skin according to manufacturer recommendations. Control samples were not exposed to therapeutic laser light, but removed from the incubator for a similar amount of time as treated samples. Bars represent the median and each datapoint represents measurements from cells of individual horses (n=4).

Scratch scores were as follows:

1 = zero cells in gap

2 = 1-3 cells in gap

3 = 4-10 cells in gap

4 = >10 cells in gap

Discussion

To the author's knowledge, this is the first study to look at the effects of LLLT on equine tendon fibroblasts in vitro. There is minimal literature available on the use of LLLT for tendinopathies in horses, with no consistent indication that this modality offers benefit for treatment of these conditions in equine patients. This study did not identify any significant differences in cell numbers, cellular metabolism, or cell migration of tendon fibroblasts harvested from grossly normal equine superficial digital flexor tendons treated with LLLT at manufacturer recommended settings through light, medium, and dark pigmented skin when compared to non-treated controls.

It is unknown what laser energy dose, or fluence, is appropriate for use on horse tenocytes. There is no standard dose of laser energy, or fluence, in the veterinary literature for animals; however the World Association of Laser Therapy has published dose recommendations for human patients. Scientific justification for these dose recommendations are not provided and adjustments for skin pigmentation are not suggested. For Achilles tendinopathies, at wavelengths between 780 nm and 904 nm, a total dose of 8 J at a maximum of 100 mW/cm² was recommended (World Association of Laser Therapy, 2010). Studies on human fibroblasts and keratinocytes in monolayer culture treated with laser therapy at powers of 5 - 100 mW for 10 – 120 seconds with an energy fluence of 0.12 – 4.24 J/cm² identified no differences in cell numbers, cell migration, or cell adhesion (Pogrel et al., 1997).

Positive effects have been recognized with LLLT of porcine and murine tenocytes in vitro which include increased cell proliferation, increased type I collagen

mRNA expression, increased decorin mRNA expression, increased cell migration, and inhibition of COX-2 expression (Chen et al., 2009; Tsai et al., 2012; Tsai et al., 2014). Porcine tendon fibroblasts treated with LLLT at 1 J/cm², 2 J/cm², and 3 J/cm² in vitro responded with increased cell proliferation (Chen et al., 2009). The highest cell proliferation effect was seen in cells treated with 2 J/cm² while the other two groups had similar responses indicating that the dose response range may be limited, and a higher laser energy dose does not necessarily correspond to increased cell proliferation (Chen et al. 2009). Rat tenocytes subjected to LLLT at 1.0 J/cm², 1.5 J/cm², and 2.0 J/cm² displayed increased cell migration via transwell migration assay in a dose-dependent manner with the tenocytes treated with 2.0 J/cm² having the highest migration (Tsai et al., 2012). A different group of rat tenocytes treated with 1.0 J/cm², 1.5 J/cm², 2.0 J/cm², and 2.5 J/cm² in vitro identified that laser therapy at a dose of 2.0 J/cm² resulted in the most significant cell proliferation further corroborating that a dose of 2.0 J/cm² appears to be the most ideal for stimulation of effects in vitro (Tsai et al., 2014). We did not reach a dose of 2.0 J/cm² in treatment of the cells in our study when using manufacturer recommended settings through cadaver skin samples. It is conceivable that minimal effects were seen due to an insufficient dose being administered to the cells. It is also possible that time points and/or response variables selected for measurement in our study happen to be times or variables that show no change, and determination of alterations in gene expression after laser treatment would be of interest to potentially allow a more sensitive indicator of tendon fibroblast response to laser energy.

It is possible that equine tendon fibroblasts are less responsive to LLLT compared to other species. An in-vivo surgically induced model of tendon healing in rat Achilles tendons determined that tendons treated with 1 J/cm^2 had increased levels of tissue hydroxyproline and improved tendon biomechanics (Demir et al., 2004), while another in-vivo induced Achilles tendonitis model in rats treated with 5 J/cm^2 resulted in reduced scarring and increased superoxide dismutase activity (Fillipin et al., 2005). It is difficult to extrapolate these results to the horse, as rat skin is much thinner and the Sprague-Dawley rats utilized in these studies is an albino rat with light colored skin which would allow greater penetration of laser light.

One study in horses suggests there may be a beneficial effect of LLLT for treatment of superficial digital flexor tendonitis in racehorses with 83.4% of horses returning to racing within 120 days of treatment (McKibbin and Paraschak, 1983). However there was no control group included in the study and no discussion of other treatments performed (McKibbin and Paraschak, 1983). It is possible that these horses would have healed with rest and controlled return to exercise, and the single LLLT treatment had no or just a negligible effect. No beneficial effects of laser therapy were noted histopathologically for surgically induced superficial digital flexor tendon lesions when compared to control tendons (Kaneps et al., 1984), and no improvement in outcomes was noted after treatment with LLLT compared to a rest and rehabilitation program alone in a retrospective study of 73 racehorses with superficial digital flexor tendonitis (Marr et al., 1993). Although our preliminary findings have not identified any potential positive effects after LLLT on equine tendon fibroblasts in vitro for the variables that we measured, a direct correlation to in

vivo LLLT cannot be made due to the multitude of extraneous variables that could have an influence on outcome in a live animal. Additional in vitro and in vivo studies would be required to confirm if equine cells do not exhibit the same response to this modality as other species.

It is unlikely that the absence of differences in cell number, cell metabolism, or cell migration identified in this study were due to a lack of penetration of the laser light through equine skin. It has been shown that laser light (810 nm, 500 mW) was transmitted through equine skin and superficial digital flexor tendons in 17 horses in one study, and penetration was improved by clipping the hair and cleaning the skin with alcohol (Ryan and Smith, 2007). Skin samples used in our study had been clipped and cleaned with alcohol prior to the laser treatments, and were stored in between treatments in a -20° F freezer. In addition, it has previously been shown that LLLT penetrates previously frozen light, medium, and dark colored equine skin, although there was greater penetration through light colored equine skin compared to medium and dark colored equine skin (Duesterdieck-Zellmer et al., 2016).

It is possible that no differences between treatment groups were identified due to the small number of horses investigated in this study. There was also a significant impact of horse on multiple variables, including cell viability, resazurin salt reduction, transwell migration, and scratch scores. This may indicate that there is variability in each horse's response to laser energy, or that individual horses' cells vary in their response to cell culture conditions. It may also indicate significant differences in individual horse's ability to heal tendon injuries. Ideally a larger study

population would allow for more accurate evaluation of outcomes at a population level, and reduce each individual horse's effect on statistical results.

It is known that penetration depth is wavelength dependent, and as such infrared laser light which has a longer wavelength is used for treatment of deeper structures (Kolari, 1985; Kolárová et al., 1999; Chung et al., 2012). Previous work in our lab has shown that a wavelength of 800 nm allowed greater penetration through light colored skin, while a wavelength of 970 nm allowed greater penetration through medium and dark colored skin (Duesterdieck-Zellmer et al., 2016), which was explained by greater absorption of laser light by melanin at 800 nm in darker skin and greater absorption of laser light by water at 970 nm in lighter skin. In our study all three wavelengths (660 nm, 800 nm, and 970 nm) were combined for all treatments, which means that an equal amount of each wavelength was delivered to the cells during the treatment time period. Although this may decrease some absorption of laser energy at less effective wavelengths, it also would not enhance penetration at more optimal wavelengths. As such it may be recommended to adjust wavelength to maximize penetration based on skin color.

Resazurin is a blue dye which becomes pink when reduced, and can be used to monitor cell viability as well as provide an estimate of cell number based on metabolic activity (O'Brien et al., 2000). The resazurin salt is metabolized by live cells and this activity is detected by the fluorescence of the byproduct. The time for reduction of resazurin is proportional to the cell number. Manual cell counts were performed in this study in addition to the resazurin assay due to the potential for LLLT to increase redox activity of cells (Chen et al., 2011). Manual cell counts and

viability determination were performed in this study after staining with Trypan Blue which is based on the principle that live cells do not take up the dye. Since these are performed manually there is the risk of human counting error, however this was attempted to be reduced by performing all counts in duplicate. A more accurate evaluation of cell division in response to laser treatment may have been better accomplished with cell counts at multiple time points and creation of a growth curve, however this was not logistically feasible in this study due to the fact that this would have required an extensive number of treated dishes.

Limitations of this study include the small number of horses studied and the lack of a positive laser control group without the use of skin in-between the laser and the cells. Time points for evaluation may not have been ideal, and it is possible that changes may have been more ideally measured at less or greater than 24 hours. Additionally, the tendon fibroblasts studied were obtained from grossly normal tendons, and it is possible that cells from injured tendons may respond differently to LLLT. Previous *in vitro* studies in other species, however have identified responses after exposure to LLLT when evaluating normal tenocytes (Chen et al., 2009; Tsai et al., 2012; Tsai et al., 2014). Future research goals should include evaluation of cellular response to LLLT via assays for growth factors and collagen production, determining which (if any) dose of laser energy *in vitro* without attenuation by cadaver skin may be effective at increasing cell proliferation and cell migration *in vitro*, evaluating whether tenocytes harvested from naturally occurring or induced tendonitis respond differently to LLLT, and establishing how to better account for

laser energy losses through the skin in order to more accurately deliver an effective dose to the affected tendon.

Conclusion

The current study was unable to identify any beneficial effects of LLLT administered according to manufacturer recommendations for tendon healing in horses, and the hypothesis that manufacturer recommended laser energy doses would increase cell proliferation and cell migration in equine tendon fibroblasts in vitro was not proven. It is possible that effects were not seen due to low doses of energy being delivered to cells as a consequence of attenuation of laser light by skin, especially the medium and dark colored skin groups. Although no increases were noted in cell proliferation or cell migration, there may have been effects of LLLT within the cell that were not measured such as increased production of growth factors or collagen synthesis. Further studies should investigate assays for growth factors and collagen production, determine whether different energy settings would result in measurable cellular effects, evaluate effects of higher energy doses, and whether there is an effect on equine tendon fibroblasts in vitro without the complicating factor of cadaver skin. Clinical efficacy of this modality for treatment of tendon injury in equine athletes remains unproven.

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Appendix A

ANOVA Table for Cell Counts

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Squares	F-Ratio	P Value
Treatment	0.3059	3	0.102	1.713	0.2654
Individual	0.04359	3	0.01453	0.2441	0.8635
Residual (random)	0.5357	9	0.05953		
Total	0.8853	15			

ANOVA Table for Cell Viability

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Squares	F-Ratio	P Value
Treatment	0.7833	3	0.12611	0.9330	0.4326
Individual	3.5	3	1.167	4.170	0.0416
Residual (random)	2.519	9	0.2798		
Total	6.802	15			

ANOVA Table for Resazurin Salt Reduction

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Squares	F-Ratio	P Value
Treatment	18.7	3	6.233	1.814	0.2613
Individual	184.7	3	61.55	0.2441	0.0004
Residual (random)	30.92	9	3.435		
Total	234.3	15			

Two-Way ANOVA Table for Transwell Migration

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Squares	F-Ratio	P Value
Treatment	6939	3	2313	1.672	0.2416
Horse	895122	1	895122	27.83	0.0133
Interaction: Treatment x Horse	2339	3	779.8	0.3519	0.7890
Interaction: Treatment x Subjects	12449	9	1383		
Interaction: Horse x Subjects	96494	3	32165		
Subjects	61453	3	20484		
Residual	19944	9	2216		

ANOVA Table for Scratch Scores

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Squares	F-Ratio	P Value
Treatment	0.575	3	0.1917	0.6738	0.5129
Individual	4.185	3	1.395	4.904	0.0274
Residual (random)	2.56	9	0.2844		
Total	7.32	15			

ANOVA Table for Scratch Width

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Squares	F-Ratio	P Value
Treatment	95.31	3	31.77	0.3288	0.6685
Individual	14.33	2	7.166	0.07415	0.9294
Residual (random)	579.8	6	96.64		
Total	689.5	11			

Appendix B: Assessment of Efficacy of IL-1 Small Interfering RNA (siRNA) on Equine Articular Chondrocytes In Vitro

Introduction

Osteoarthritis is a disease process whereby articular cartilage, subchondral bone, and other joint tissues undergo degenerative changes resulting in loss of articular cartilage, bone remodeling, and loss of normal function which may manifest clinically as discomfort (Frisbie, 2012). Osteoarthritis is a significant cause of joint pain among many species, and is estimated to amount to > \$185 billion in human health care expenditure annually (Kotlarz et al., 2009). In the horse, it is a common source of lameness and poor performance affecting all breeds and disciplines and results in significant pain for the animal and economic losses for the equine industry (United States Department of Agriculture, 2002).

Normal synovial joint biology balances catabolic or degradative processes with anabolic or reparative processes, and in osteoarthritis the balance of these processes is tipped towards degradation of articular cartilage. The pathophysiology of osteoarthritis involves the interaction of complex biological pathways with multiple mediators. These mediators include metalloproteinases, prostaglandins, interleukins, tumor necrosis factor, free radicals, and nitric oxide. Interleukin-1 β (IL-1 β) has been identified as the major pro-inflammatory and catabolic cytokine in development and progression of osteoarthritis (Morris, 1990; Tortorella and Malfait, 2003; Daheshia and Yao, 2008). Secretion of IL-1 β from chondrocytes and synoviocytes is upregulated in osteoarthritis which promotes expression and activation of proteases,

including matrix metalloproteinases (MMPs), aggrecanases and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs), prostaglandin E₂ (PGE₂), and nitric oxide which leads to cartilage degradation (Frisbie, 2012; Benito et al., 2005; Morris, 1994; Caron et al., 1996b; Freen et al., 1997). Upregulation of IL-1 β has also been shown to decrease synthesis of interleukin-1 receptor antagonist (IL-1 Ra) and tissue inhibitor of matrix metalloproteinase (TIMP) that antagonize cartilage degradation in normal joints (Martel-Pelletier et al., 1991).

Inhibition of IL-1 β through interleukin-1 receptor antagonist protein has shown promise in humans and animal models with both rheumatoid arthritis and osteoarthritis (Caron et al., 1996a; Cohen et al., 2002; Chevalier et al., 2005; Yang et al., 2008). Intra-articular treatment with interleukin-1 receptor antagonist has resulted in improved lameness scores and histologic variables in horses with experimentally induced osteoarthritis compared to controls (Frisbie et al., 2002; Frisbie et al., 2007).

Small interfering RNAs (siRNAs) are short, double stranded RNA molecules that are complementary and thus highly specific RNA transcripts of specific genes. This is a relatively new method of gene therapy that has been effective at silencing genes post transcription in several in vitro experiments, animal models of disease processes, and a few clinical studies. There is an abundance of literature from recent years examining siRNA gene therapy for inhibition of various neoplasms (Ellermeier et al., 2013; Wu et al., 2013; Kong et al., 2015; Devi, 2006; Li et al., 2015). The vast majority of this work has been done in vitro, although some studies have been performed in animal models, and the focus appears to have shifted to identifying a safe and effective delivery system to transport the siRNA into the cell. In addition to

cancer therapy, siRNA has been evaluated for inhibition of progression of ophthalmic diseases (Chen et al., 2013; Courtney et al., 2014; Li et al., 2014; Katakai et al., 2015), amelioration of allergic airway inflammation (Wu et al., 2014; Chen et al., 2014; Asai-Tajiri et al., 2014; Xie and Merkel, 2015), reduction of progression of rheumatoid arthritis (Zhang et al., 2013; Lee et al., 2014; Mehta et al., 2015; Kim et al., 2015a;), and regeneration of muscle and stimulation of muscle hypertrophy (Mosler et al., 2014; Kim et al., 2015b).

Gene therapy via inhibitory RNA has been recently investigated for amelioration of osteoarthritis symptoms. Intra-articular injection of siRNA to inhibit expression of hypoxia-inducible factor 2α (HIF- 2α), a factor produced by chondrocytes that promotes cartilage degradation, in an osteoarthritis model in mice resulted in downregulation of HIF- 2α , MMP-13, MMP-9, and VEGF in chondrocytes, decreased IL- 1β concentration in synovial fluid, and improved cartilage integrity (Pi et al., 2015). Short hairpin RNA intended to reduce transcription of IL- 1β delivered via viral vector resulted in significant knockdown of IL- 1β (> 90%) in cultured chondrocytes from guinea pigs (Santangelo and Bertone, 2011). A lentivirus mediated ADAMTS-5 siRNA resulted in decreased ADAMTS-5 gene expression by 80% in rat chondrocytes in culture, and a single intra-articular injection into the tibiofemoral joint after induced osteoarthritis prevented degradation of articular cartilage (Chu et al., 2013). MMP-13 siRNA injected into the knee of a mouse model of osteoarthritis resulted in a 55% decrease in MMP-13 levels in cartilage 48 hours after injection and at 8 weeks the histological score of articular cartilage was significantly reduced compared to controls (Akagi et al., 2014). These

studies have shown significant promise that siRNA treatment may be effective at reducing the catabolic processes that drive osteoarthritis.

Objectives

The objectives of the study were to optimize in vitro conditions to subsequently evaluate the efficacy of 3 different IL-1 β siRNAs to inhibit IL-1 β gene expression in equine chondrocytes in culture, using a challenge with recombinant equine IL-1 β protein as an in vitro model of osteoarthritis.

There were multiple specific aims investigated with this project. These included:

- 1) Optimize chondrocyte cell culture techniques, cell passage, and cell dissociation time.
- 2) Evaluation of the effect of antibiotic free media on chondrocytes to determine whether antibiotic free media during lipofectamine transfection would have a negative effect on cells.
- 3) Determine ideal concentrations of IL-1 β to simulate osteoarthritis without excessive effects on chondrocyte viability when experiencing a time period of culture without FBS to simulate the transfection period.
- 4) Investigation of ideal concentration of lipofectamine for transfection.
- 5) Explore effect of 3 IL-1 β siRNAs on knockdown of IL-1 β .

Materials and Methods

Cell Culture

Articular cartilage was harvested aseptically within 12 hours of euthanasia from grossly normal femorotibial and femoropatellar joints of 6 horses (aged 3-12 years) euthanatized for reasons unrelated to this study or joint disease. The excised cartilage pieces were placed immediately into cooled chondrocyte media for up to 30 minutes. Chondrocyte media included a 50:50 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Hams F-12 with 10% fetal bovine serum (FBS), 21.5 $\mu\text{g/ml}$ ascorbic acid, 130 $\mu\text{g/ml}$ L-glutamine, 13 $\mu\text{g/ml}$ α -ketoglutaric acid, 2.3% 1M HEPES buffer, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin. Cartilage was then cut into small pieces of approximately 1-2 mm using aseptic technique. The cartilage pieces were put into 250 ml flasks with chondrocyte media and collagenase (Worthington Collagenase Type II) at 1.5 mg/ml and placed on a covered rotator that was set at 37° C and 220 rpm to digest for 16-18 hours. After the digestion period the cell suspension was filtered through sterile cheese cloth, 70 μm cell strainers, and finally 40 μm cell strainers into 50 ml conical tubes. The suspension was centrifuged at 500 $\times g$ for 5 minutes. The supernatant was removed and cells were resuspended in chondrocyte media.

Cell number and viability were determined using 0.4% Trypan Blue (Ameresco, VWR) and a hemocytometer. 50 μl of cell suspension was removed and diluted with 950 μl PBS in a 2 ml Eppendorf tube. 50 μl of diluted cell suspension was combined with 400 μl PBS and 50 μl 0.4% Trypan Blue (Ameresco, VWR) and allowed to sit for 5 minutes. After 5 minutes the suspension was gently mixed before

10 μ l was loaded onto each side of a hemocytometer. Using a standard microscope at 40X, all cells in each of the 4 outer squares were counted on both sides of the hemocytometer. Viable cells were unstained, and nonviable cells were stained blue.

Cell count and viability were determined using the following equations:

$$\text{Cell count (cells/ml)} = \left(\frac{\text{viable cells}}{\text{number of squares}} \right) (\text{dilution factor}) (10,000)$$

$$\text{Cell viability (\%)} = \frac{\text{cell count (viable)}}{\text{total cell count (viable+nonviable)}}$$

Cells were plated at 2×10^6 cells/cm² and maintained at 37° C in a humidified incubator with 5% CO₂ and 95% air. Media changes were performed every 48 hours until confluence. Cells that were not being used immediately were prepared for freezing. Cells were frozen in cryovials at 10×10^6 cells/ml with 1 ml of freeze media (95% FBS + 5% DMSO) and placed in a freezing container (Nalgene® Mr. Frosty, Sigma Aldrich) which contained isopropyl alcohol and maintained at -80° F for 5-24 hours before transfer to liquid nitrogen.

Cell Passage and Optimization of Cell Dissociation Time

Cell passage was performed as needed when cells reached confluence. Cells were dissociated from the culture surface with TrypLE™ Express cell dissociation reagent (Gibco, Life Technologies). To determine optimum cell dissociation time with maximum cell recovery and highest viability, several exposure times (2, 4, 6, 10, 20 minutes) were evaluated both with flasks in the incubator and at room temperature with light tapping of the flasks to loosen cells. After each experiment cell viability

was determined as described above, and culture flasks were evaluated to determine the number of cells remaining.

For cell passage, cells were dissociated from the culture surface with TrypLE™ Express cell dissociation reagent (Gibco, Life Technologies) which was placed directly into the culture flask and allowed a total of 4 minutes for maximal cell detachment at room temperature with gentle tapping. After 4 minutes, 2 ml chondrocyte media was added to the suspension, and the suspension was placed in a 15 ml conical tube. To ensure all cells were removed from the culture flask, an additional 2 ml of chondrocyte media was used to rinse the dish and also added to the conical tube. The tube was centrifuged at 500 xg for 5 minutes. The supernatant was removed from the tube and cells were gently resuspended in 2 ml chondrocyte media. A cell count was performed using 0.4% Trypan Blue (Ameresco, VWR) and a hemocytometer as previously described. Cells were again plated at 2×10^6 cells/cm² and maintained under previously described culture conditions.

Evaluation of FBS and Antibiotic Free Media on Chondrocytes

When performing siRNA transfection of chondrocytes with Lipofectamine® Transfection Reagent (Invitrogen, Life Technologies), the cells must be cultured in FBS and antibiotic free media around the time of transfection. To ascertain cell viability of cells cultured without FBS and antibiotics, the following experiments were performed. Cells were passaged, resuspended in media, and plated in 24 well cell culture plates (Sigma-Aldrich) at a density of 30,000 cells and a total of 500 μ l media per well. Cell viability was evaluated after incubation in chondrocyte media

without antibiotics for 24 hours, followed by removal of FBS for 4, 6, and 8 hours. Cells were inspected subjectively for morphology, comparing them to cells cultured in media containing both antibiotics and FBS.

Evaluation of Different Concentrations of IL-1 β on Chondrocytes

In order to determine concentrations of IL-1 β high enough to simulate osteoarthritis but without an excessive effect on chondrocyte viability (especially when experiencing a time period of culture without FBS to simulate the transfection period), the following experiment was performed. Cells were passaged, resuspended in media, and plated in 24 well cell culture plates (Sigma-Aldrich) at a density of 30,000 cells and a total of 500 μ l media per well. Cells were incubated for 24 hours in chondrocyte media without antibiotics or IL-1 β . At 24 hours the cells were exposed to media containing 0 (control), 0.02, 0.1, and 0.5 ng/ml IL-1 β (R&D Systems). Once wells were 50-60% confluent, a simulated transfection period was performed. The media containing FBS and IL-1 β was removed for 6 hours during which FBS and antibiotic free media was used, and then FBS and IL-1 β media was replaced. After 72 hours, cell viability was determined by removing media from wells and replacing with 10% Trypan Blue in PBS. Percentages were based on counts of 100 cells at 40X.

Evaluation of Different Concentrations of IL-1 β for Simulation of Osteoarthritis

Simulation of osteoarthritis in vitro was accomplished by the addition of IL-1 β (R&D Systems) to the media, as has previously been described (Takafuji et al. 2002). Cells were passaged, resuspended in media, and plated in 24 well cell culture plates (Sigma-Aldrich) at a density of 30,000 cells and a total of 500 μ l media per well. Cells were incubated for 24 hours in chondrocyte media without antibiotics or IL-1 β . At 24 hours the cells were exposed to media containing 0 (control), 0.001, 0.0025, 0.005, 0.01, 0.02, 0.025, 0.0375, 0.05, 0.075, and 0.1 ng/ml IL-1 β . A media change was performed at 48 hours after the addition of IL-1 β . Media samples for the initial 48 hour period of exposure to IL-1 β were collected to evaluate PGE₂ concentration via a commercially available assay PGE₂ enzyme immunoassay (Cayman Chemical). Assay was performed in accordance with the manufacturer's instructions on samples of nondiluted media.

Forward Transfection of Chondrocytes with Positive Control

To determine which concentration of Lipofectamine® RNAiMAX Reagent (Invitrogen, Life Technologies) would be effective for transfection in chondrocytes with and without IL-1 challenge, a nonspecific fluorescently labeled siRNA (Block-iT Alexa Fluor Red Fluorescent Control, Thermo Fischer Scientific) was used as a visual control for successful transfection. Cells were passaged, resuspended in antibiotic and FBS free media, and plated in 24 well cell culture plates (Sigma-Aldrich) at a density of 30,000 cells and a total of 500 μ l media per well. Cells were

incubated for 24 hours before 0.1 ng/ml IL-1 β (R&D Systems) was added to half the wells to simulate osteoarthritis conditions. At 30-50% confluence the positive control forward transfection experiment was performed with 0.15, 0.3, 1.5, and 3 μ L Lipofectamine $^{\text{®}}$ RNAiMAX Reagent (Invitrogen, Life Technologies) per well with 10 nM Block-iT (Thermo Fischer Scientific) over 6 hours. At 24 and 48 hours following transfection fluorescent microscopic images were captured under UV using TRITC filter set to determine if positive control RNA entered the cell.

siRNA Forward Transfection

The knockdown effect of 3 different IL-1 siRNAs (#1-AB1RU6R, #2-AB0IW6A, #3-AB1RVCI, Invitrogen, Life Technologies) on IL-1 gene expression was determined using two housekeeping genes; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and succinate dehydrogenase complex flavoprotein subunit A (SDHA). A commercially available negative control (Silencer $^{\text{®}}$ Select Negative Control, Invitrogen, Life Technologies) was used, also. Cells were passaged, resuspended in media, and plated in 24 well cell culture plates (Sigma-Aldrich) at a density of 30,000 cells and a total of 500 μ l media per well. Cells were incubated for 24 hours before 0.1 ng/ml IL-1 was added to half the plates to simulate osteoarthritis conditions. At 60-80% confluence forward transfection was performed over 6 hours. During the transfection period media containing FBS and antibiotics was removed. 3 μ l Lipofectamine $^{\text{®}}$ was diluted in 50 μ l of antibiotic free media, and the siRNA (3, 10, 30, 100 nM) was also diluted in 50 μ l of antibiotic free media. The diluted Lipofectamine $^{\text{®}}$ and diluted siRNA mixtures were combined and incubated for 5

minutes. After 5 minutes the 24 well cell culture plates were treated as outlined in Table 1 and Table 2, which allowed evaluation of 4 concentrations of siRNA and no treatment and Lipofectamine® only controls. The plates were organized as follows:

Plate 1: siRNA #1 with 0.1 ng/ml IL-1

Plate 2: siRNA #1 without IL-1

Plate 3: siRNA #2 with 0.1 ng/ml IL-1

Plate 4: siRNA #2 without IL-1

Plate 5: siRNA #3 with 0.1 ng/ml IL-1

Plate 6: siRNA #3 without IL-1

Plate 7: Negative control with 0.1 mg/ml IL-1

Plate 8: Negative control without IL-1

Plate 9: No treatment and Lipofectamine® only controls

Table 1: Diagram of 24 well plates 1-8 for siRNA experiment; X = empty wells

X	X	X	X	X	X
30 nm siRNA only	3 nm siRNA with 3 uL Lipofectamine® RNAiMAX Reagent	10 nm siRNA with 3 uL Lipofectamine® RNAiMAX Reagent	30 nm siRNA with 3 uL Lipofectamine® RNAiMAX Reagent	100 nm siRNA with 3 uL Lipofectamine® RNAiMAX Reagent	X
30 nm siRNA only	3 nm siRNA with 3 uL Lipofectamine® RNAiMAX Reagent	10 nm siRNA with 3 uL Lipofectamine® RNAiMAX Reagent	30 nm siRNA with 3 uL Lipofectamine® RNAiMAX Reagent	100 nm siRNA with 3 uL Lipofectamine® RNAiMAX Reagent	X
30 nm siRNA only	3 nm siRNA with 3 uL Lipofectamine® RNAiMAX Reagent	10 nm siRNA with 3 uL Lipofectamine® RNAiMAX Reagent	30 nm siRNA with 3 uL Lipofectamine® RNAiMAX Reagent	100 nm siRNA with 3 uL Lipofectamine® RNAiMAX Reagent	X

Table 2: Diagram of 24 well plate 9 for siRNA experiment; X = empty wells

No treatment with 0.1 ng/ml IL-1	Lipofectamine only with 0.1 ng/ml IL-1	X	No treatment without IL-1	Lipofectamine only without IL-1	X
No treatment with 0.1 ng/ml IL-1	Lipofectamine only with 0.1 ng/ml IL-1	X	No treatment without IL-1	Lipofectamine only without IL-1	X
No treatment with 0.1 ng/ml IL-1	Lipofectamine only with 0.1 ng/ml IL-1	X	No treatment without IL-1	Lipofectamine only without IL-1	X
X	X	X	X	X	X

Following the transfection procedure, the 24 well cell culture plates (Sigma-Aldrich) were incubated for 6 hours after which the reagents were removed and chondrocyte culture media containing FBS and antibiotics with or without IL-1 was replaced into the wells. At 24 hours cells were collected from each well for qPCR, using the previously described trypsinization protocol. RNA from chondrocytes was purified with the Total RNA Purification Micro Kit (Norgen Biotek Corp). A DNase incubation (Norgen Biotek) was included to decrease the chance of DNA contamination of samples. RNA quantity and quality were determined with the Nanodrop (ND-1000) and Bioanalyzer (Agilent 2100) respectively, and all purified RNA had RIN numbers greater than 8.5. RNA was stored at -80°C . Reverse transcription reactions (High Capacity RNA to cDNA, Applied Biosystems) were set up with 225 ng total RNA in 20 μl reaction volumes and incubated for 60 minutes at 37°C followed by 95°C for 5 minutes. 3 μl cDNA was loaded in duplicate into a 96 well MicroAmp reaction plate (Applied Biosystems) along with a mixture of TaqMan[®] Gene Expression Master Mix, 0.9 μM TaqMan[®] primers and 0.25 μM TaqMan[®] probes (Applied Biosystems) for either Interleukin-1 (IL-1), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), or succinate dehydrogenase complex flavoprotein subunit A (SDHA). Samples were run on Step One Plus

(Applied Biosystems) with the following program: 50 °C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Threshold was set at 0.1 and C_T values of genes of interest were compared to reference gene(s) C_T values using the $\Delta \Delta C_T$ method.

Calibrator samples were the respective untreated samples with and without IL-1 challenge. Calculation of gene knockdown was performed using the following formula:

$$\Delta C_T (\text{sample}) = C_T \text{ target gene} - C_T \text{ reference gene}$$

$$\Delta C_T (\text{calibrator}) = C_T \text{ target gene} - C_T \text{ reference gene}$$

$$\Delta \Delta C_T = \Delta C_T (\text{sample}) - \Delta C_T (\text{calibrator})$$

$$\text{Normalized target gene expression in sample} = 2^{-\Delta \Delta C_T}$$

Results

Cell Culture

Initial cultures were performed with DMEM with 10% fetal bovine serum (FBS), 21.5 $\mu\text{g/ml}$ ascorbic acid, 130 $\mu\text{g/ml}$ L-glutamine, 13 $\mu\text{g/ml}$ α -ketoglutaric acid, 2.3% 1M HEPES buffer, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin. The first few batches of cells failed to survive. Gram stain and culture of media failed to grow any organisms. Media was altered to a 50:50 mixture of DMEM and Hams F-12 with 10% FBS and the same additives. Cell cultures obtained after the media was altered all survived, and were able to be successfully frozen.

Cell Passage and Optimization of Cell Dissociation Time

Cell viability for dissociation times and conditions are included in Table 3. Optimal cell dissociation with TrypLETM Express cell dissociation reagent (Gibco, Life Technologies) was determined to be 4 minutes with gentle tapping of culture flasks under tissue culture hood.

Table 3: Cell viability and cell recovery after different trypsinization protocols

Dissociation Time and Condition	Cell Viability	Cells Remaining in Flask
2 mins, incubator	98.1%	Many
2 mins, tapping under hood	99.4%	Some
4 mins, incubator	99.2%	Few
4 mins, tapping under hood	99.8%	None
6 mins, incubator	97.6%	Few
6 mins, tapping under hood	98.5%	None
10 mins, incubator	96.1%	None
10 mins, tapping under hood	96.8%	None
20 mins, incubator	92.5%	None
20 mins, tapping under hood	91.0%	None

Evaluation of FBS and Antibiotic Free Media on Chondrocytes

Chondrocyte cell viability was good (> 95%), and no difference was noted after either 6 or 8 hours incubation in FBS and in antibiotic free media (Table 4).

Table 4: Cell viability after different times of incubation in media without FBS or antibiotics

Incubation Time	Cell Viability
6 hours	98 %
8 hours	97 %

Evaluation of Different Concentrations of IL-1 β on Chondrocytes

All concentrations of IL-1 β evaluated resulted in > 95% viable cells (Table 5).

Table 5: Cell viability after challenge with different concentrations of IL-1 β

IL-1 Concentration	Cell Viability
0	97.3%
0.02 ng/ml	98.2%
0.1 ng/ml	98.9%
0.5 ng/ml	96.2%

Evaluation of Different Concentrations of IL-1 β for Simulation of Osteoarthritis

PGE₂ concentrations were determined via enzyme immunoassay (Table 6). In order to consistently obtain PGE₂ concentrations in osteoarthritis simulated conditions that were 5x higher than baseline, a concentration of 0.015 ng/ml was selected for use in future experiments.

Table 6: PGE₂ concentrations measured after 48 hours of IL-1 β challenge

IL-1 Concentration (ng/ml)	PGE ₂ Concentration (pg/ml)
0	4982
0.001	5337
0.0025	7589
0.005	10237
0.01	17101
0.02	34479
0.025	38754
0.0375	< curve
0.05	< curve
0.075	< curve
0.1	< curve

Forward Transfection of Chondrocytes with Positive Control

Forward transfection with Block-iT Alexa Fluor Red Fluorescent Control (Invitrogen, Life Technologies) was successful, and images were obtained of TRITC staining in cytoplasm surrounding the nucleus (Figure 1).

siRNA Forward Transfection

Forward transfection was successful, and qPCR data for gene knockdown is listed in Table 7.

Table 7: qPCR Data with C_T values for housekeeping genes, GAPDH and SDHA, and IL-1 with knockdown calculation

Treatment	GAPDH C _T	SDHA C _T	IL-1 C _T	2 ^{-$\Delta\Delta$C_T}	Knockdown
siRNA #1	18.580	22.454	33.525	0.884	11.57%
siRNA #2	18.814	22.779	33.722	0.937	6.34%
siRNA #3	18.964	22.849	34.365	0.647	35.27%
Negative	18.641	22.629	33.721	0.838	16.18%
None	18.584	22.498	33.372	1.00	0%

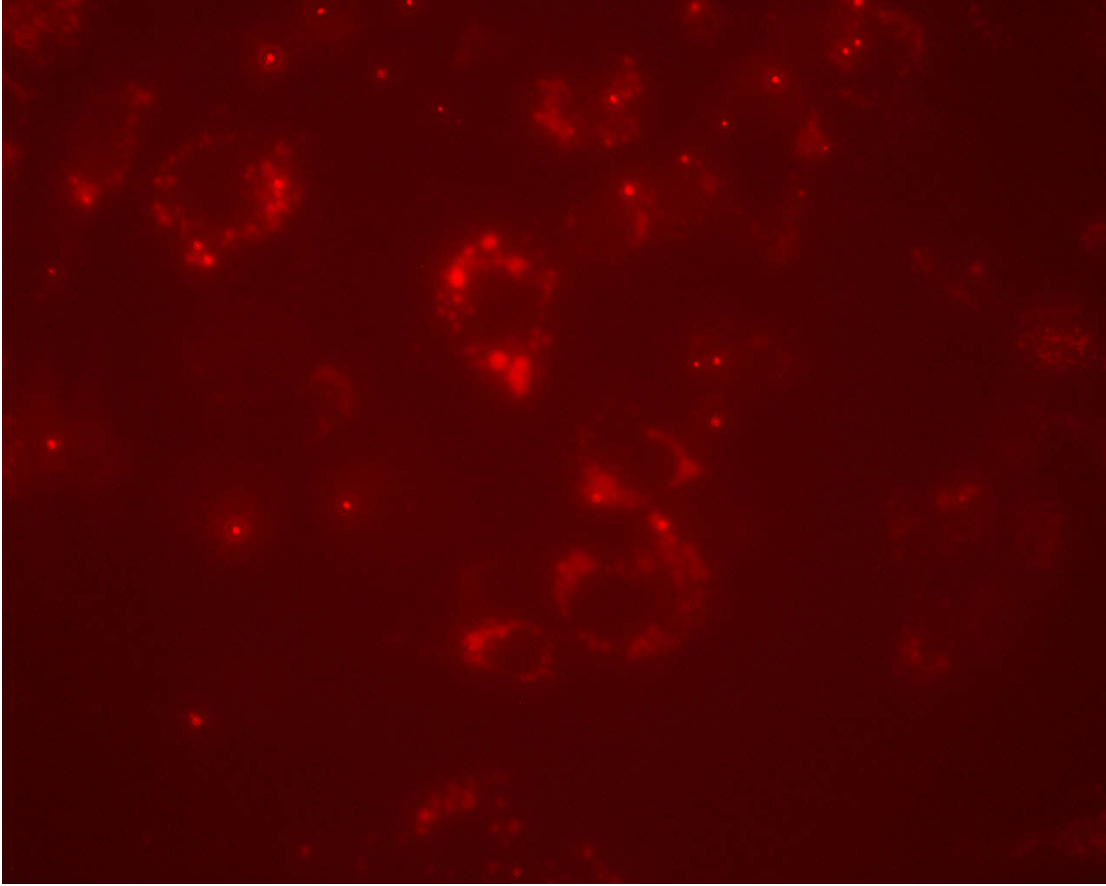


Figure 1: TRITC stained image (100X) with fluorescent signal localized in cytoplasm surrounding the nucleus

Discussion

Cell Culture

Cell culture methods used were based on previous laboratory experience and published chondrocyte culture protocols (Takafuji et al., 2005; Ahmed et al., 2007; Busschers et al., 2010), however adjustments were made to the media used after the first few attempts at cell culture failed to maintain live cells. Initial cultures were performed with DMEM with 10% fetal bovine serum (FBS), 21.5 µg/ml ascorbic acid, 130 µg/ml L-glutamine, 13 µg/ml α-ketoglutaric acid, 2.3% 1M HEPES buffer, 100 U/ml penicillin, and 100 µg/ml streptomycin. Media was ultimately altered to a 50:50 mixture of DMEM and Hams F-12 with 10% FBS and the same additives. It is possible that the learning curve associated with cell culture could have been responsible for the failure of the first few batches of cells to survive, as opposed to the change in cell culture media.

Cell Passage and Optimization of Cell Dissociation Time

Cell passage was perfected over several weeks, and determination of optimal cell dissociation time was identified by evaluation of various time lengths from 2 minutes to 20 minutes. Effects of leaving culture flasks in incubator during dissociation time or gentle tapping of culture flasks under tissue culture hood was also evaluated. Optimal cell dissociation was determined to be 4 minutes with gentle tapping of culture flasks under tissue culture hood.

Evaluation of FBS and Antibiotic Free Media on Chondrocytes

Antibiotic and FBS free media was required for transfection protocols, and thus it was necessary to prove that chondrocytes could be grown in monolayer without the addition of antibiotics or FBS. After both 6 and 8 hours of antibiotic and FBS free media there was no difference in cell viability, which meant that when performing RNA transfection procedures either time frame would be acceptable.

Evaluation of Different Concentrations of IL-1 β on Chondrocytes

All concentrations of IL-1 β resulted in viable cells, and thus appropriate concentrations of IL-1 could be used to simulate osteoarthritis of chondrocytes in vitro without causing cell death.

Evaluation of Different Concentrations of IL-1 β for Simulation of Osteoarthritis

Simulation of osteoarthritis conditions in vitro with the addition of IL-1 β has been previously described (Takafuji et al., 2002, Duesterdieck-Zellmer, 2012). It has been previously reported that concentrations of IL-1 β as low as 0.01 ng/ml can effectively result in prolonged release of proteoglycans, such as what occurs in naturally occurring osteoarthritis (Duesterdieck-Zellmer, 2012). Determination of an appropriate concentration for use in the experimental protocol was based on obtaining PGE₂ concentrations that were five times the baseline level. Cells that were not challenged with IL-1 β had a PGE₂ concentration of approximately 5000 pg/ml, and a

five-fold increase fell in between 0.01 ng/ml and 0.02 ng/ml of IL-1 β . Thus, we elected to use a concentration of 0.015 ng/ml IL-1 β to simulate osteoarthritis conditions in our cell population.

Forward Transfection of Chondrocytes with Positive Control

Forward transfection with a positive control was successful, and images were obtained with TRITC with fluorescent signal localized in the cytoplasm surrounding the nucleus. Previous work has shown that siRNA will selectively localize to the cytoplasm or nucleus depending on the location of the target RNA, however if there is no actual target the siRNA can be distributed throughout the nucleus and cytoplasm (Berezhna et al., 2006).

siRNA Transfection

The 3 siRNA constructs evaluated in this study did not produced adequate knockdown of the gene of interest, IL-1. siRNA #3 had the greatest knockdown of IL-1 at 35.27%. It was thought that degree of knockdown was not adequate, and the project was discontinued. It is possible that greater knockdown could be obtained with minor alterations in the sequence of siRNA, or this degree of knockdown may be adequate to modify continued cartilage degeneration in osteoarthritis.

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