

*In Vitro* Equine Flexor Tendonitis: New Model Development and Therapeutic Investigation

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Thesis has been submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science

In

Biomedical and Veterinary Sciences

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July 8, 2009

Blacksburg, Virginia

Keywords: Tendonitis, model, prostaglandin E<sub>2</sub>, horse, in vitro

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## ABSTRACT

Flexor tendonitis is a common cause of lameness and wastage in the equine athlete. Current techniques for tendonitis therapy provide limited success, and horses that do recover tend to return at a decreased level of performance. Current treatment techniques have begun to focus on regenerative medicine to improve tissue healing. Investigations of new treatments are made difficult by the lack of reliable *in vitro* models that allow for accurate comparison of treatment protocols. New techniques are often implemented into the clinical setting prior to thorough investigation for safety and efficacy.

*In vitro* testing is an important step in the development of new therapeutic agents. However, results of *in vitro* tests should only be deemed as useful if the model used is one that is reliable and mimics the clinical situation that the researchers are attempting to investigate. Equine flexor tendonitis is believed to be the result of microdamage caused by cyclic loading of tendons. Cyclic loading of fibroblasts results in increased production of the inflammatory cytokine prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). Thus the exposure of tendon fibroblasts to exogenous PGE<sub>2</sub> may induce metabolic changes in the cells similar to what is seen in clinically affected animals making this a useful model for the investigation of therapeutic techniques.

Currently a variety of techniques exist for treatment of flexor tendonitis; however, no single treatment has separated itself as superior. A new technique using autogenous conditioned serum (ACS) in humans for treatment of muscle injury has been shown to speed tissue regeneration. ACS produced from human blood has been shown to contain significantly increased levels of

growth factors that may improve tendon fibril formation and strength. We propose to investigate the effect of ACS on cellular metabolism in equine tendon fibroblast monolayers. This will involve cell culture, PGE<sub>2</sub>-induced cellular injury, and analysis of the cellular response to injury when treated with ACS. Controls will include fetal bovine serum, normal equine serum, and ACS without PGE<sub>2</sub>-induced cellular injury. The cellular response will be investigated biochemically by quantification of DNA, glycosaminoglycan, and soluble collagen levels and by real time PCR to assess gene expression for matrix metalloproteinases (MMP)-1, MMP-3, and MMP-13, collagen types I and III, and the non-collagenous proteins cartilage oligomeric matrix protein (COMP) and decorin. Data will be analyzed by analysis of variance and post-hoc comparisons. Significance will be set at  $p < 0.05$ .

We hypothesize that the addition of exogenous PGE<sub>2</sub> to culture media for monolayers of equine tendon fibroblasts will insight alterations in cellular metabolism that will generate a suitable model for the *in vitro* study of fibroblast response to novel therapies. We then hypothesize that the addition of ACS to PGE<sub>2</sub>-treated fibroblasts will result in increased gene expression for collagen types I and III, cartilage oligomeric matrix protein, and decorin. ACS will also stimulate increased protein production of collagen and glycosaminoglycans, and stimulate increased cell proliferation. The use of ACS will decrease gene expression of inflammatory molecules important in tendon degradation, namely matrix metalloproteinases -1, -3, and -13.

## Acknowledgments

I would like to acknowledge the help and support that the members of my committee, Dr. Linda Dahlgren, Dr. Jennifer Barrett, and Dr. John Robertson, have provided to me throughout the duration of my project. I owe much of the completion of this project to Dr. Dahlgren. Without her knowledge, patience, and general enthusiasm for this field of research this project would never have grown to fruition. Additionally her support as my mentor and friend throughout my surgical residency has been invaluable. I would also like to thank Dr. Jolynne Tschetter who served on my Master's Committee and provided me with critical help during the early stages of my project, but was unable to continue with me during the second half of the project after moving to North Dakota.

I would like to thank Stewart Harvey for all of her assistance with the bench top portion of this project. Without Stewart's knowledge and patience my ability to complete this project would have been severely compromised. In addition to Stewart, I would also like to thank both Robyn Shaffer and Shelley Stewart for their assistance with the bench top portions of this project.

I thank the technicians of the Virginia-Maryland Regional College of Veterinary Medicine, who treated the horses used for this study with the utmost in care and compassion. Additionally many of these technicians, Tony Huffman, Jamie Monroe, and Jennifer Stanley, have been good friends to me throughout my residency, making the last three years much easier.

Finally, I would like to express my deepest thanks to my wife, Emily. Without her moral and emotional support I would not have been able to dedicate myself fully to this project. In addition to my wife, I would like to also thank my children Zachary and McKenzie. It is the

continual love and support of my family at home that allows me to get through each day at work.

I have always told people that veterinary medicine is what I do, a father and husband is who I really am.

I would like to thank the American College of Veterinary Surgeons for their funding of the project through the Surgeon-in-Training grant.

## Attributions

Several colleagues and coworkers aided in the writing and research behind the chapters of this thesis. A brief description of their background and their contributions are included here.

**Linda Dahlgren** – DVM, PhD Diplomate ACVS (Department of Large Animal Clinical Sciences, Virginia-Maryland Regional College of Veterinary Medicine) is the primary Advisor and Committee Chairperson. Dr. Dahlgren's primary research interest is equine tendonitis, and has earned both a Master's degree and Ph.D. while focusing on this interest. Dr. Dahlgren through her previous knowledge and experience in the field of equine tendonitis research was vital in the overall project design and general benchtop work. Furthermore, Dr. Dahlgren also aided extensively in the writing of the thesis.

**Chapter 2:** Investigation of a Prostaglandin E<sub>2</sub>-Induced Model of *In Vitro* Tendon Injury

**Stewart Harvey** – BS (Veterinary Medicine Experiment Station, Virginia Tech) is a member of the author's lab and a co-author for Chapter 2. Stewart has vast experience in a research laboratory, previously working for companies such as Fort Dodge Pharmaceuticals. Stewart aided in both the performance and analysis of the gene expression and biochemical assays.

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## **Introduction**

### **Thesis Organization**

This thesis is presented in a format that contains a journal publication as the central portion of the document. The publication is entitled “Investigation of a Prostaglandin E<sub>2</sub>-Induced Model of *In Vitro* Tendon Injury.” This paper contains its own Introduction, Materials and Methods, Results, Discussion, and References. The following Introduction provides a brief overview of significant issues in equine tendonitis that are directly relevant to this project. The Literature Review is a summary of the pertinent literature that was used to stimulate the ideas, and plan the design of this project. The Literature Review also serves as an in-depth review of the topics of anatomy, tendon physiology, pathophysiology, therapies for tendonitis, and the current models of tendon degeneration. Additionally this thesis includes some final comments on the results of the study along with possible ideas for improvement of the model.

### **Introduction**

Flexor tendonitis is a common cause of lameness in all disciplines of equine performance. Flexor tendonitis represents a difficult issue for equine practitioners due to the extended length of time required for healing and the high rate of recurrence. Current therapies for tendonitis are primarily conservative or surgical, but new focus has moved to a regenerative approach. The shift in therapeutic focus is due to the limited success with current reparative techniques. Regenerative medicine aims to aid the tendon in healing so that it returns to a more normal architecture and strength. This is in comparison to current reparative techniques that are designed to protect the tissue from further damage during the normal healing process, and that ultimately result in the formation of scar tissue.

A major difficulty in the development of new therapeutic techniques is the lack on a reliable *in vitro* model for flexor tendonitis. This lack of a model prevents the accurate comparison of various therapies in a controlled environment. As a result, many new treatment protocols are utilized in equine practice without scientific backing. Thus a model that closely mimics the *in vivo* characteristics of flexor tendonitis is needed to validate new promising therapies.

Prostaglandin-E<sub>2</sub> (PGE<sub>2</sub>) is an inflammatory mediator that has been shown to induce changes in tendon tissue of other species similar to those seen in equine clinical cases.<sup>1,2</sup> Thus the use of exogenous PGE<sub>2</sub> may induce metabolic changes in cultured equine flexor tendon fibroblasts consistent with clinical tendonitis. The development of this model would allow for the future controlled study of therapeutic agents.

A new therapy being used in equine practice is autogenous conditioned serum (ACS). ACS has been shown to contain significantly increased levels of growth factors that may improve healing of various tissues following damage. Currently ACS is used for the treatment of osteoarthritis, tendonitis, and desmitis. However, the use of ACS has not been investigated in equine tendonitis, and thus lacks scientific support for its use. Prior to recommending the use of ACS clinically, it should be tested in controlled *in vitro* experiments to validate its effects and benefits.

The aim of this Master's Project was two-fold:

1. To develop and validate the use of exogenous PGE<sub>2</sub> as an *in vitro* model of equine flexor tendonitis.
2. To use this new model to investigate the effects of ACS on equine flexor tenocytes cultured in monolayer.

## Chapter 1

### Literature Review

Flexor tendonitis is a common ailment diagnosed in all disciplines of equine athletes.<sup>3,4</sup> Tendonitis of the superficial digital flexor tendon is also referred to as “bowed tendon”. Tendonitis is, however, not classically characterized by infiltrates of inflammatory cells within the tendon parenchyma,<sup>1</sup> but by signs of degeneration that result in disruption of the normal collagen fiber pattern. Currently the molecular pathways for both the degenerative and healing processes have yet to be completely elucidated.

#### Anatomy

Tendons are collagenous tissues that attach muscles to bones. Through these attachments tendons translate muscle contraction into movement of the body. Tendons are composed of a small population of resident fibroblasts whose function is to maintain the extracellular matrix that composes the tendon’s parenchyma.

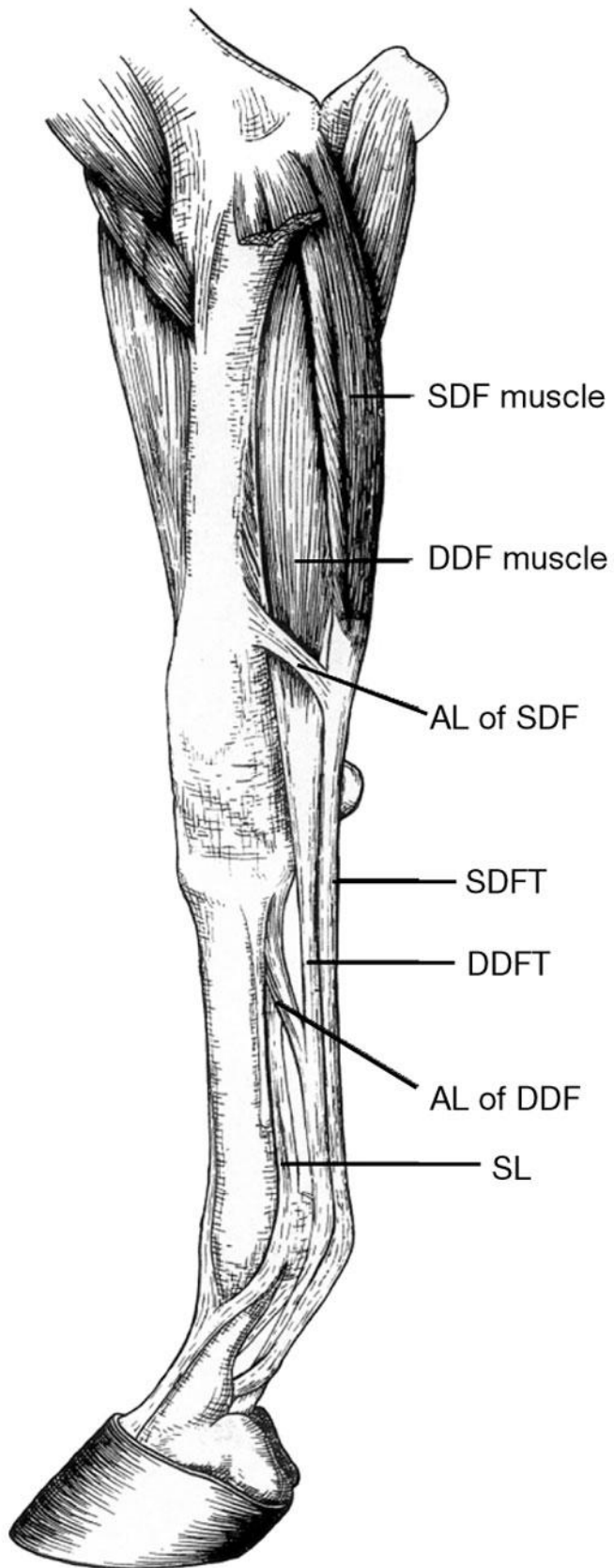
The two primary tendons in which tendonitis is most commonly diagnosed in the horse are the superficial and deep digital flexor tendons. The most common site of flexor tendonitis is the superficial digital flexor tendon (SDFT) of the forelimb,<sup>5</sup> thus the study described by this Master’s Project involved the use of tendon fibroblasts from the superficial digital flexor tendon. In the forelimb, the superficial digital flexor muscle (SDF) arises from the caudal portion of the humerus and courses distally along the palmar aspect of the limb (Figure 1). The muscle belly occupies a central location between the larger deep digital flexor muscle group and the flexor carpi ulnaris muscle. The SDFT arises proximal to the carpal joints, and

continues its course distally until it inserts on the palmar eminences of the second phalanx with minor attachments to the distal portion of the palmar aspect of the first phalanx. The SDFT shares a common synovial sheath with the deep digital flexor tendon both at the level of the carpus (carpal sheath) and the fetlock (digital sheath).

Diagnosis of SDF tendonitis is based primarily on history, physical examination, and diagnostic imaging. The degree of lameness can vary from mild to severe, and is more related to the degree of active inflammation than to the degree of damage to the tendon.<sup>6</sup> It is important to note however, that the inflammation that occurs during clinical tendonitis is not believed to be the primary cause of tendon damage, but instead the result of mechanical disruption of collagen fibers, which is secondary to repetitive strain-induced damage.<sup>7,8</sup> SDF tendonitis can be a focal or diffuse condition, and commonly manifests as a centrally located region of damage within the tendon, which is commonly referred to as a core lesion. Damage to the SDFT most commonly occurs in the mid-metacarpal region, or tensile region of the tendon.<sup>4,6</sup>

### Tendon Physiology

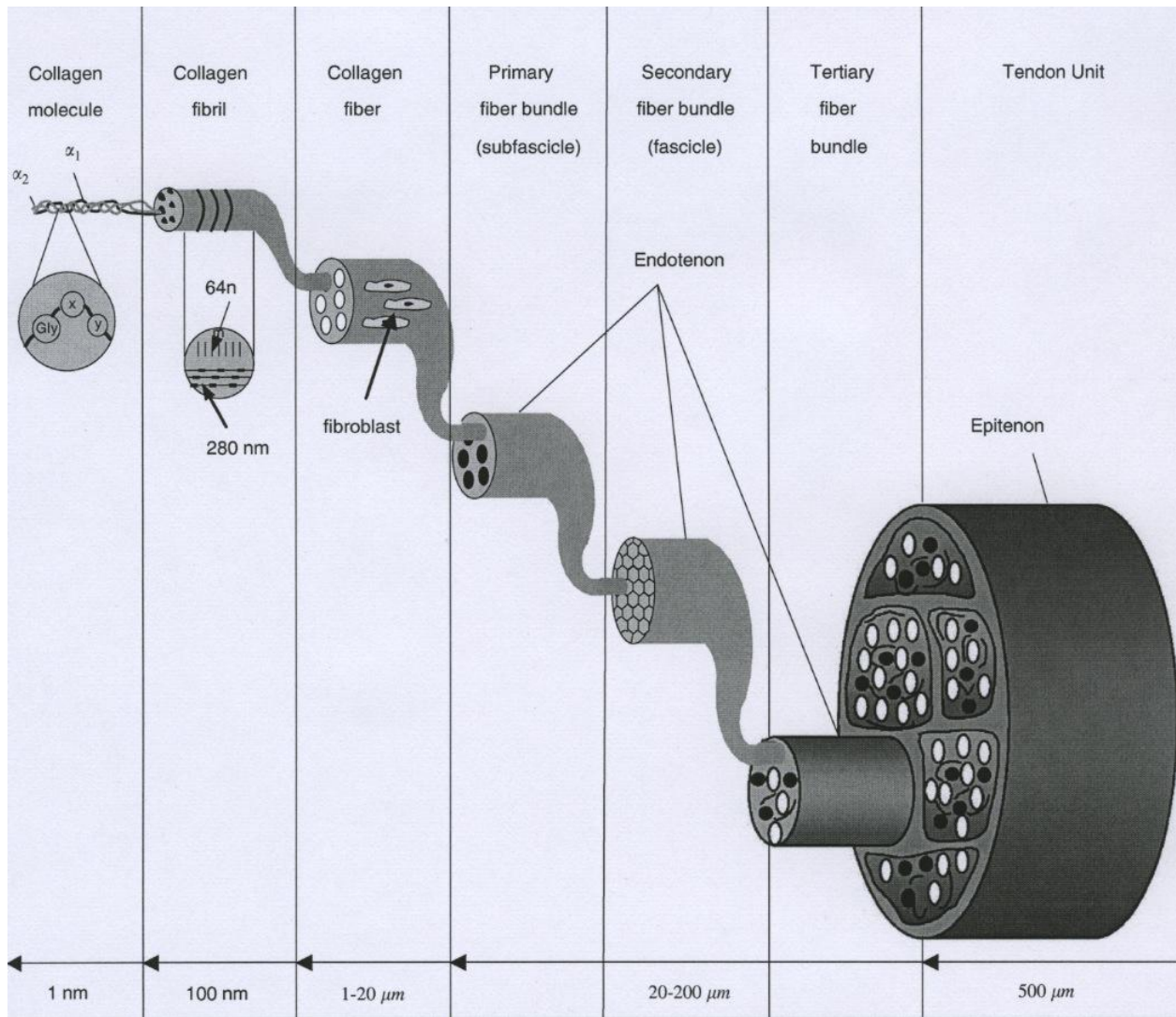
Tendons function in a variety of ways during locomotion; transmitting tensile forces generated by muscle contraction, providing support to the skeleton, and acting as energy stores. Tendons are composed of water, collagen, proteoglycans, glycoproteins, elastin, and a limited number of tendon fibroblasts. Water accounts for nearly 70% of the overall weight of a tendon,<sup>6</sup> while the remaining 30% (the dry weight of the tendon) is composed primarily of collagen.<sup>6</sup> Type I collagen accounts for 80-86% of the dry weight of the tendon<sup>9,10</sup> and 95% of the total collagen located within a tendon.<sup>11</sup> Other collagen types primarily include III and V. The



**Figure 1.1** Anatomy of the Superficial Digital Flexor Muscle and Tendon. The superficial digital flexor tendon (SDFT) arises from the caudal humerus and courses distally along the palmar aspect of the limb.<sup>12</sup>

collagen fibrils in a tendon are formed into successively larger bundles that are surrounded by a thin layer of connective tissue called epitenon (Figure 2). The epitenon is primarily composed of type III collagen<sup>13</sup> and is continuous with internal connective tissue called endotenon. The endotenon houses the tendon's vasculature, lymph vessels, and nerve supply, aids in alignment of larger fiber bundles along the lines of tension, and serves as a low friction surface, allowing the tendon's fiber bundles to slide relative to each other during loading and unloading of the tendon.<sup>14</sup> This structural design conveys the high tensile strength, elasticity, and flexibility characteristic of tendons. The elastic nature of the tendon allows it to store energy during locomotion, thus increasing the efficiency of locomotion. When the tendon is unloaded, nearly 95% of the kinetic energy stored within the tendon is converted to mechanical energy in the form of joint motion, while the remainder is lost as heat into the tendon.<sup>15,16</sup>

Tendons are designed to withstand two primary types of forces; tension and compression. Due to this variation in applied forces, there are two distinct regions of tendons. Regions of tendons that pass over bony prominences or other sites of friction (compressive regions) have a different molecular composition than those that travel along a straight line (tensile regions). The compressive region has a higher type V collagen content and lower cartilage oligomeric matrix protein levels (COMP) than the tensile regions.<sup>17</sup> Molecular composition also varies between tendons,<sup>17</sup> suggesting that the type of load applied to a tendon helps determine its molecular composition. Additional differences between the tensile and compressive regions of a tendon are demonstrated in their healing abilities. Tendons were originally believed to only undergo extrinsic type healing, i.e. fibroblasts migrate into the damaged tissues from the surrounding tendon sheath or from the paratenon, which is the loose connective tissue surrounding the



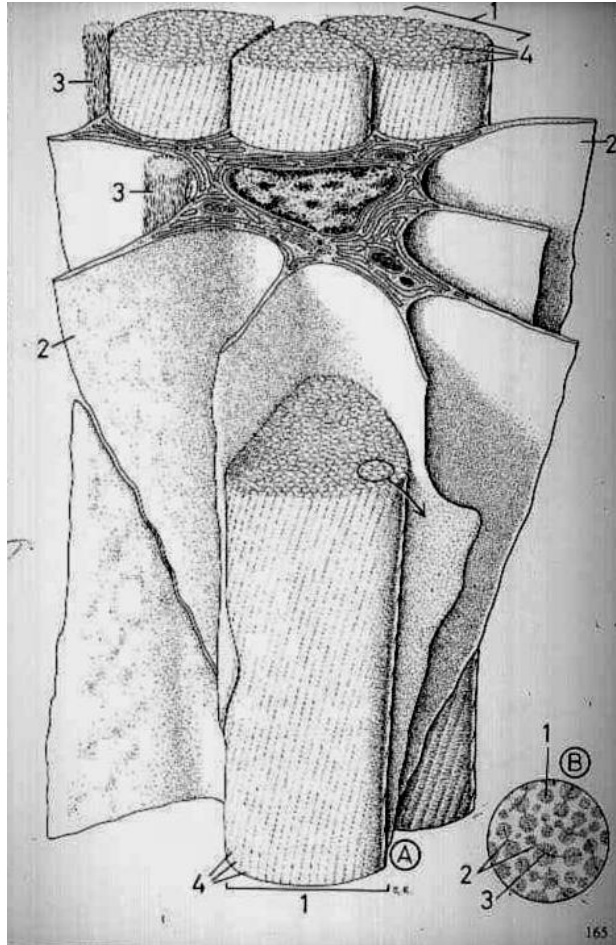
**Figure 1.2** Structure of a tendon. Schematic of the multi-unit hierarchical structure of the tendon.<sup>11</sup>



tendon. Although this mechanism represents a major source of cells involved in the healing process, it is now also known that tendons do have an intrinsic mechanism for healing.<sup>18</sup> Fibroblasts originating in the tendon parenchyma itself along with progenitor cells from the endotenon, migrate into the damaged regions following neovascularization. Other factors that can contribute to differences in the healing process include the presence of synovial fluid in the sheathed portions of the tendon, variations in the amount of motion through that region of the tendon, and the potential for the development of adhesions between the tendon and the surrounding tissues.

At the cellular level, tendons are composed of a low number of highly differentiated resident fibroblasts. These cells are located within the extracellular matrix (ECM) and are responsible for ECM synthesis and organization.<sup>19</sup> Tendon fibroblasts have vast cytoplasmic extensions that allow them to communicate with each other and respond to the mechanical forces applied to the tendon (Figure 3). Fibroblasts communicate with each other and the ECM through desmosomal attachments between their cytoplasmic extensions.<sup>20</sup> Cell-to-ECM interactions allow the fibroblasts to produce the appropriate components for the local ECM. In normal healthy tendon, fibroblasts remain in a balanced state of production of new matrix components and the degradation of old or damaged components. It is when this balance is disrupted that damage can exceed the reparative abilities of the fibroblast and therefore result in the development of pathology.

The ECM produced by fibroblasts, is composed of amorphous ground substance (proteoglycans and glycoproteins) and fibrous elements (collagen and elastin). Collagen accounts for 80-86% of the dry weight of the tendon,<sup>9,10</sup> while elastin only accounts for approximately 1%. However, elastin makes a very important contribution to the elastic nature of



**Figure 1.3** Schematic of a tendon fibroblast. A representation of a tendon fibroblast with its cytoplasmic extensions (2) surrounding individual collagen fiber bundles (1).<sup>14</sup>

the tendon by allowing the tendon to deform and stretch without damage.<sup>21</sup> Proteoglycans, a vital part of the ground substance, account for less than 5% of the tendon's dry weight.<sup>22-24</sup> The connective tissue glycoproteins found in the ECM account for a small percentage of the tendon's dry weight, and consist of a heterogeneous group of molecules that include fibronectin, cartilage oligomeric matrix protein (COMP), laminin, thrombospondin, and tenascin.

There are two main types of proteoglycans present within tendons: small proteoglycans with one or more glycosaminoglycan (GAG) side chains attached to a single core protein and large proteoglycans. The large proteoglycans consist of multiple proteoglycans molecules each with their own GAG side chains. In the large proteoglycans, the GAG molecules are aggregated around a hyaluronic acid core. Small proteoglycans predominate in the tendon ECM of the tensile regions, while the large proteoglycans predominate in the areas of compressive forces.<sup>23</sup> Although the exact mechanical roles of the proteoglycans in tendons are unknown, it is likely that the large proteoglycan aggregates function similarly to those in articular cartilage, binding extracellular water to create a gel-like material that helps to resist compressive forces.<sup>20</sup> Regulation of water content also provides lubrication, spacing between fibrils, and flexibility to the tissues. The smaller proteoglycans, decorin, biglycan, and fibromodulin, serve to modulate the tendon's structure through regulation of collagen fibrillogenesis and fibril diameter.<sup>20,23,25-27</sup> Of the proteoglycans present within the tendon, decorin is the most abundant.<sup>28</sup> Decorin is capable of binding to the cysteine-free portion of the core protein of almost all types of collagen, and through this binding aids in the determination of fibril diameter.<sup>28</sup> A lack of the decorin gene results in increased collagen fibril fragility and abnormal fibril morphology.<sup>26</sup> These defects occur in dermal and tendon collagen fibrils, but not in corneal collagen fibrils, thus indicating tissue specificity of decorin. This level of specificity would suggest that there may not

be a compensatory mechanism for defects in the functionality of decorin, therefore reinforcing its level of importance.

The final major component of the ECM is the connective tissue glycoproteins that make up a very small, but necessary, portion of the tendon ECM. These glycoproteins are found with other matrix molecules and are believed to play a role in both cell-to-cell and cell-to-matrix interactions.<sup>22</sup> Fibronectin is a disulfide-bonded dimer that is capable of binding collagen,<sup>29</sup> and will interact with cell surface integrins. Fibronectin is a necessary mediator of collagen fibril formation; however, the site of the interactions during fibrillogenesis is unknown.<sup>29</sup> Fibronectin does increase in concentration one week following tendon injury, and remains in an elevated concentration for two months following injury.<sup>30</sup>

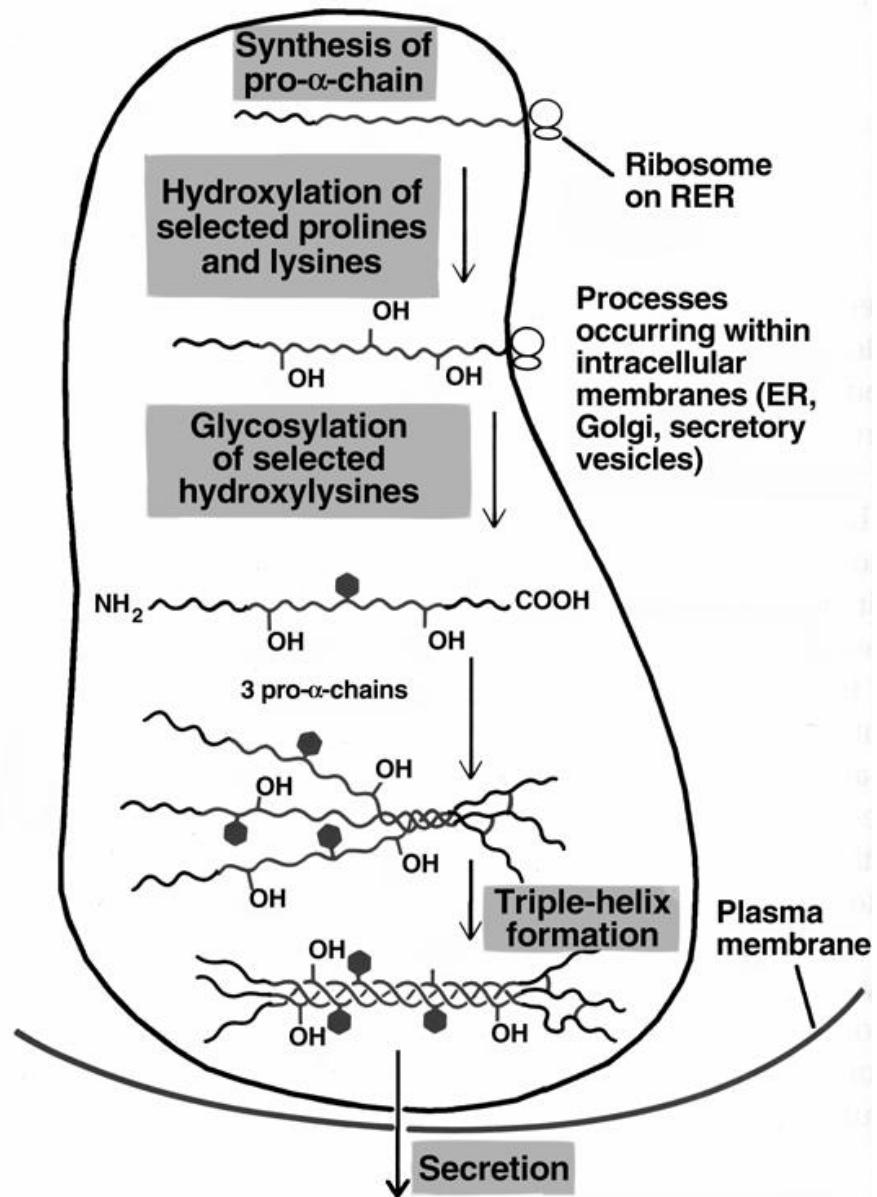
In addition to fibronectin, another major connective tissue glycoprotein is COMP, which is one of the more abundant noncollagenous proteins found within tendon. COMP is a five-armed protein bound via disulfide bonds at their N-termini and contains globular C-terminal domains. COMP is structurally related to the thrombospondin family of proteins.<sup>31</sup> The precise function of COMP has not yet been fully determined but it is known to bind to fibrillar collagen (type I and II collagen), and be involved in fibril organization. Additionally COMP is known to be capable of binding to the more complex collagen type IX through a zinc-dependent mechanism.<sup>32</sup> COMP's ability to bind type IX collagen is important despite the low concentration of type IX collagen within the tendon, since this particular collagen type plays a role in the formation of hydrogen cross-bridges located between tendon fibers.<sup>33</sup> These cross-bridges are needed to help stabilize the tendon structure. COMP levels are evenly distributed throughout the length of the equine SDFT, but variation can occur based on the amount of weight bearing forces applied to the tendon.<sup>17</sup> A decrease in the amount of weight bearing forces applied to the equine SDFT,

results in a decrease in the expression of COMP.<sup>17</sup> Age also has an effect on COMP levels within equine SDFT. Levels of COMP continue to increase until approximately two years of age. Following this peak in COMP levels there is a general decline with age and the cessation of growth.<sup>17</sup> The actual importance of COMP in the development of the equine digital flexor tendon is suggested by the correlation between the level of COMP at the time of skeletal maturity and the ultimate tensile strength of the tendon.<sup>34</sup>

Just as the array of molecules associated with collagen fibrillogenesis is complex, so is the arrangement and actual production of a collagen fibril. Collagen is the most abundant protein of mammals, and is the primary structural component of tendon. Several types of collagen are present within tendons, including types I, II, III, V, IX, and X.<sup>35</sup> A collagen chain's primary structure is defined by its amino acid sequence. It is this sequence that determines the folding pattern of the collagen chain into its unique helical structure. The smallest component of a collagen fibril is the individual  $\alpha$  chain. This chain is arranged into a left-handed helix with three amino acids per turn. Glycine is the most abundant amino acid, with every third residue being a glycine residue. Glycine's small size allows the amino acid chain to form its helical shape by not inducing steric hindrance with other amino acids. Additionally, glycine is responsible for creating the hydrogen bonds found between chains within the superhelix.<sup>21</sup> While glycine accounts for 33% of the amino acid residues in collagen's sequence, the imino acids proline and hydroxyproline together account for 20-30%.<sup>21</sup> Glycine is commonly followed by a proline residue, which is then often followed by hydroxyproline. Hydroxyproline is a derivative of the proline imino acid formed by hydroxylation of proline following protein synthesis, and is found almost exclusively in collagen.<sup>21</sup>

Once the  $\alpha$  chain has been produced within the fibroblast, it binds through hydrogen bonds to two other  $\alpha$  chains, to form a right-handed superhelix, and is then secreted from the cell as a pre-topocollagen molecule. The hydrogen bonds that hold the superhelix together are located between the glycine residues and the side chains of the other amino acids. These hydrogen bonds serve to stabilize the superhelix, thus providing strength to the molecule. The structure of the triple helix is further stabilized by steric repulsion of the pyrrolidine rings of the hydroxyproline and proline residues.<sup>21</sup> Although the triple helical structure is maintained for all collagen types, the individual chains making up the helix contain some variation in amino acid content and sequence. Type I collagen consists of two  $\alpha 1$  chains and one  $\alpha 2$  chain, while type III collagen is a homotrimer consisting of three unique  $\alpha 1$  chains. The triple helix forms the building block for a collagen fibril. The collagen fibril is produced through a complex series of substrate specific steps, that occur both intra- and extracellularly. Collagen protein synthesis is carried out on a ribosome on the rough endoplasmic reticulum (RER) (Figure 4). Initially, hydroxylation of both proline and lysine occurs to generate the hydroxyproline and hydroxylysine residues, respectively. Following hydroxylation, glycosylation of selected hydroxylysine residues occurs, which is then followed by the formation disulfide bonds between the carboxy terminal propeptides. These disulfide bonds not only direct self assembly of the three  $\alpha$  chains into the superhelix, but also aid in further stabilization of the helix. The superhelix is then transported out of the RER and packaged into secretory vesicles that are secreted into the extracellular space.

Following release of the procollagen molecule into the extracellular space, the amino and carboxy portions of the procollagen molecule are enzymatically removed and the collagen



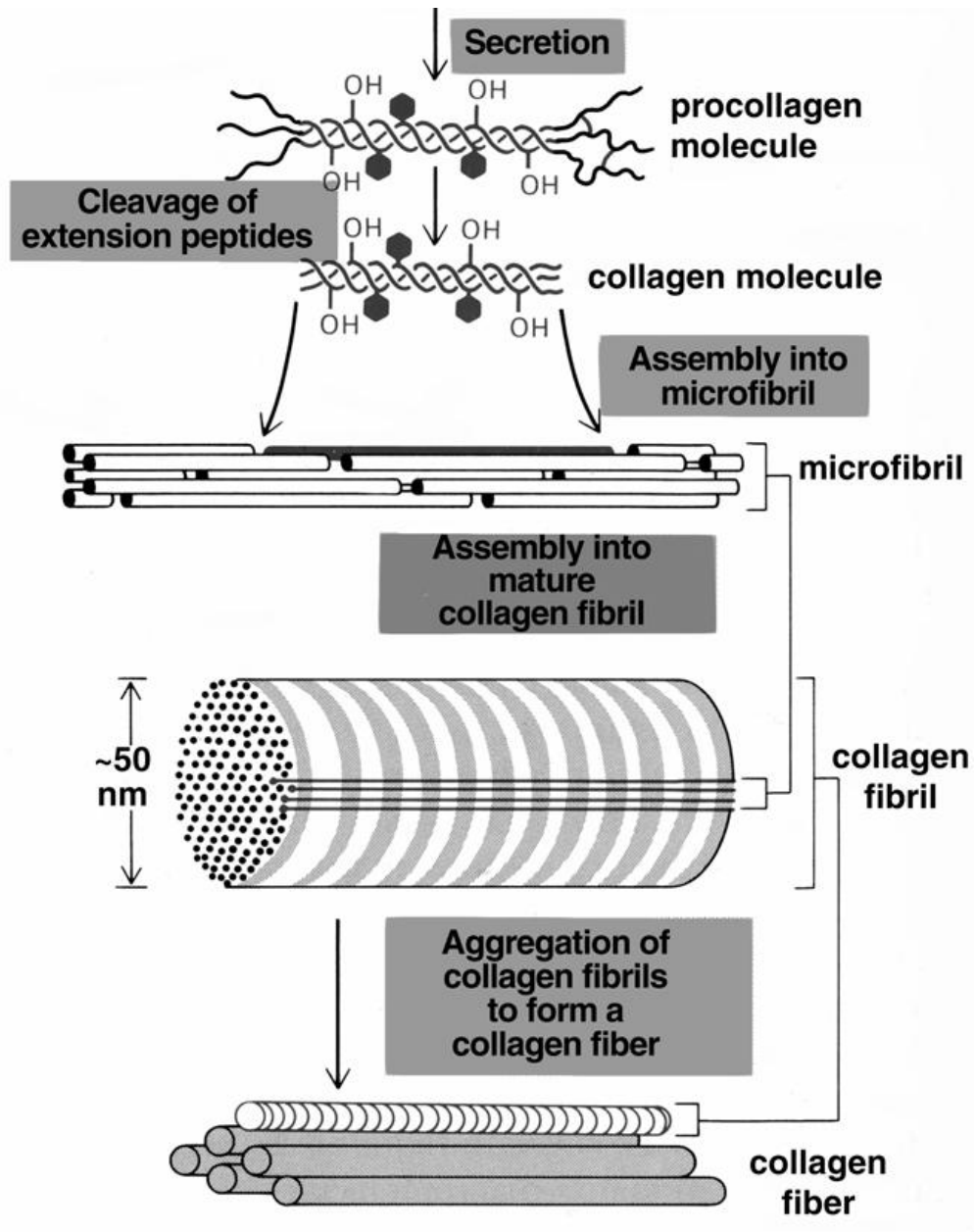
**Figure 1.4** Diagram of the intracellular production of collagen superhelix synthesis.<sup>21</sup>

molecules align themselves to form a collagen fibril (Figure 5). The collagen molecules are aligned with each other in a quarter-stagger pattern and are held together through intra- and inter-molecular cross-links between adjacent lysine residues. These cross-links are only found in collagen and elastin, and contribute to the high tensile strength of mature tendon. There are three types of cross-links found in collagen; disulphide bridges, lysyl oxidase mediated crosslinks, and those formed by non-enzymatic glycosylation of lysine and hydroxylysine residues.<sup>22</sup> It is the lysyl oxidase mediated cross-links that aid in binding the collagen molecules together to form the fibril. Initially, this enzyme catalyzes the hydroxylation of the lysine residues at the non-helical N- and C-terminals of each  $\alpha$  chain. This converts the lysine residues to aldehyde groups, which then participate in the formation of the cross-links. Once cross-linking has occurred, the newly formed collagen fibrils combine to form fibers, then subfascicles, fascicles, tertiary fiber bundles, and finally the tendon unit. Water, proteoglycans, connective tissue glycoproteins, and elastin are then incorporated in between the fibrils to create the ECM.

### Tendon Pathophysiology

Flexor tendonitis is a common cause of lameness among horses and is difficult to treat successfully. The incidence of flexor tendon injuries among Thoroughbred race horses has been reported to range from 8 to 35%<sup>4,36</sup> with a rate of re-injury reported to be as high as 43-93%.<sup>37,38</sup> Tendons can sustain strain injuries or be injured through percutaneous damage such as puncture wounds or lacerations. Tendon strain injuries are believed to occur through one of two mechanisms, either a sudden overload, or by chronic degeneration due to microdamage. Evidence that supports the theory of microdamage includes the presence of “asymptomatic”





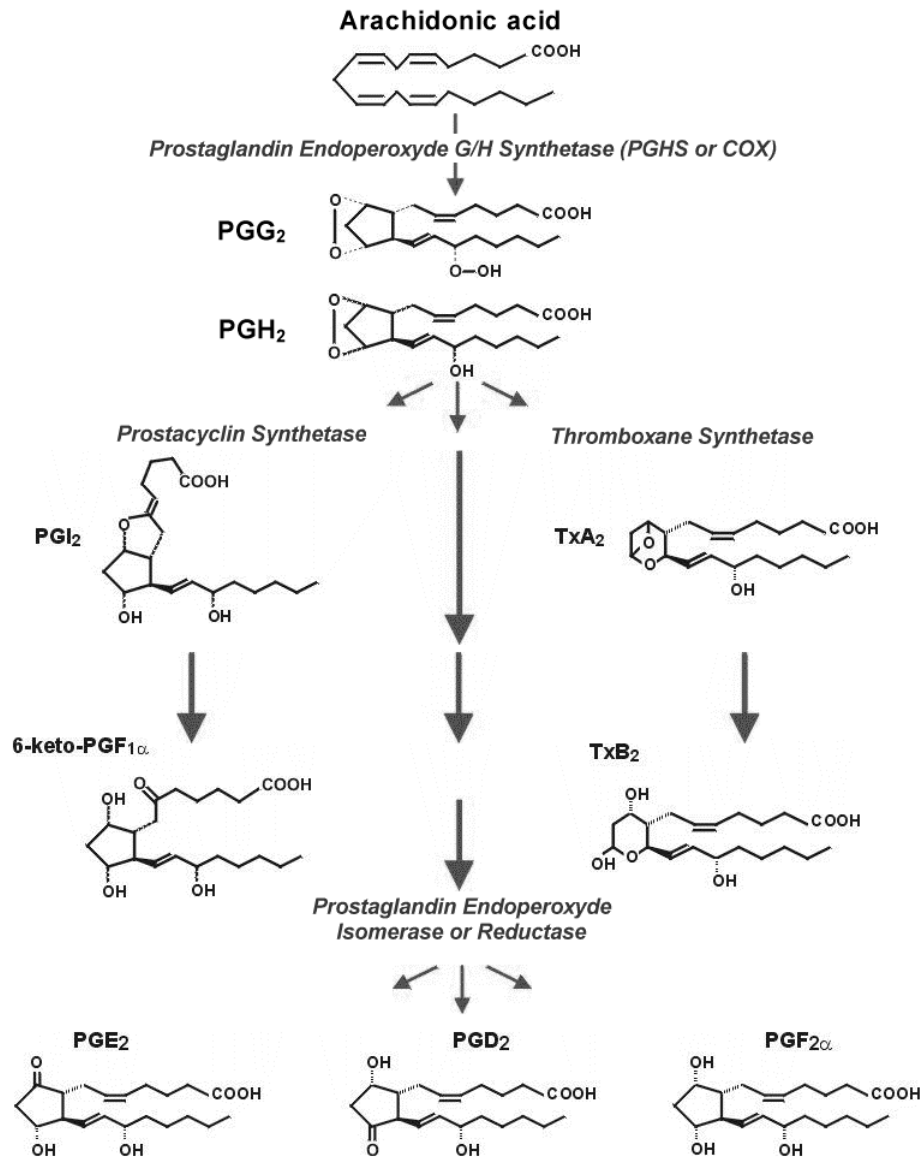
**Figure 1.5** Extracellular formation of a collagen fiber. The completion of collagen fiber production following secretion from within the fibroblast. The structure of the pre-topocollagen molecule located at the top of this diagram is the same as that at the bottom of Figure 4.<sup>21</sup>

lesions is cadaver horses,<sup>25</sup> bilateral involvement for most clinical strain-induced tendonitis lesions, equine epidemiological studies that demonstrate a strong association between age and injury rates (suggesting degeneration of the tendon with age),<sup>39,40</sup> and experimental exercise studies and post mortem examinations that have revealed tendon degeneration instead of tendon adaptation as age increases.<sup>8</sup> Although the exact mechanism behind strain-induced microdamage is not yet fully understood, cumulative microdamage is thought to be the result of tendon matrix damage and the failure or limited response of the resident population of cells to respond. Several etiologies to explain this pathology have been postulated including mechanical overstretching, repeated microstrains, focal hyperthermia, and ischemia/hypoxia.<sup>4,22,41</sup>

Like all tissues, tendon is in a continual balance of matrix production and degradation, and under normal conditions, the tenocytes within the tendon are able to maintain this balance. When this normal balance is disrupted, then fiber damage can occur, making the tendon susceptible to further damage. Normal strains measured in the SDF tendon range from 3-8% at the walk, 7-10% at the trot, and 12-16% at the gallop.<sup>42</sup> *In vitro* the SDF can sustain strains of up to 12-20% prior to rupture,<sup>4</sup> thus it becomes obvious that a horse's tendon is operating at or near maximal capacity while at the gallop. However, equine tendons do not obey Hooke's law, meaning that they do not increase in length in direct proportion to all applied loads. When a tendon is initially loaded, the crimp in the collagen fibers becomes straight, resulting in a large increase in the length of the tendon in response to a small load. Further loading of the tendon causes a linear increase in length when compared to the load applied to it. At a point prior to failure, if the load is removed from the tendon, it will regain its normal length and shape. This is due to the elastic nature of tendon. However, if the load was not removed, then the continued loading of the tendon exceeds the elasticity of the tendon and rupture occurs. This is known as

the yield point of the tendon. Because of a tendon's elastic nature, it is capable of storing energy during movement. However, when a tendon is relaxed, not all of the stored energy is released for locomotion. Five to seven percent of the energy stored within a tendon is released as heat within the core of the tendon.<sup>15,16</sup> This release of heat within the tendon can cause temperatures within the tendon core to reach 45°C during galloping.<sup>41</sup> Exposure of equine flexor tendon fibroblasts to temperatures of 45°C for 10 minutes results in a statistically insignificant reduction in cell viability.<sup>43</sup> Although the decrease in viability was not significant after 10 minutes, tendons do have a very small population of resident cells, thus any loss of cells, could result in a decreased ability to respond to matrix damage. Additionally, human fibroblast cultures subjected to micro-strain increase their production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>).<sup>44</sup> Cyclic loading results in increased production of cyclooxygenase (COX), matrix metalloproteinase (MMP) -1, -3, and -13, along with the inflammatory mediator interleukin 1β (IL-1β) by human tendon fibroblasts.<sup>45-</sup><sup>49</sup> PGE<sub>2</sub> is known to cause degradation of tendon matrix due to its inflammatory effects and its ability to cause up regulation of the matrix metalloproteinases.<sup>1</sup>

PGE<sub>2</sub> is a specific type of prostanoid that is involved in the regulation of a variety of processes in the body. These processes include blood pressure regulation, blood clotting, sleep, labor and inflammation. PGE<sub>2</sub> is generated by the arachidonic acid cascade following cellular stimulation in either a physiological or pathological manner (Figure 6). Arachidonic acid is initially liberated from the phospholipid membrane of the cell by phospholipase A<sub>2</sub>, and is then converted to prostaglandin H<sub>2</sub> (PGH<sub>2</sub>) by prostaglandin H synthase. PGH<sub>2</sub> is the common substrate for a number of different synthases that produce the major prostanoids including PGD<sub>2</sub>, PGE<sub>2</sub>, prostacyclin (PGI<sub>2</sub>) and thromboxane (TXA<sub>2</sub>). PGE<sub>2</sub> concentrations of 10 ng/ml and 100 ng/ml result in decreased collagen synthesis and fibroblast proliferation.<sup>2</sup> This demonstrates the



**Figure 1.6** Arachidonic acid cascade. Arachidonic acid is released from the phospholipid membrane of the cell by phospholipase A<sub>2</sub> and is then converted to either prostaglandin G<sub>2</sub> or H<sub>2</sub> (PGG<sub>2</sub> or PGH<sub>2</sub>) by prostaglandin G or H synthase, respectively. These two prostaglandins serve as the substrates for a series of enzymes to produce the major prostanoids including PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2α</sub>, prostacyclin (PGI<sub>2</sub>), and thromboxanes A<sub>2</sub> and B<sub>2</sub>.

([www.gremi.asso.fr/mediator\\_inflammation-uk.html](http://www.gremi.asso.fr/mediator_inflammation-uk.html))

negative effects that PGE<sub>2</sub> can have on the matrix of a tendon. Additional affects of PGE<sub>2</sub> include its ability to directly increase the levels of MMP-1 and -3, both at the protein and mRNA levels in human patellar tendon fibroblasts cultured in monlayer.<sup>50</sup>

The mammalian family of MMPs consists of 24 related genes.<sup>51</sup> MMPs are a family of highly homologous zinc (Zn<sup>++</sup>) endopeptidases that collectively cleave most, if not all, of the constituents of the extracellular matrix. They are secreted or anchored to the cell surface, thereby confining their activity to membrane proteins and proteins in the secretory pathway or extracellular space.<sup>51</sup> The MMPs are involved in both normal tendon maintenance and in tendon pathology. MMPs -1 and -13 have the ability to specifically target type I fibrillar collagen for degradation,<sup>51</sup> while MMP-3 targets aggrecan present in the ECM of various tissues.<sup>52</sup>

Mechanical stretching of human patellar tendon fibroblasts induces upregulation of COX-2, MMP-1, and PGE<sub>2</sub>.<sup>53</sup> Additionally, mechanical stretching upregulates the production of IL-1 $\beta$ ,<sup>54</sup> which can cause increased production of MMPs -1, -3, and -13.<sup>48</sup> PGE<sub>2</sub> also induces upregulation of the production of MMP -1 and -3.<sup>50</sup> MMP-13 has an additional positive feedback mechanism on the inflammatory cascade by having the ability to directly activate MMP-1.<sup>55</sup> The inducible increase in MMP production by mechanical stretching and by various inflammatory mediators, combined with the degredative function of the MMPs suggests that these enzymes may play a significant role in the development of tendonitis.

Tendon is inherently slow to heal due to its limited number of resident cells, and its heavy collagenous nature. Injured tendons progress through all phases of healing; inflammation, repair, and maturation similar to that of other tissues. The inflammatory phase of healing following tendon injury begins immediately, and lasts for approximately 10 days. Initially, the site of damage is filled with blood, which then forms a fibrin clot rich in both platelets and

growth factors. The fibrin clot also serves as a scaffold for migratory cells and for collagen deposition. Neutrophils and macrophages are migratory cells that arrive following tendon injury, and release collagenases and proteolytic enzymes to begin the process of removing damaged tissue. The macrophages phagocytize necrotic debris, but more importantly they release chemotactic and angiogenic factors that attract fibroblasts and endothelial cells to the site of injury. The onset of the repair and proliferative phases of healing are marked by the arrival of fibroblasts that produce and deposit collagen within the damaged area. As immature collagen is deposited, the fibrin clot becomes early granulation tissue, a tissue of low tensile strength. The repair and maturation phases of healing in a tendon last for six months or more.<sup>30,56</sup> During the remodeling phase, collagen fibrils increase in diameter, while the tenocytes and collagen fibrils align along lines of tension. Additionally, cross-links are created between the collagen fibrils as they mature, providing additional strength to the tendon. Early in the healing process, the amount of collagen type I gene expression within the tendon decreases, with a relative increase in collagen type III expression.<sup>57</sup> However, contrary to early beliefs, type I collagen remains the predominant collagen type throughout the healing period, with its concentrations never accounting for less than 66% of the total soluble collagen within the tendon when tendonitis was induced using a bacterial collagenase model.<sup>58</sup> Despite collagen type I remaining the predominant form of collagen following healing of the tendon, type III fibrils persist. Due to their smaller diameter, type III collagen fibers result in decreased tensile strength of the healed tendon.<sup>30,56</sup>

The current effects of proteins that aid in the regulation of collagen fibril formation such as COMP and decorin are poorly understood in normal tendons, and their roles are less understood in damaged tendons. Targeted disruption of the decorin gene in newborn mice

resulted in abnormal skin fragility with significantly reduced tensile strength, and abnormal collagen morphology.<sup>26</sup> This disruption lead to more irregular and coarse collagen fibrils that had abrupt changes in fibril diameter in both skin and tendon samples.<sup>26</sup> These findings demonstrate the relative importance of decorin in collagen fibrillogenesis during tissue development. Injection of decorin antisense oligodeoxynucleotides enhances the healing of rabbit medial collateral ligament injuries.<sup>59</sup> Inhibition of decorin production resulted in the formation of larger collagen fibrils earlier in the healing process. Additional effects included a significant improvement in scar failure strength and a significant decrease in irrecoverable creep.<sup>59</sup> These findings suggest that decorin may have a deleterious effect on the healing of fibrous connective tissues by limiting the size of new collagen fibrils, and resulting in decreased strength of the healed tendon unit.

Little is known about the mechanism of action of COMP in both normal and damaged tendons. COMP initially increases in expression as a horse ages, and peaks at 3 years of age.<sup>60</sup> Additionally there is variation in expression between the superficial and deep digital flexor tendons in equine, with higher amounts of COMP found within the SDFT. COMP immunolabeling has been positively correlated with the presence of small, <60nm in diameter, collagen fibrils, which accounts for a higher percentage of overall fibrils in the SDFT as a horse ages.<sup>60</sup> The variation in COMP concentrations, not only between the DDFT and the SDFT, but also within the SDFT itself, is likely due to the forces applied to the tendon.<sup>17</sup> The variation in concentrations of COMP may be related to multiple factors first being that the SDFT is an energy storing tendon, and therefore may accumulate more microdamage than the DDFT. Since COMP is involved in fibril formation, it is found in higher concentrations in areas of tendon that are experiencing higher fibril turnover.

## Therapies for Tendonitis

Tendonitis is a common injury in both race and sport horses.<sup>3,4,38</sup> These injuries can result in a significant economic loss to the equine industry. When severe in nature or recurrent, they can lead to the early retirement or euthanasia of affected horses.<sup>61</sup>

Tendon is slow to heal for multiple reasons; limited number of highly differentiated resident fibroblasts, dense collagenous nature of the surrounding ECM, and slow collagen turnover rate.<sup>62</sup> The extended duration of rehabilitation combined with the high recurrence rate make these lesions difficult to treat successfully. Current standard therapies are based on the principles described by Asheim in 1964 including cooling, support wraps, and rest.<sup>63</sup> Although these principles make up the basis for current treatment protocols, they still have limited success in returning equine athletes to their previous level of performance because they are designed simply to decrease further damage, without truly addressing the enhancement of tendon healing. Both surgical and conservative protocols are available for the treatment of tendon lesions. The decision to approach a tendon lesion surgically compared to conservatively is based on the type of lesion present and surgeon preference. Lesions that are found to be anechoic on ultrasonography would be treated surgically, while ones found only to have an increase in the cross-sectional diameter are treated conservatively. Surgical techniques include tendon splitting and desmotomy of the accessory ligament of the SDFT. The main benefits of surgical therapy over the conservative approach are the induction of neovascularization into the damaged region of tendon, a reduction in strain on the tendon, or removal of damaged fibers depending on the technique used. It should be noted that while there are additional benefits to surgical management, some previously recommended techniques have been found to result in complications. For example, desmotomy of the accessory ligament of the SDFT results in



increased strain on the SDFT, and increases the risk of suspensory desmitis in the treated limb in Thoroughbred racehorses.<sup>64,65</sup> Tendon splitting can result in the formation of excessive granulation tissue within the tendon, and the persistence of lameness.<sup>66</sup> Tendon splitting is now only used in acute anechoic lesions, and aids in the decompression of the core lesion by evacuating serum or hemorrhage, then facilitating vascular ingrowth.<sup>67</sup> Despite the difference in therapeutic approaches, success rates in affected horses remained limited, with re-injury rates ranging from 43-93%.<sup>37,38</sup> As a result of the limited success of current therapeutic techniques, recent research has shifted away from a simple conservative or surgical approach to tendonitis, and has focused on regeneration versus repair of the damaged tissue.

The concept of reparative and regenerative medicine is of significance for tendon therapies. Reparative techniques such as those listed previously are directed at healing of the tissue, in this case the tendon. However, as with most tissues, tendon heals by the formation of scar tissue. This is recognized ultrasonographically by irregular fiber pattern and structurally by a reduction in overall tendon strength. In comparison, regenerative medicine focuses on healing of the tissue in a manner such that the tissue is returned to its pre-injury biochemical and biomechanical characteristics. Ideally this would include normal orientation of collagen fibers, normal cross-link formation, and a return to pre-injury strength levels. In an attempt to focus on tendon regeneration, injectible therapies have become and continue to be the focus of tendonitis therapy. Previously the pharmacological approach has been limited to the use of intravenous medications such as non-steroidal anti-inflammatories, steroids, and dimethyl sulfoxide (DMSO) which were used primarily as anti-inflammatories. Intra-lesional therapies including polysulfated glycoaminoglycans (PSGAGs),<sup>38,68</sup> hyaluronic acid (HA),<sup>69,70</sup> and beta-aminopropionitrile fumarate (BAPN)<sup>71</sup> have been investigated in an attempt to improve tendon healing. Additional

therapies investigated include the use of growth factors such as insulin-like growth factor-1 (IGF-1)<sup>72</sup> and transforming growth factor- $\beta$  (TGF- $\beta$ ),<sup>6</sup> the use of mesenchymal stem cells,<sup>73,74</sup> platelet-rich plasma,<sup>75-77</sup> and acellular bioscaffolds (ACell™).<sup>78,79</sup> Recently a new therapeutic, autogenous conditioned serum (ACS), known by the trade name IRAP™, has made its way into the clinical setting of equine practice for use in the treatment of tendonitis. ACS was initially used in equine practice to treat osteoarthritis, but despite a complete lack of investigation to its efficacy, is now also used to treat tendonitis.

Each of the previously mentioned therapies has been found to have potential advantages in tendon regeneration, but they have also been noted to have significant limitations. PSGAGs inhibit collagenases, MMPs, and the activation of macrophages.<sup>80</sup> PSGAGs however have no effect on proteoglycan production by fibroblasts.<sup>68</sup> There is variability in reports involving the evaluation of treatment with PSGAGs. Success rates vary from a 76% return to athletic function for horses treated with PSGAGs while only 46% of control horses returned to function,<sup>81</sup> to no difference between treated and control animals.<sup>82</sup> The use of intralesional HA failed to improve case outcome when compared to conservative management.<sup>82</sup> The use of intrathecal HA however, does decrease the inflammatory cell infiltrate, intralesional hemorrhage, and intrathecal adhesions.<sup>69,83</sup> BAPN inhibits the lysyl oxidase enzyme that is responsible for the formation of collagen crosslinks, thus it was thought that its use may allow for the formation of more normal parallel fibers instead of a haphazard arrangement. The use of BAPN results in a normal ultrasonographic appearance of tendon architecture in 80% of horses at 20 weeks post treatment.<sup>84</sup> BAPN also causes a reduction in collagen synthesis in equine tendon explants *in vitro*, and the addition of IGF-1 to culture medium did not abate these effects.<sup>71</sup>

The use of various growth factors has been investigated in an attempt to improve tendon healing following injury. IGF-1 is a mitogen that stimulates ECM synthesis, and causes an initial decrease in swelling when compared to controls. Additional benefits to the use of intralesional IGF-1 include an increase in DNA and collagen synthesis, improved echodensity of tendon parenchyma, and a trend towards increased stiffness.<sup>72</sup> Currently there are no studies that evaluate IGF-1's effect on re-injury rate, and further studies are required before it can be recommended as a primary therapeutic protocol. Similar to the use of IGF-1, the use of TGF- $\beta$  has also been investigated for intralesional use.<sup>6</sup> Treated horses show gross enlargement of the tendon following injection, and re-injury rates are similar to that of conservatively managed horses.<sup>6</sup>

The use of bone marrow aspirate (BMA) has been investigated as a source of autologous stem cells, but its use may more closely resemble the effects of a mixture of growth factors, as the number of actual stem cells within the bone marrow is relatively low.<sup>85</sup> Its use has been investigated in suspensory ligament lesions, and was found to have a success rate of 84%, with these horses returning to full work.<sup>73</sup> Although the numbers of stem cells present within BMA are low, there is currently no data that defines a minimum number of cells needed to achieve a therapeutic effect. Therefore BMA may achieve its increased success rate by combining the effects of stem cells and concentrated growth factors.

The use of platelet-rich plasma (PRP) has been investigated based on the numerous growth factors present within the  $\alpha$ -granules of platelets. These granules contain platelet derived growth factor (PDGF), transforming growth factor- $\beta$  (TGF- $\beta$ ), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and epidermal growth factor (EGF), and are released once the platelet is activated in a wound.<sup>76</sup> Tendon explants cultured in 100% PRP

showed enhanced gene expression of the matrix molecules collagen type I, type III, and COMP with no associated increase in the catabolic molecules MMP-3 and MMP-13.<sup>76</sup>

An additional therapy includes the use of an acellular matrix (ACell™) that is derived from porcine urinary bladder basement membrane. It has been proposed that ACell™ delivers extracellular matrix components and growth factors to the damaged region of the tendon, and may attract mesenchymal stem cells to the site through the release of bioactive breakdown products.<sup>86</sup> The use of ACell™ has been investigated following a collagenase induced tendinitis model, but found no significant differences between the treatment and control groups for any of the response variables evaluated.<sup>78</sup>

A new therapy that has gained popularity in equine medicine is the use of autogenous conditioned serum (ACS) for the treatment of tendon lesions. The use of this treatment modality has been extrapolated from human sports medicine<sup>87</sup> and its previous use for the treatment of equine osteoarthritis.<sup>88</sup> ACS is currently available commercially to equine practitioners, and is known by the trade name IRAP™, which is meant to denote its ability to increase the production of the anti-inflammatory mediator interleukin-1 receptor antagonist protein (IL-1Ra). The effects of ACS are based on a physio-chemical reaction that results in increased levels of both anti-inflammatory mediators and growth factors in the serum.<sup>87,89</sup> The conditioning process consists of incubating autogenous blood in a syringe containing chromium sulfate coated medical grade glass beads for a period of 24 hours.<sup>90</sup> This process stimulates leukocytes to increase production of certain growth factors and anti-inflammatory mediators that may be beneficial in tendon healing. The serum produced by the incubation process results in levels of interleukin-1 receptor antagonist (IL-1Ra) protein that are increased by 140 fold in human subjects.<sup>90</sup> This increase in protein production is the basis for the use of ACS to treat osteoarthritis. In addition

to the conditioning process's effects on the IL-1Ra protein, it also results in levels of fibroblast growth factor-2 (FGF-2) and TGF- $\beta$ 1 that are increased up to 750% and 182% in human subjects.<sup>87</sup> It is the increase in these growth factors that have lead to the investigation of ACS for the treatment of soft tissue injuries. Treatment of muscle injuries in both mice and humans by local administration of ACS resulted in accelerated muscle regeneration.<sup>87,89</sup> Currently in veterinary medicine, ACS has been investigated for use as an intraarticular therapy for equine degenerative joint disease, but literature evaluating ACS's effects on tendinitis in equine practice is currently limited,<sup>91</sup> thus further evaluation is required prior it being recommended as a primary treatment protocol.

### Models of Tendon Degeneration

Due to the limited success of current tendonitis therapies, new therapeutic protocols are constantly being investigated. Unfortunately many new therapies are initially tested *in vivo* and the *in vitro* phase of research is omitted. Although enticing to manufacturers and researchers alike, bypassing the *in vitro* phase of investigation neglects a critical step in the scientific process. As a result, research dollars and horse lives may be wasted while testing what eventually are found to be unsuccessful therapies. As public awareness for the ethical use of animals and the costs for *in vivo* studies rise, it is becoming increasingly important to develop suitable models for the *in vitro* screening of promising new therapies. To ensure that the *in vitro* phase of testing is not bypassed, it is necessary to develop reliable models that closely mimic clinical pathology.

Current models used to investigate the pathophysiology of tendonitis can be divided into one of two categories: the first being those that alter tendon fibroblast metabolism by creating

changes in the mechanical environment through the application of strain to the cells. The second induces damage by the use of a chemical agent.

Of the currently available models, the mechanical stretch model is advantageous for research on tendonitis since its ability to generate repetitive stretching of the cultured fibroblasts would mimic closely the *in vivo* nature of clinical injury. However, the mechanical stretching model can result in some variation in testing results. Mechanical stretching of cultured human patellar tendon fibroblasts results in a significant up-regulation in the production of both the COX-1 and -2 enzymes, along with increased production of PGE<sub>2</sub> and leukotriene-B<sub>4</sub> (LTB<sub>4</sub>).<sup>45</sup> However, the use of mechanical stretching on rabbit fibroblasts did not result in an increase in production of the COX-2 enzyme<sup>92</sup> as was demonstrated in human patellar tendon fibroblasts.<sup>45,54</sup> Additional concerns about the use of mechanical stretching as a valid *in vitro* model are supported by the *in vivo* finding that mechanical loading did not result in a significant increase in PGE<sub>2</sub> concentrations.<sup>93</sup> The lack of an *in vivo* elevation in PGE<sub>2</sub> suggests that the cellular alterations found in the mechanical stretching model may not accurately reflect the clinical disease. It is possible that the variation in results found within the literature in regards to mechanical stretching is the result in variation of technique. A wide range of strain rates and loading frequencies is present in the literature where this modality is utilized. Without a standardized protocol, it becomes difficult to compare accurately results from various studies.

Chemically induced modalities have been used both *in vivo* and *in vitro*. A common and well described model of chemical induction of tendonitis in the equine athlete is the use of collagenase.<sup>30,58,68,94-97</sup> This model is not compatible with *in vitro* use since the target tissue for the enzyme, collagen, is not present initially. Much of the focus on *in vitro* chemically induced tendonitis has involved the use of various inflammatory cytokines such as, IL-1 $\beta$  and PGE<sub>2</sub>. The

use of a species-matched mixture of inflammatory cytokines, referred to as cell-activating factor (CAF), has been utilized *in vivo*.<sup>98</sup> The use of CAF causes increased cellularity that resolves 16 weeks following injection and a decrease in overall tendon strength. The CAF utilized by Stone et al<sup>98</sup> was produced by synovial fibroblasts, and involved the dialysis of harvested media into medium containing IL-1 $\alpha$ . IL-1 is a potent mediator of inflammation produced throughout the body. The two main pro-inflammatory members of the IL-1 superfamily are the alpha and beta proteins.<sup>99</sup> In comparison to the use of CAF and IL-1 $\alpha$ , the addition of IL-1 $\beta$ , which has similar effects to IL-1 $\alpha$ ,<sup>99</sup> to cell culture media results in increased production of COX-2, IL-6, MMP-1, and MMP-3.<sup>100</sup> However studies involving torn human rotator cuff tendons from clinical patients revealed increased levels of IL-1 $\beta$  within the synovium, but not within the tendon itself suggesting that the synovium was the source of IL-1 $\beta$ .<sup>101</sup>

PGE<sub>2</sub> is another inflammatory mediator that has been utilized *in vitro* to induce tendonitis. PGE<sub>2</sub> is one of the most abundant prostaglandins in many tissues, is synthesized from arachidonic acid, and mediates tendon and ligament inflammation.<sup>102</sup> The exposure of human patellar tendon fibroblasts to PGE<sub>2</sub> resulted in decreased cellular proliferation and collagen production.<sup>2</sup> Additionally, PGE<sub>2</sub> results in an increase in the production of MMP-1 and -3, both at the mRNA and protein levels,<sup>50</sup> and is found in the peritendinous space of humans undergoing intermittent exercise.<sup>93</sup> These findings suggest that PGE<sub>2</sub> might be involved in the development of tendonitis due to repetitive mechanical loading of the tendon.

Like IL-1 $\beta$ , PGE<sub>2</sub> is easy to use as an induction agent for the *in vitro* study of tendonitis. An added benefit of the use of PGE<sub>2</sub>, is that it allows for the investigation of potential therapeutic agents that are directed at the inhibition of the effects of IL-1 $\beta$  specifically such as ACS. This study was aimed at the validation of PGE<sub>2</sub> as an *in vitro* model for equine tendonitis.

## Conclusions

Based on the limited success rates of current therapies utilized to treat equine flexor tendonitis, new treatment techniques are becoming available to practitioners. As with any treatment technique, it is important that new techniques be adequately scrutinized prior to their inclusion into mainstream practice. ACS is one such treatment technique that requires further investigation prior to its recommendation for the treatment of flexor tendonitis. ACS has been studied as a treatment for osteoarthritis,<sup>88</sup> but its success for that process should not be blindly extrapolated as meaning that it will be successful in the treatment of flexor tendonitis. Therefore this study was designed with the major goal being the investigation of ACS on cellular metabolism of equine SDFT fibroblasts. In an attempt to closely mimic the clinical patient, the study was designed so that the fibroblasts would be stressed, so as to avoid the determination of ACS's effects on quiescent cells.

As seen in the previous literature discussion on available models, a simple to use and relevant model that mimics closely the clinical situation would be valuable but is not currently available. Thus an attempt was made as part of this study to develop a model for altered cellular metabolism that would mimic the disease state of tendonitis. Based on both human and animal literature, the use of PGE<sub>2</sub> was selected for validation as a new model for *in vitro* equine flexor tendonitis. PGE<sub>2</sub> is a known mediator of inflammation,<sup>103</sup> but also has significant effects on the metabolism and proliferation of human fibroblasts.<sup>2</sup> Additionally, as previously stated, the selection of PGE<sub>2</sub> instead of IL-1 $\beta$  would allow for the investigation of ACS, which exerts its primary effects in the mammalian joint by blocking IL-1 $\beta$  specifically. The use of IL-1 $\beta$  as a cellular stressor may have confounded the results and made data interpretation difficult. The design of the portion of this study directed at the validation of the PGE<sub>2</sub> model was based on



literature describing significant effects of specific concentrations of PGE<sub>2</sub>.<sup>1,2</sup> The details of the design of this project are outlined in Chapter 2 of this thesis.

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## **Objectives and Hypothesis**

### **Objectives**

Our objective in performing this study was to design and validate an easy to repeat *in vitro* model of altered fibroblast metabolism that would mimic those seen in clinical cases of equine flexor tendonitis. The information will be useful for future research projects, by limiting the number of live test subjects necessary, thus decreasing research expense. Most importantly, this project will reduce the number of horses euthanized for research purposes.

Following validation of this model, the project aims to utilize the model to assess the effects of the treatment on equine flexor tendon fibroblasts with autogenous conditioned serum (ACS).

### **Hypothesis**

1. The administration of exogenous PGE<sub>2</sub> to monolayers of equine tendon fibroblasts will insight alterations in cellular metabolism that will generate a suitable model for the *in vitro* study of fibroblast response to novel therapies.
2. The addition of ACS to PGE<sub>2</sub>-treated fibroblasts will result in increased gene expression for collagen types I and III, cartilage oligomeric matrix protein, and decorin. ACS will also stimulate increased protein production in collagen and glycosaminoglycans, and stimulate increased cell proliferation. The use of ACS will decrease gene expression of inflammatory molecules important in tendon degradation, namely MMP-1, -3, and -13.

## **Chapter 2**

### Investigation of a Prostaglandin E<sub>2</sub>-Induced Model of *In Vitro* Tendon Injury

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ACVS

#### **Abstract**

**Objective**—To assess the use of PGE<sub>2</sub> treatment of monolayers of equine tendon fibroblasts as an *in vitro* model for investigating new drugs aimed at the treatment of equine tendonitis.

**Study Design**—*In vitro* cell culture study.

**Animals**—Six young adult horses (2-7 years of age) free of flexor tendonitis.

**Methods**—Superficial digital flexor tendon fibroblasts were isolated via collagenase digestion and grown in monolayer in varying concentrations of PGE<sub>2</sub> for 48 hours (0, 10, 50, 100 ng/ml in growth medium). Cells and medium were harvested for gene expression via real-time polymerase chain reaction for collagen types I and III, cartilage oligomeric matrix protein (COMP), decorin, and matrix metalloproteinases (MMP)-1, -3, and -13, biochemical analysis (glycosaminoglycan, DNA, and collagen content), and cytological staining.

**Results**—Gene expression for collagen type I was significantly increased at 100 ng/ml PGE<sub>2</sub> compared to 10 and 50 ng/ml. No significant changes were detected for biochemical content or gene expression of collagen type III, COMP, decorin or MMP-13. Unexplained moderate cell detachment occurred at the highest dose in two horses.

**Conclusion**—At concentrations less than 100 ng/ml, PGE<sub>2</sub> did stimulate alterations in metabolic activity; however, these changes failed to reach statistical significance. Variable cell detachment at the highest concentration suggests that PGE<sub>2</sub> concentrations of 100 ng/ml may be too high to induce injury without causing cell loss.

**Clinical Relevance**—Further investigation may prove PGE<sub>2</sub> treatment to be a useful model of tendon injury *in vitro*; however, the conditions described in this report resulted in inconsistent results.

**Key Words:** Tendonitis, model, prostaglandin E<sub>2</sub>, horse, in vitro



## Introduction

Flexor tendonitis is a common cause of lameness among horses and is challenging to treat successfully. The incidence of flexor tendon injuries among Thoroughbred race horses has been reported to range from 8 to 35%<sup>1,2</sup> and the rate of re-injury can be as high as 43-93%.<sup>3,4</sup> The initial rate of occurrence coupled with the high rate of re-injury results in a significant economic impact on the equine industry in lost time in work, decreased return to previous level of performance and cost of treatment and rehabilitation.<sup>1,3</sup>

Treatment of flexor tendonitis can be difficult due to the slow native healing response of dense fibrous connective tissues such as tendon, and the high rate of recurrence of tendon injuries. An ever-increasing number of treatment options are available to treat tendon and ligament injuries in the horse.<sup>5-8</sup> Conservative management and a controlled exercise regimen monitored by serial ultrasound examinations form the basis for all treatments. Novel therapies for the treatment of flexor tendon injuries continue to be developed and require *in vitro* screening prior to their testing *in vivo* and/or commercialization for use in clinical cases.

In the current environment, new treatments for equine tendonitis may or may not be tested *in vitro* initially prior to their evaluation *in vivo* in a controlled study or clinical trial. Although enticing to manufacturers and veterinarians alike, bypassing the *in vitro* phase of investigation neglects a critical step in the scientific process. The risks include wastage of research dollars and horses through the completion of terminal studies for what turn out to be unsuccessful therapies. As public awareness for the ethical use of animals and the costs for *in vivo* studies rise, it is increasingly important to develop suitable models for the *in vitro* screening of promising new therapies. Reliable *in vitro* models of tendon injury for such investigations are

currently not available, therefore preventing controlled efficacy and comparative studies for both current and new therapies.

An ideal model for *in vitro* research would be easy to perform, cost effective and reliable in the nature and degree of injury or degeneration. Cells or tissue explants from normal horses can be useful; however, the cellular response may not accurately reflect the *in vivo* response due to differences in cell metabolism and gene expression in normal (potentially quiescent cells) compared to stimulated cells from injured tissues. A normal joint responds differently to treatments than does an inflamed joint.<sup>9,10</sup> A useful model should attempt to mimic critical aspects of the clinical disease.

The pathophysiology of tendon injury is multifactorial and incompletely understood; however repetitive microdamage is believed to cause tendon degeneration and play a significant role in the development of clinically relevant tendon lesions.<sup>1,11,12</sup> Cyclic mechanical strain of tendon tissues stimulates tendon cells to produce inflammatory mediators involved in tendon injury.<sup>1,11,13</sup> Infiltrates of inflammatory cells are not a typical histological hallmark of tendonitis;<sup>11</sup> however, inflammatory mediators released from tendon fibroblasts play a role in tendon overuse injuries, and are not detectable using standard staining techniques.<sup>13-15</sup> Cyclic loading of tendon and ligament fibroblasts results in significant increases in proinflammatory cytokines such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), matrix metalloproteinases (MMP)-1, -3 and -13, and interleukin-1 $\beta$  in a magnitude dependent manner.<sup>15-23</sup> Proinflammatory mediators directly degrade tendon extracellular matrix in a manner similar to that seen in clinical patients.<sup>11,24,25</sup> PGE<sub>2</sub> has been shown to decrease fibroblast proliferation and collagen production *in vitro*<sup>24</sup> and repeated exposure of tendon tissue to exogenous PGE<sub>2</sub> resulted in increased tendon cellularity and greater fiber disorganization *in vivo*.<sup>11</sup> Prostaglandin E<sub>2</sub> is synthesized from arachidonic acid

via the action of cyclooxygenase and is a potent mediator of tissue inflammation.<sup>26-28</sup> Based on these findings, the authors suggest that PGE<sub>2</sub> may participate significantly in the pathology of chronic, degenerative tendon lesions and that the application of exogenous PGE<sub>2</sub> to cultures of tendon tissue or monolayers of fibroblasts would be a useful *in vitro* model to alter cellular metabolism in a manner similar to that seen *in vivo*.

The purpose of this study was to assess the utility of PGE<sub>2</sub> treatment of monolayers of equine tendon fibroblasts as an *in vitro* model for investigating new drugs aimed at the treatment of equine tendonitis. We hypothesized that treatment of monolayers with exogenous PGE<sub>2</sub> would induce a clinically relevant model of cellular injury *in vitro* characterized by altered metabolism of extracellular matrix proteins critical to the structure and function of tendon.

## Materials & Methods

### *Tissue Harvest and Cell Isolation*

Six young adult horses (2-7 years of age (mean 3.8); 3 geldings, 2 stallions, 1 mare) donated to the teaching hospital for reasons other than flexor tendonitis were used for the study. All horses were free of lameness due to forelimb superficial digital flexor (SDF) tendons based on observation at a walk, palpation of the tendons, and gross appearance of the tendons at the time of harvest. All protocols were approved by the Institutional Animal Care and Use Committee.

Following euthanasia via barbiturate overdose, the tensile region of the superficial digital flexor tendons was harvested aseptically from both forelimbs and transported to the laboratory in ice cold Hank's Balanced Salt Solution (HBSS) for isolation of tendon fibroblasts by collagenase digestion.<sup>29</sup> Briefly, the paratenon was removed and the central portion of the tendon was minced into 1-2 mm pieces, digested in 0.075% w/v collagenase in digest medium (Dulbecco's Modified Eagle Medium, high glucose (DMEM), 10% fetal bovine serum (FBS), 50 µg/ml ascorbic acid, 30 µg/ml α-ketoglutaric acid, 25 mM HEPES buffer, and 100 units/ml sodium penicillin and 100 µg/ml streptomycin sulfate ) overnight at 37°C, 5% CO<sub>2</sub>, and 95% humidity with stirring. Following overnight digestion, the digest medium was prefiltered through cheesecloth, rinsed with DMEM, filtered through 40 µm nylon mesh (Small Parts, Inc., Miramar, FL), and centrifuged at 1500 rpm for 10 minutes at 25°C. The cell pellet was resuspended in growth medium (DMEM, high glucose, 10% FBS, 50 µg/ml ascorbic acid, 30 µg/ml α-ketoglutaric acid, 25 mM HEPES, and 100 units/ml sodium penicillin and 100 µg/ml streptomycin sulfate) and fibroblasts were plated in 75cm<sup>2</sup> tissue culture flasks, incubated until

they reached approximately 95-100% confluence, trypsinized (0.25% trypsin/2.21 mM EDTA in HBSS without sodium bicarbonate, calcium and magnesium) and split 1:4. Following expansion, cells were suspended in freeze medium (90% FBS and 10% dimethylsulfoxide), slowly cooled to  $-80^{\circ}\text{C}$ , and stored in the vapor phase over liquid nitrogen. Equine tendon fibroblasts subcultured for 4 to 6 passages were used for all experiments. Each horse's tendon fibroblasts were maintained as a separate batch of cells (n=6 for all assays except DNA where n=5).

### *Cell Culture*

At the time of each experiment, fibroblasts were thawed and plated in growth medium at a density of  $2 \times 10^4$  cells/cm<sup>2</sup> for 3 to 4 days in 75 cm<sup>2</sup> culture flasks at  $37^{\circ}\text{C}$ , 5% CO<sub>2</sub>, and 95% humidity until they reached >90% confluence, trypsinized, and seeded in 24 well plates. Wells for cell staining and DNA content were seeded at a density of  $4.5 \times 10^4$  cells per well. Cells to be used for soluble collagen and glycosaminoglycan content, and PCR analysis were seeded at a density of  $6.5 \times 10^4$  cells per well. The lower seeding density was selected for DNA quantification so that the cells would be in an actively dividing state that would give an estimate of the effect of PGE<sub>2</sub> on cell proliferation. The higher seeding density was used for protein analysis and gene expression assays to purposefully achieve a confluent monolayer early in the process, selecting for a greater impact on matrix production rather than proliferation. Cells were allowed to equilibrate and reach a predetermined level of confluence (~75% for lower density cells and ~90% for the higher density cells) over a 48 hour period. Growth medium was exchanged for culture medium containing 0, 10, 50, or 100 ng/ml PGE<sub>2</sub> (MP Biomedicals, Solon, OH) for 48 hours.<sup>24,28</sup> Each treatment was repeated in triplicate. Following the 48 hour

incubation with PGE<sub>2</sub>, monolayers for cell staining and DNA analysis were harvested (see below). The medium in the wells designated for collagen and glycosaminoglycan content and gene expression was replaced with growth medium, the plates were incubated for an additional 72 hours and subsequently harvested (see below).

### *Cell Morphology*

Cell morphology and health were assessed subjectively via phase contrast microscopy (CKX41, Olympus Imaging America, Inc., Center Valley, PA) prior to and following treatment with PGE<sub>2</sub>. Monolayers of cells were fixed for 24 hours at room temperature in 10% buffered formalin, rinsed with distilled water and allowed to air dry. A routine Diff-Quick<sup>®</sup> protocol was then used to stain the cells. Eosin and methylene blue were added alternately in dropwise fashion to the wells and left in contact with the monolayers for 15 seconds each. Excess stain was removed by rinsing the monolayers with distilled water. The monolayers were rapidly dehydrated in absolute alcohol and coated in an aqueous stain preservation solution (Crystal/Mount, Biomedica Corporation, Foster City, CA).

### *Biochemical Analysis*

Cell monolayers were trypsinized, transferred to centrifuge tubes, and centrifuged at 15,000 rpm for 10 minutes at 25°C. The cell pellet was washed with phosphate buffered saline and re-centrifuged as above, and the cell pellet stored at -80°C for determination of DNA content. Medium for collagen and glycosaminoglycan content was aspirated and stored at -80°C for quantification of glycosaminoglycan and collagen content. The mean value from triplicate wells was used for statistical analysis. Glycosaminoglycan content of the medium was

determined using the dimethylmethylene blue dye binding assay and spectrophotometric analysis.<sup>30,31</sup> DNA content of monolayers was determined following digestion in 0.5% papain for 20 hours at 65°C using bisbenzamide binding and fluorometric assay.<sup>31,32</sup> Soluble collagen levels in the medium were quantified spectrophotometrically using the Sirius red dye-binding method (Sircol Collagen Assay, Biocolor Ltd., Belfast, N. Ireland).<sup>33</sup>

### *Gene Expression*

Total RNA was isolated from monolayers of cells using the guanidinium chloride-phenol extraction method (Life Technologies, Grand Island, NY) and column purification (RNeasy<sup>®</sup> spin columns, Qiagen, Valencia, CA).<sup>34</sup> Total RNA from triplicate wells was combined and purified across the same column. Purity of RNA was assessed by agarose gel electrophoresis. RNA was quantified using a fluorescent nucleic acid stain (Quant-iT<sup>™</sup> RiboGreen<sup>®</sup> RNA reagent, Invitrogen, Carlsbad, CA). First strand complementary DNA was synthesized by use of oligo(dT) priming and a commercial reverse transcriptase kit (High Capacity cDNA Archive Kit, Applied Biosystems, Foster City, CA). Complementary DNA was amplified by fluorescent real-time PCR (Absolute Quantitative PCR: ABI PRISM 7300 Sequence Detection System, Applied Biosystems, Foster City, CA) using custom primers and MGB probes designed using equine-specific sequence data (Primer Express Software Version 3.0, Applied Biosystems, Foster City, CA). (Table 1) All primer/probe sets were verified using cDNA made from RNA with known expression for the gene of interest. Relative gene expression was calculated using the comparative threshold cycle method ( $\Delta\Delta C_t$ ) using 18s ribosomal RNA expression as the endogenous housekeeping gene<sup>34,35</sup> and the 0 ng/ml PGE<sub>2</sub> as the control sample for normalization.<sup>36,37</sup> Genes of interest included those important in the biochemical composition or

organization of the extracellular matrix of tendon (collagen types I and III, cartilage oligomeric matrix protein (COMP), and decorin), and those important in the degradation and/or reorganization of the extracellular matrix (matrix metalloproteinases (MMP)-1, -3, and -13).

### *Statistical Analysis*

Treatments were set up in triplicate for each horse and each assay. The mean of the 3 wells (biochemical analysis) or the 3 wells combined (gene expression) was used to increase the precision of the data obtained for statistical analysis (n=6 horses). Treatment effects were analyzed by One-way ANOVA and Tukey's post-hoc comparison using a commercial software program (Statistix, version 8.1, Analytical Software, Tallahassee, FL). Significance was set at  $P \leq 0.05$ .



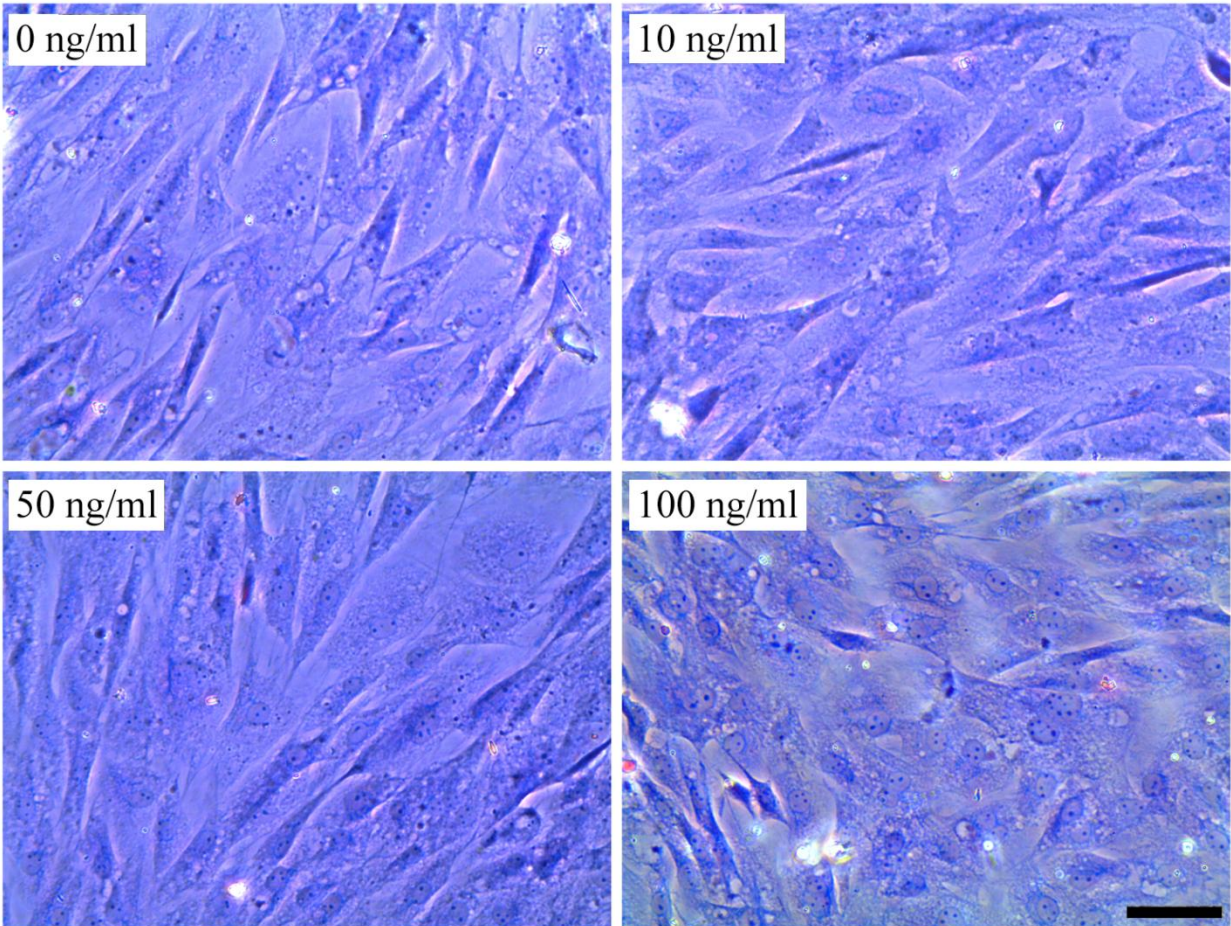
## Results

### *Cell Morphology*

Prior to cell harvesting, an increased number of detached cells were noted to a variable extent in two horses. Cell detachment was evidenced by holes in the cell monolayer and a marked number of floating cells. In some cases, cells were noted to reattach to the vacated areas of plastic. The cells from one horse in particular seemed particularly sensitive to the PGE<sub>2</sub>, with approximately 60 and 50% of the cells detaching in the 100 and 50 ng/ml groups respectively and only a small number at 10 ng/ml. No detachment was noted in the control group. Approximately 40 and 25% of the monolayer was lost in a second horse at 100 and 50 ng/ml respectively with none at either 10 or 0 ng/ml. No cell detachment was noted in any of the four other horses. The degree of cell detachment was variable between replicate wells. Subjectively, cell morphology was unchanged by treatment with PGE<sub>2</sub> (Fig 1). All cells maintained a normal stellate shape, granular cytoplasmic appearance, and multiple nucleoli.

### *Biochemical Analysis*

The collagen content of the medium was increased at all PGE<sub>2</sub> concentrations (Table 2) with 10 ng/ml being highest; however, there were no significant differences between groups ( $p = 0.41$ ). There were no significant differences between groups for glycosaminoglycan content of the medium ( $p = 0.81$ ) (Table 2). DNA content of the wells was decreased at all concentrations of PGE<sub>2</sub> compared to the control group (Table 2); however, these differences did not reach statistical significance ( $p = 0.52$ ).



**Figure 2.1** Diff Quick<sup>®</sup> stain of equine tendon fibroblasts. The cells were grown in monolayer culture and treated with varying concentrations of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in the medium. There were no detectable changes in cell morphology between groups, suggesting that the toxic effects of PGE<sub>2</sub> on tendon fibroblasts were minimal or absent. Bar = 50µm.

<b>PGE<sub>2</sub> (ng/ml)</b>	<b>Collagen (ng/well)</b>	<b>GAG (µg/well)</b>	<b>DNA (µg/well)</b>
0	50 ± 30	0.83 ± 0.10	1.93 ± 0.57
10	120 ± 20	0.86 ± 0.11	1.83 ± 0.34
50	100 ± 30	0.81 ± 0.11	1.53 ± 0.32
100	90 ± 40	0.86 ± 0.09	1.63 ± 0.52

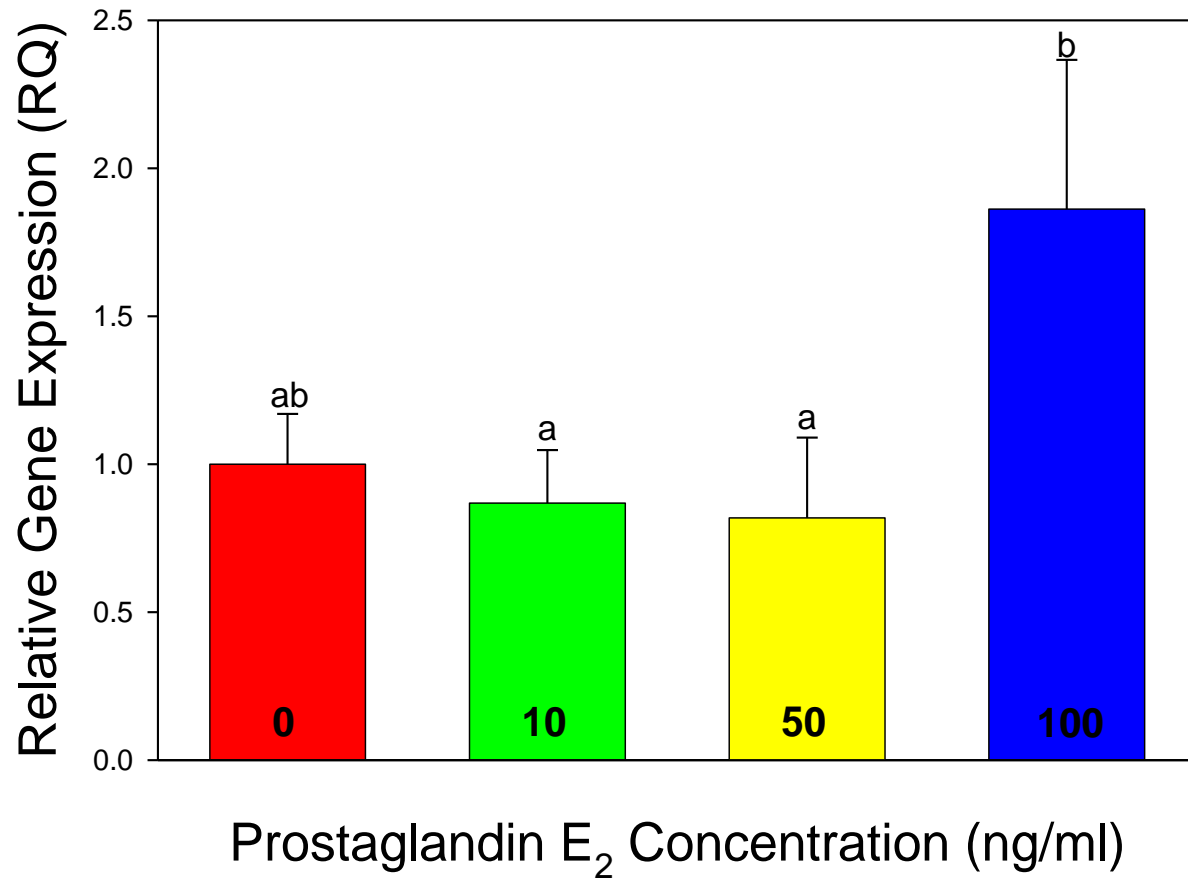
**Table 2.1** Biochemical content of monolayers (n=5) and culture medium (n=6). Effects of varying concentrations of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) for 48 hours on equine tendon fibroblasts grown and harvested at 48 (monolayer) and 120 hours (media) following the addition of PGE<sub>2</sub>. Mean ± SD. No statistically significant changes were found as a result of treatment with PGE<sub>2</sub>. GAG = glycosaminoglycan.

### *Gene Expression*

Gene expression for collagen type I was significantly increased in the 100 ng/ml PGE<sub>2</sub> treatment group compared to 10 and 50 ng/ml ( $p = 0.02$ ); however, it was not significantly different from the 0 ng/ml group (Figure 2). Treatment with 10 and 50 ng/ml PGE<sub>2</sub> resulted in decreased gene expression compared to control. Gene expression for collagen type III was decreased at 10 and 50 ng/ml and increased at 100 ng/ml of PGE<sub>2</sub> compared to the control; however, these changes were not statistically significant ( $p = 0.14$ ) (Figure 3). The ratio of collagen type I to collagen type III was not significantly different between treatment groups and was essentially 1:1 ( $0.96 \pm 0.06$  (0 ng/ml),  $0.93 \pm 0.04$  (10 ng/ml),  $0.96 \pm 0.04$  (50 ng/ml),  $0.95 \pm 0.03$  (100 ng/ml). Gene expression for COMP showed a dose dependent increase that was not significantly different from the control ( $p = 0.21$ ) (Figure 4). Decorin gene expression was decreased at 10 ng/ml and increased at 50 and 100 ng/ml compared to the control group but was not statistically significant ( $p = 0.09$ ) (Figure 5).

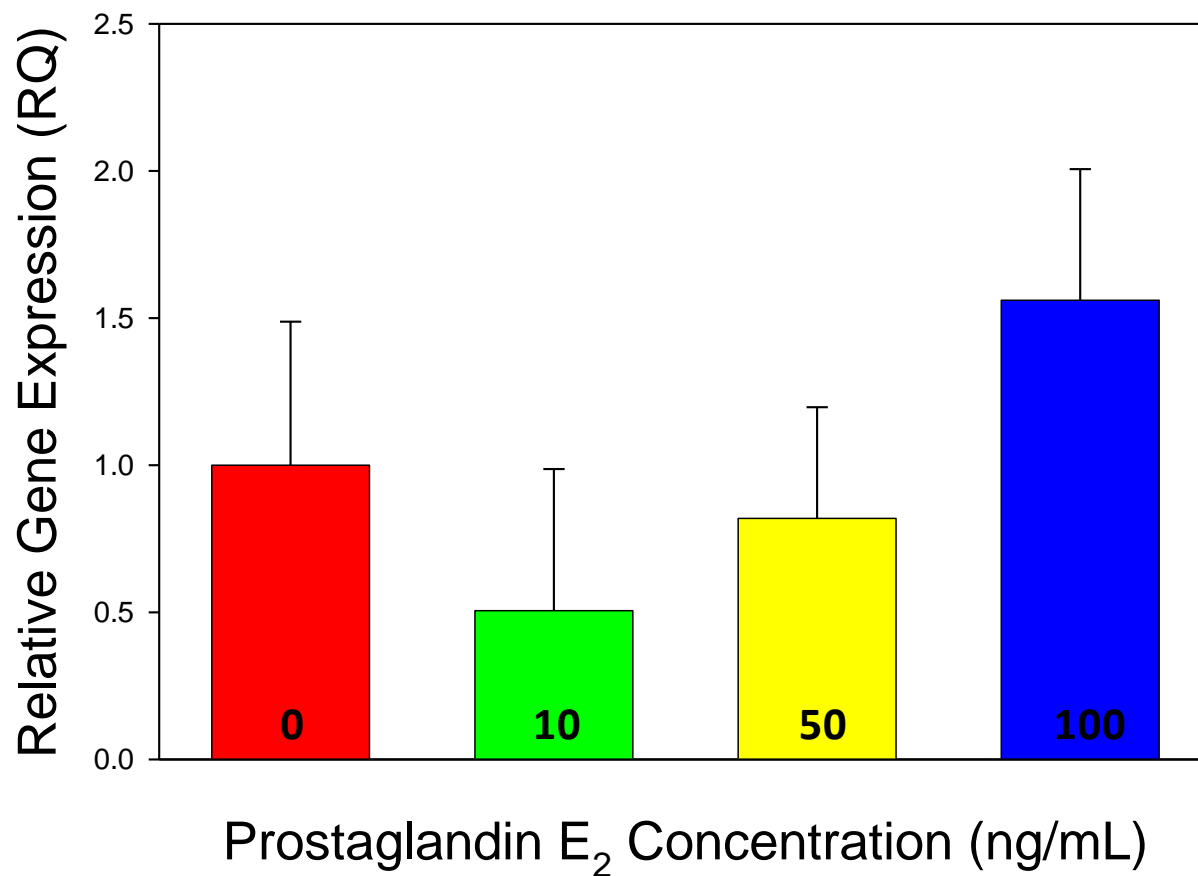
Gene expression for the three MMPs was extremely low in all samples. Primer/Probe sets for all MMPs were validated using cDNA synthesized from RNA samples that showed adequate expression to generate an appropriate standard curve. MMP-1 and -3 were not expressed in measurable quantities in any of the 6 horses in this study. MMP-13 was detectable in very low quantities in only 2 of the 6 horses, and undetectable in the remaining 4 horses (Figure 6). Statistical analysis for the MMP genes was not performed due to the low or absent expression.

# Collagen Type I



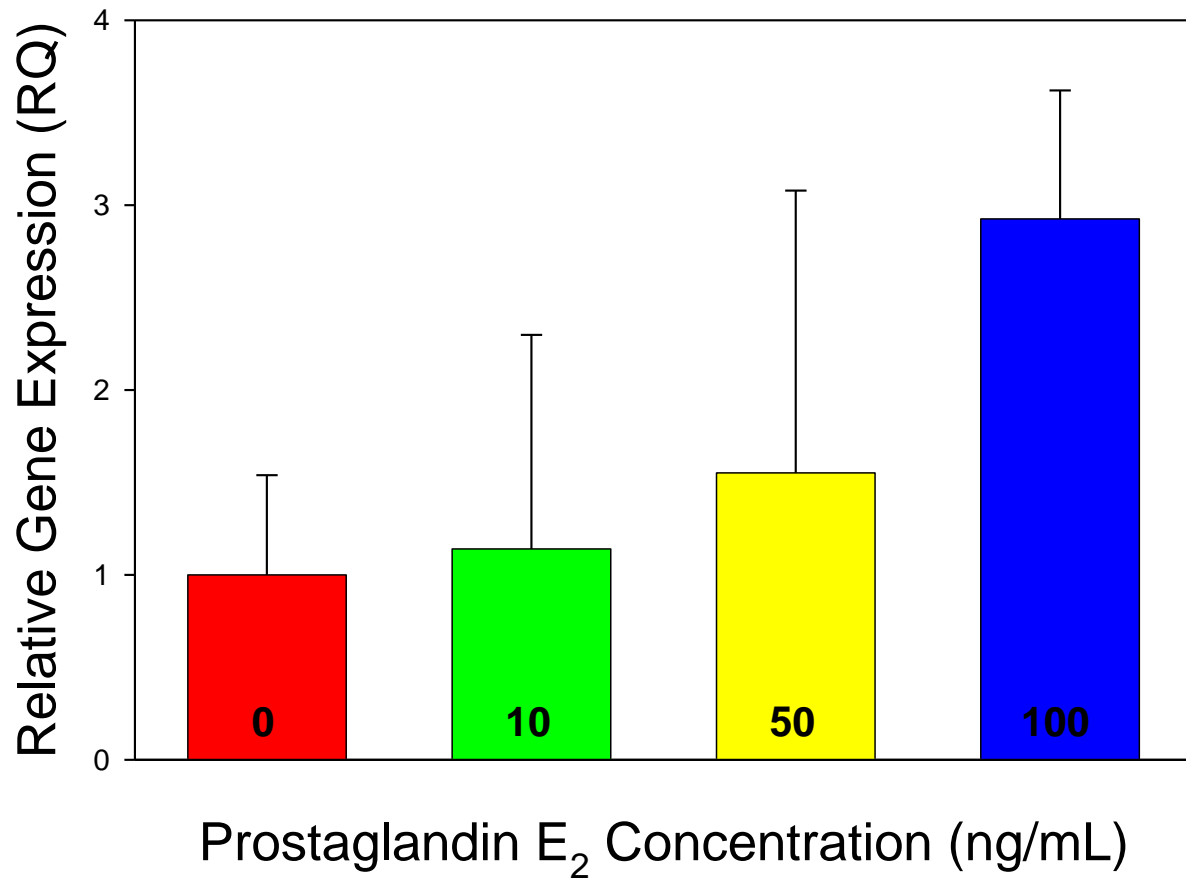
**Figure 2.2** Relative collagen type I gene expression. Expression determined by fluorescent real-time polymerase chain reaction calculated by the comparative threshold cycle method ( $\Delta\Delta C_t$ ) using 18s ribosomal RNA expression as the endogenous control. ( $p = 0.02$ )

## Collagen Type III



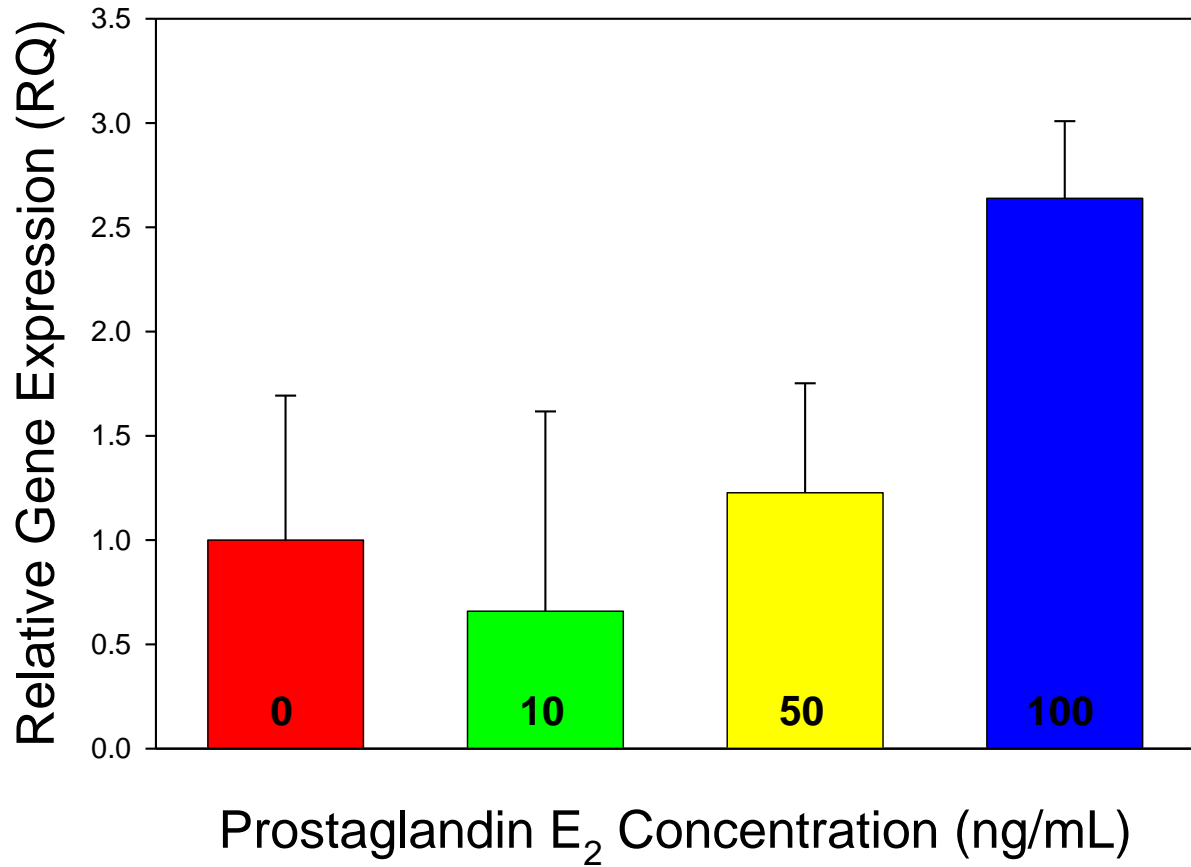
**Figure 2.3** Relative collagen type III gene expression. Expression determined by fluorescent real-time polymerase chain reaction calculated by the comparative threshold cycle method ( $\Delta\Delta C_t$ ) using 18s ribosomal RNA expression as the endogenous control. ( $p = 0.14$ )

# Cartilage Oligomeric Matrix Protein



**Figure 2.4** Relative COMP gene expression. Expression determined by fluorescent real-time polymerase chain reaction calculated by the comparative threshold cycle method ( $\Delta\Delta C_t$ ) using 18s ribosomal RNA expression as the endogenous control. ( $p = 0.09$ )

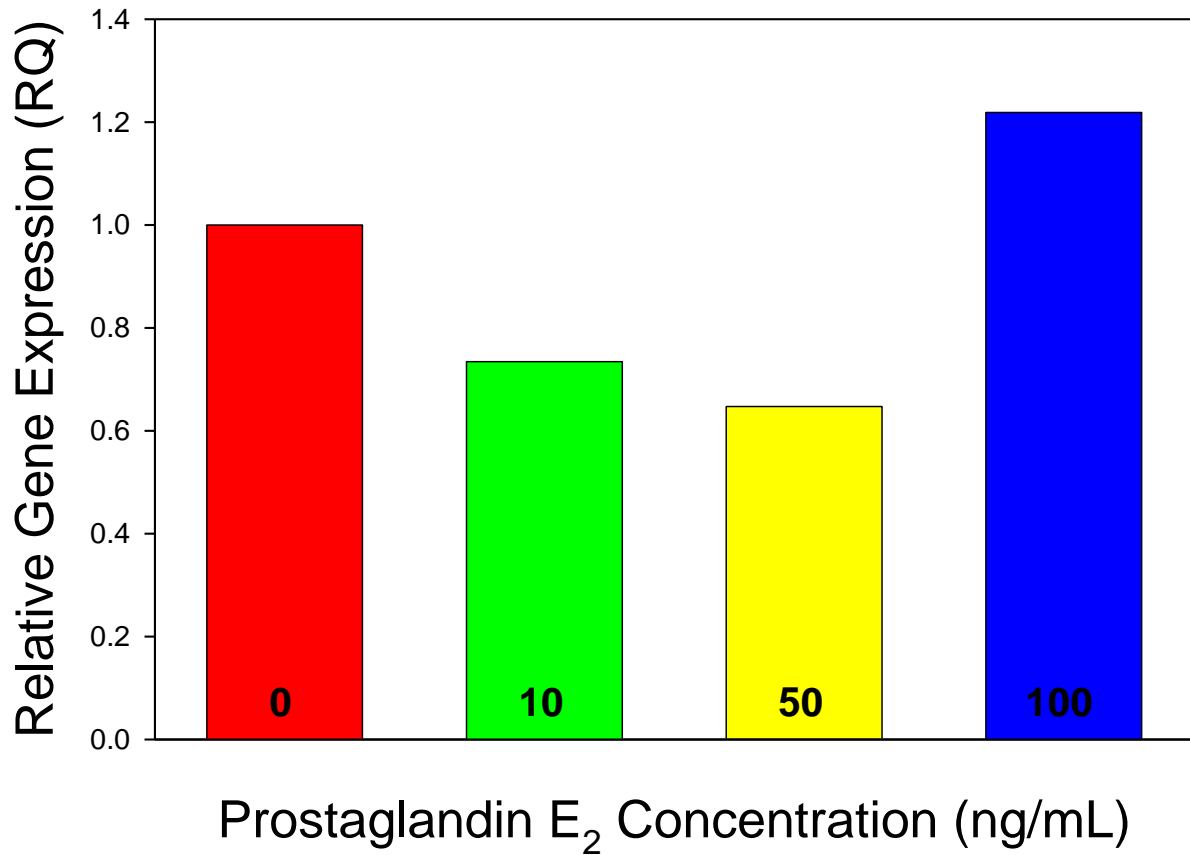
# Decorin



**Figure 2.5** Relative decorin gene expression. Expression determined by fluorescent real-time polymerase chain reaction calculated by the comparative threshold cycle method ( $\Delta\Delta C_t$ ) using 18s ribosomal RNA expression as the endogenous control. ( $p = 0.21$ )



# MMP-13



**Figure 2.6** Relative MMP-13 gene expression. Expression determined by fluorescent real-time polymerase chain reaction calculated by the comparative threshold cycle method ( $\Delta\Delta C_t$ ) using 18s ribosomal RNA expression as the endogenous control. n=2. Mean.

## Discussion

Treatment of monolayers of equine tendon fibroblast with the PGE<sub>2</sub> protocol described in this study resulted in statistically insignificant alterations in cell numbers, collagen production, and gene expression for the extracellular matrix proteins of interest. Although these differences were small, they may represent an adequate degree of alteration in cell metabolism to fulfill the goal of creating an “abnormal” cell for *in vitro* investigations. The variable degree of cell detachment in two of the horses associated with the highest dose of PGE<sub>2</sub> is undesirable and suggests that modified doses and exposure times may need to be explored to optimize this model for future investigations.

Matrix metalloproteinases and cell morphology were either minimally affected or not at all; an indication that the toxic effects of PGE<sub>2</sub> were not excessive. Direct measurement of cytotoxicity and quantitative growth kinetics were not evaluated, however and could have proved a useful means of further characterizing the potential cause of cell detachment. The goal of the model is to induce changes in gene expression and cell metabolism without abolishing the general phenotype of the tendon fibroblasts. The results of the study reported here do in part accomplish this goal and provide a basis from which to continue to develop the model.

Gene expression for all target genes was highest in response to 100 ng/ml PGE<sub>2</sub> and ranged from a 1.6 fold increase for collagen type III to a nearly three-fold increase for COMP. At the lower doses of PGE<sub>2</sub> the gene responses were variable with some decreasing and some increasing. The significant increase in expression of collagen type I was expected since this is the primary type of collagen in the extracellular matrix of tendon. Expression of collagen type I has been demonstrated to become elevated immediately following injury, and to remain elevated

for up to 24 weeks post injury.<sup>33</sup> The lack of detectable levels of both MMP-1 and -3, and the variable levels of MMP-13, are inconsistent with the literature. Treatment of monolayers of human patellar tendon fibroblasts with 100 ng/ml of PGE<sub>2</sub> resulted in a significant increase in both gene expression and protein production of MMP-1 and -3.<sup>28</sup>

The cell detachment that was noted in two of the six horses during this study was unexpected and of unknown cause. These cells may have become detached due to rapid cell proliferation and the formation of an overly dense monolayer of cells, apoptosis or cell death, or for another undetermined reason. Monolayers were assessed daily by phase contrast microscopy throughout the course of the study and at no time appeared stressed or on the verge of impending cell death. Cell detachment occurred in the 24 hours prior to harvesting of samples for DNA analysis, during the time of exposure to PGE<sub>2</sub> and in a dose response manner in the two horses affected. This suggests that cells from individual horses may vary in their response to PGE<sub>2</sub> exposure and that the duration of exposure and dosage of PGE<sub>2</sub> may have been too high for the desired application and that successful application of the model may require modification to a lower time of exposure to PGE<sub>2</sub> and/or a slightly lower dose of PGE<sub>2</sub>. A cell viability assay performed on the detached cells at the time of harvest may have been useful in elucidating the cause of cell detachment.

The cell detachment complicated data analysis and interpretation in this study and required the omission of the one horse that was most severely affected by cell loss to be omitted from the data analysis for the DNA assay. In addition, it is unknown whether cell loss from the monolayers associated with the medium harvested for collagen and glycosaminoglycan content affected the data for these assays. Analysis of the raw data and the standard deviations indicate that this was not the case and that the data are representative of the changes affected by PGE<sub>2</sub>.

The additional 72 hours of culture prior to media harvest may have lessened the impact of the cell loss in the two horses affected. The PCR data would have been unaffected because the use of an endogenous control gene, 18s RNA, automatically normalizes for cell numbers. Collagen and glycosaminoglycan content could not be normalized based on DNA content because they were harvested from different sets of wells.

There are a number of possible explanations for the lack of agreement between our results and those of other studies. Our study design was similar to that of Cilli in the use of monolayers of tendon fibroblasts, the culture conditions, and the dosages of PGE<sub>2</sub> selected.<sup>24</sup> Differences in the anatomic source of the cells and/or species differences may have contributed to the difference in response by the equine SDF tendon fibroblasts.<sup>38-41</sup> Cells from different sources have been shown to respond differently to identical stimuli. Fibroblasts from the human anterior cruciate (ACL) and medial collateral ligaments (MCL) showed significant differences in expression of pro-matrix metalloproteinase-2 following injury.<sup>38</sup> Collagen gene expression is higher in the rabbit MCL than the ACL in the normal and injured states.<sup>39</sup> Growth factors are also more highly expressed in rabbit MCL than ACL following injury.<sup>40</sup> Species differences may also play a role in the response of the tendon fibroblasts to PGE<sub>2</sub>. Equine chondrocytes cultured in monolayer treated with PGE<sub>2</sub> also showed decreased expression of MMP-1, -3, and -13.<sup>41</sup>

The use of monolayers of cells, as described in this study, rather than tissue explants could be an additional source for variability in response. Tissue explants contain both tendon fibroblasts and their surrounding extracellular matrix. The extracellular matrix may protect fibroblasts by decreasing the cell surface area that is exposed to PGE<sub>2</sub> or by reducing the amount of PGE<sub>2</sub> to which the cells are actually exposed. The metabolic rate of cells embedded in

extracellular matrix may be different from that of cells in monolayer. Monolayers were utilized for this study in an effort to select the least complicated environment in which to manipulate and assess the cells. Maintaining the protection offered by the extracellular matrix may prove to be a more suitable model for equine studies and result in more reproducible responses to PGE<sub>2</sub> injury. Repeated injections of 50 and 500 ng PGE<sub>2</sub> into rabbit patellar tendons resulted in increase cellularity at the site of injection based on histopathology.<sup>11</sup> Although the 500 ng dose was significantly higher than those used in our study, the precise level of exposure at the cellular level following injection into the tendon parenchyma is unknown. Additionally, the origin of the cells observed in these tissue sections was not determined and may have been migratory cells rather than primary tendon fibroblasts.

The dosages of PGE<sub>2</sub> selected for this study were based on a previous publication using dosages of 1, 10, and 100 ng/ml to treat monolayers of human patellar tendon fibroblasts.<sup>24</sup> There were no differences in response between the 10 and 100 ng/ml groups and the 100 ng/ml concentration was selected as optimal for further studies.<sup>28</sup> Our results do show a difference in cellular response between the 10 and 100 ng/ml test groups, supporting the thought that human patellar tendon fibroblasts and equine flexor tendon fibroblasts do not respond in the same manner to PGE<sub>2</sub> exposure. This may be in part due to potential species differences in the four receptor subtypes through which PGE<sub>2</sub> exerts its cellular effects. Each of the receptor subtypes is believed to cause a different response when stimulated. It is possible that the predominant receptor subtype in equine SDF tendon fibroblasts is different from that in human patellar tendon fibroblasts. To the authors' knowledge, this information is not available in the literature.

Although most of the results reported in this study failed to reach statistical significance, treatment with PGE<sub>2</sub> does appear to cause changes in gene expression, protein production, and

cell proliferation that may adequately reproduce the injury to tendon fibroblasts that occurs following repetitive strain. Further development of this model of tendon injury to establish a more predictable response of suitable magnitude is warranted. Additional fine-tuning of the duration of exposure to PGE<sub>2</sub>, the dosage selected, and the optimal amount of time following PGE<sub>2</sub> treatment to apply therapeutic agents may result in a useful model. Tissue explants may be a more suitable model for equine studies.

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## Final Comments

This thesis has investigated the development of a new model of *in vitro* equine flexor tendonitis using exogenous PGE<sub>2</sub> to alter cellular metabolism. The intent was to validate this model so that controlled experiments could be performed on both current and future therapeutic drugs for this condition. PGE<sub>2</sub> was selected for use based on its efficacy in other species; however, we have demonstrated that its use in monolayers of equine flexor tendon fibroblasts is not the same as what has previously been reported in other species. As stated earlier, this may be the result of multiple factors; use of monolayers versus tissue explants, species variation, potential for the presence of different PGE<sub>2</sub> receptor type amongst different species, or inappropriate study design. Alterations in study design may include changes in PGE<sub>2</sub> concentrations used, duration of exposure, or potentially the use of a repeated exposure protocol to improve the efficacy of this model. The results of this study have shown changes in the metabolic response of these fibroblasts due to exposure to exogenous PGE<sub>2</sub>, however with the current study design the results were insignificant. Future work with this model is warranted and may entail the use of tissue explants or alterations in PGE<sub>2</sub> concentration or duration of exposure.

As one of the ultimate goals of this project was to validate an *in vitro* model of equine flexor tendonitis, it is worth noting that there are other options that may meet this criterion. Currently much work has been performed in other species utilizing cyclic strain culture techniques. Although there is still much work to be done with these techniques to optimize strain rates and frequencies, this model may be useful to mimic the clinical situation of microdamage secondary to cyclic fatigue. In comparison to the model proposed by this study, a model of cyclic strain would serve to “exercise” the cells where the PGE<sub>2</sub> model was intended to

alter cell metabolism in a way to mimic damage. Currently further investigations of both models are needed to make them more consistent and reliable for future use.

In scientific design it is important not only to know what works, but also what does not. This study has demonstrated that the current proposed model does not serve as a reliable model for investigation. Due to this, the testing of ACS on equine tendon fibroblasts was not completed. As ACS is currently being used clinically in equine practice, it is still important that the effects of ACS be investigated. A new goal for this project is to serve as a basis for the design of a model that will allow for controlled testing of therapeutics such as ACS.

## Appendix A

Sequence information for primers and MGB probes for real time PCR analysis.

Gene of Interest	Forward Primer	Reverse Primer	Probe
MMP 1	AGCTGCTTATGAGGTTTCCCATAG	TGCCCCTTAACAGCCCAGTA	TCCGGTTTTTCAAAGGTAATA
MMP 3	TGTCGGTCACTTCACTACATTTCC	CAACGTCAGAATCAACAGCATCT	ACTTACAGGATTGTGAATTATACAC
MMP 13	AAGCCACTTTGTGCTTCCTGAT	GGATCGCATTTGTCTGGTGTT	TCTCTATGGTCCAGGAGAT
Collagen Type I	GCCAAGAAGAAGGCCAAGAAGAA	TGAGGCCGTCCTGTATGC	ACATCCCAGCAGTCACCT
Collagen Type III	CTGCTTCATCCCACTTATTCTG	ATCCGCATAGGACTGACCAAGAT	AACAGGAAGTTGCTGAAGG
COMP	GAGATCGTGCAAACAATGAACAG	GACCGTATTCACGTGGAACGT	CTGGCTGTGGGTTACA
Decorin	AAGTTGATGCAGCTAGCCTGAGA	GGCCAGAGAGCCATTGTCA	ATTTGGCTAAATTGGGACTG
18S RNA	GAGGCCCTGTAATTGGAATGAG	CGCTATTGGAGCTGGAATTACC	CAAGTCTGGTGCCAGCA