Healing in Horses
Biomechanical properties of equine digital flexor tendons

A report for the Rural Industries Research and Development Corporation


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Foreword

The aim of this project was to evaluate the biomechanical and biochemical properties of normal and healing equine superficial digital flexor tendons. In addition the effect of a novel product, equine somatotropin was assessed on normal and healing tendons.

Wastage within the horse industry is extremely high especially within the racing sector. Musculoskeletal injury has been identified as the second most common cause of wastage (Bailey et al 1996). Soft tissue injury especially tendon and ligament injury is common within all equine athletic disciplines. Superficial digital flexor tendon injury is a major cause of musculoskeletal injury affecting up to 30 % of horses in race training. Healing of SDFT injuries tends to be slow, with re-injury common (Silver et al 1983). It has been reported that only approximately 20-60 % of affected horses may successfully return to racing with up to 80 % of horses sustaining re-injury (Silver et al 1983; Bramlage 1986; Genovese et al 1996; Sawdon et al 1996; Genovese et al 1997).

Recent investigations in tendon pathology have found age and exercise related changes to occur in equine tendons (Gillis et al 1995; Patterson-Kane et al 1997a; Patterson-Kane et al 1997b; Patterson-Kane et al 1997c; Patterson-Kane et al 1997d; Patterson-Kane et al 1998). These degenerative alterations may predispose the tendon to injury. The mechanisms of age related changes in musculoskeletal tissue composition and mechanical behaviour are complex. However, the role of declining circulating concentrations of various growth factors has been incriminated (Buckwalter et al 1993; Rudman et al 1990).

Growth hormone has been reported to have positive effects on various collagenous tissues (Christensen & Flyvbjerg 1992; Christensen & Oxlund 1994; Jørgensen et al 1989; Walls et al 1995; Creis et al unpublished data). It was hypothesised that eST may modify the properties of normal and healing tendons, thereby preventing or reversing degenerative changes associated with aging and exercise and assisting in repair and rehabilitation of tendon injuries.

This report, a new addition to RIRDCs diverse range of almost 400 research publications, forms part of our Horses R&D program, which aims to assist in developing the Australian horse industry and enhance its export potential.

Most of our publications are available for viewing, downloading or purchasing online through our website:
- downloads at www.rirdc.gov.au/reports/Index.htm

Peter Core
Managing Director
Rural Industries Research and Development Corporation
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Finally, thankyou Donna for your seemingly limitless patience.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AL-DDFT</td>
<td>Accessory ligament of the deep digital flexor tendon</td>
</tr>
<tr>
<td>AL-SDFT</td>
<td>Accessory ligament of the superficial digital flexor tendon</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblastic growth factor</td>
</tr>
<tr>
<td>BAPN-F</td>
<td>Beta-aminoproprionitrile fumurate</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulfate</td>
</tr>
<tr>
<td>COMP</td>
<td>Cartilage oligomeric matrix protein</td>
</tr>
<tr>
<td>CSA</td>
<td>Cross-sectional area</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
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<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>DDFT</td>
<td>Deep digital flexor tendon</td>
</tr>
<tr>
<td>DS</td>
<td>Dermatan sulfate</td>
</tr>
<tr>
<td>DSL</td>
<td>Distal sesamoidean ligament</td>
</tr>
<tr>
<td>EDGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>E_{max}</td>
<td>Modulus of elasticity</td>
</tr>
<tr>
<td>eST</td>
<td>Equine somatotropin</td>
</tr>
<tr>
<td>G</td>
<td>Gauge</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
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<tr>
<td>GHRH</td>
<td>Growth hormone releasing hormone</td>
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<tr>
<td>gm</td>
<td>Grams</td>
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<tr>
<td>GRF</td>
<td>Ground reaction force</td>
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<tr>
<td>GPa</td>
<td>Giga Pascals</td>
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<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
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<tr>
<td>hr</td>
<td>Hours</td>
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<tr>
<td>IGF-I</td>
<td>Insulin-like growth factor-I</td>
</tr>
<tr>
<td>IGF-II</td>
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</tr>
<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>IU.ml⁻¹</td>
<td>International units per millilitre</td>
</tr>
<tr>
<td>kD</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>kg</td>
<td>Kilograms</td>
</tr>
<tr>
<td>kN</td>
<td>KiloNewtons</td>
</tr>
<tr>
<td>MAD</td>
<td>Mass average diameter</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitres</td>
</tr>
<tr>
<td>ml.min⁻¹.100gm⁻¹</td>
<td>Millilitres per minute per 100 grams</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetres</td>
</tr>
<tr>
<td>mm²</td>
<td>Millimetres squared</td>
</tr>
<tr>
<td>mm.s⁻¹</td>
<td>Millimetres per second</td>
</tr>
<tr>
<td>MPa</td>
<td>Megapascals</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Metacarpo-sesamoidean</td>
</tr>
<tr>
<td>N</td>
<td>Newtons</td>
</tr>
<tr>
<td>N.mm⁻¹</td>
<td>Newtons per millimetre</td>
</tr>
<tr>
<td>N.kg⁻¹</td>
<td>Newtons per kilogram</td>
</tr>
<tr>
<td>N.mm²⁻¹</td>
<td>Newtons per millimetre squared</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometres</td>
</tr>
<tr>
<td>No.</td>
<td>Number</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>per os</td>
<td>By mouth</td>
</tr>
<tr>
<td>pmM</td>
<td>Proximo-middle-metacarpal</td>
</tr>
<tr>
<td>PSGAG</td>
<td>Polysulfated glycosaminoglycans</td>
</tr>
<tr>
<td>q12</td>
<td>Every 12 hours</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>SDFT</td>
<td>Superficial digital flexor tendon</td>
</tr>
<tr>
<td>sem</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SL</td>
<td>Suspensory ligament</td>
</tr>
<tr>
<td>SS</td>
<td>Sesamoido-digital</td>
</tr>
<tr>
<td>Temp²</td>
<td>Temperature at the tendon grip interface</td>
</tr>
<tr>
<td>Temp³</td>
<td>Temperature within the midsubstance of the tendon</td>
</tr>
<tr>
<td>TGFβ⁻¹</td>
<td>Transforming growth factor beta-1</td>
</tr>
<tr>
<td>µ</td>
<td>Microns</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometres</td>
</tr>
<tr>
<td>µg</td>
<td>Micrograms</td>
</tr>
<tr>
<td>µg.kg⁻¹.day⁻¹</td>
<td>Micrograms per kilogram per day</td>
</tr>
<tr>
<td>VDA</td>
<td>Video dimensional analysis</td>
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Executive Summary

Three experiments were performed examining the *in vitro* biomechanical properties of the metacarpal region of forelimb equine superficial digital flexor tendons.

Experiment 1 was aimed at determining the *in vitro* biomechanical properties of forelimb SDFT from horses without evidence of clinical tendonitis. Tendons were collected from mature horses that were euthanased for reasons unrelated to tendon injury. The *in vitro* biomechanical properties of normal forelimb tendons have been previously described however, under different experimental conditions to those used here. As such it was important to establish a range of normal values and to become familiar with experimental technique. In addition these experiments were aimed at recording the mechanical properties of the metacarpal region of the tendon only. Measured biomechanical variables included maximal load, cross-sectional area, ultimate tensile stress, ultimate tensile strain, and stiffness. All tendons were tested under quasistatic linear conditions to the point of tendon failure. No statistical comparison between tendons was made due to variation in population sampling. However, a significant positive correlation was determined between mean cross-sectional area and mean maximal load ($r^2=0.57$) ($p<0.05$). Differences in the mean values of mechanical variables, compared with those reported by other investigators could be explained by differences in population selection and experimental procedure. None-the-less a reliable and repeatable method of measuring the *in vitro* biomechanical properties of forelimb SDFT was established.

Experiment 2 examined the effect of equine recombinant somatotropin (eST) on the *in vitro* biomechanical properties of normal tendons. Nine horses were used in this experiment, 6 of which received intra-muscular eST at an initial dose rate of 10 $\mu$g.kg$^{-1}$.day$^{-1}$ by intramuscular injection for one week increasing to 20 $\mu$g.kg$^{-1}$.day$^{-1}$ for an additional five weeks. The remaining 3 horses were used as controls and received no eST. No significant differences were determined between the measured biomechanical variables of control and treated tendons. These results may be interpreted to suggest that eST, as used in this model has limited effects on the biomechanical properties of normal, mature equine SDFT.

Experiment 3 examined the effect of eST administration on the *in vitro* biomechanical properties of healing tendons. A collagenase model was used to create bilateral tendonitis in 12 horses. A total of 2000 IU of collagenase was divided into three equal volumes of 0.15ml and injected at 1cm intervals into both fore limb SDFT of each horse. Six horses received eST daily for 6 weeks as in experiment 2, and 6 horses were used as controls. At the end of the treatment period all horses were euthanased. Treated tendons had significantly larger mean cross-sectional area measurements ($502 \pm 41.8 \text{ mm}^2$) compared to control tendons ($371.8 \pm 37.8 \text{ mm}^2$) ($p<0.05$). Mean ultimate tensile stress was lower in treated tendons ($12.9 \pm 1.8$ MPa) compared to controls ($20.9 \pm 3.1$ MPa) ($p=0.05$). Control tendons were biomechanically stiffer than treated tendons ($680.7 \pm 51.7 \text{ N.mm}^{-1}$ versus $477.0 \pm 67.7 \text{ N.mm}^{-1}$) ($p<0.05$). These results indicate that under these conditions eST administration to healing tendons results in a biomechanically inferior healing response.

In conclusion the biomechanical properties of normal equine superficial digital flexor tendons vary between horses and a significant positive correlation exists between tendon cross-sectional area and maximal load. Administration of eST, as used in this study had no beneficial effect on the biomechanical properties of normal, mature tendons or healing superficial digital flexor tendons.
Introduction

Evolution and domestic selection of members of the equine family has by requirement resulted in the development of an athlete capable of great speeds. As a consequence of this requirement, the equine distal limb has become streamlined with a reduction of the number of digits to one and increased re-enforcement with strong tendons and ligaments. While these developments have contributed to the athletic ability and endurance of the horse they have increased the susceptibility of the musculoskeletal system to injury.

Injury to the superficial digital flexor tendon (SDFT) is a significant cause of wastage in racing and performance horses. The incidence of tendonitis is reported to be in the range of 8-30% (Genovese 1993; Goodship 1993). Healing of SDFT injuries tends to be slow, with re-injury common (Silver et al 1983). It has been reported that only approximately 20-60% of affected horses may successfully return to racing with up to 80% of horses sustaining re-injury (Silver et al 1983; Bramlage 1986; Genovese et al 1996; Sawdon et al 1996; Genovese et al 1997).

Tendons serve primarily as force transmitters between a muscle and its bony insertion, thereby enabling muscular contraction to result in skeletal movement. In the case of the equine flexor tendons they not only act as coaptation devices but also provide support for the distal limb and contribute to the efficiency of locomotion through their elastic properties. Early investigators found the in vitro mechanical properties of equine flexor tendons to approximate a sigmoidal curve when stress (force/unit CSA) was plotted against strain (% elongation) (Evans & Barbenel 1975). The stress-strain response is initially lax with progressive stiffening as stress increases and eventual rupture occurs at extreme levels of stress.

The SDFT has been shown to be loaded preferentially, early in the stance phase (Riemersma et al 1988a; Riemersma et al 1988b), and to experience the greatest rate of rise in load (Platt et al 1991). In vitro values for ultimate tensile strain have been shown to approximate those obtained in vivo, suggesting that at maximal athletic activity the equine SDFT is operating close to its physiological limits (Riemersma et al 1985; Stephens et al 1989; Wilson & Goodship 1990; Crevier et al 1996).

Investigations into tendon ultrastructure, response to exercise, and healing have provided new information on the pathogenesis of tendonitis and potential future treatment methods. In vitro measurement of SDFT biomechanical properties has been shown to be a repeatable and reliable method of providing objective data. Its use therefore, in objectively assessing the response of tendons to various treatment regimens should not be overlooked and indeed forms the basis of this thesis.

A variety of physical, pharmacological and surgical treatments have been advocated for use in the treatment of equine tendonitis. To date there is little objective evidence that any treatment has had consistent and long lasting beneficial effects. Recent investigations into the pharmacological modulation of intrinsic healing of collagenous
structures has lead to the investigation of various growth factors as potential therapeutic aids in the healing of musculoskeletal injuries. A number of growth factors have been investigated to date many with encouraging results. Those examined include growth hormone, the insulin-like growth factors, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), epidermal growth factor (EDF) and transforming growth factor beta-1 (TGFβ-1) (Murphy et al 1994; Duffy et al 1995; Trippel et al 1996; Spindler 1996).

Age and exercise related changes have been demonstrated to occur in equine tendons. Increased SDFT stiffness, reduction in collagen fibre diameter, and alteration of collagen fibre morphology have been reported (Gillis et al 1995; Patterson-Kane et al 1997a; Patterson-Kane et al 1997b; Patterson-Kane et al 1997c; Patterson-Kane et al 1997d; Patterson-Kane et al 1998). The mechanisms of age related changes in musculoskeletal tissue composition and mechanical behaviour are diverse and complex. However, the role of declining circulating concentrations of GH and IGF-I has been incriminated (Buckwalter et al 1993; Rudman et al 1990).

Growth hormone administration has been reported to positively influence the biomechanical properties of rat colonic anastomoses, and intact rat skin (Christensen & Flyvbjerg 1992; Christensen & Oxlund 1994; Jørgensen et al 1989). Similarly, positive effects on skin flap survival and fibroplasia of surgically created wounds have been reported (Walls et al 1995; Creis et al unpublished data). The effect of insulin-like growth factor I (IGF-I) on the in vitro metabolism of normal equine SDFT has been investigated. Positive effects on collagen synthesis, synthetic rate, cellularity and cellular metabolic activity were demonstrated (Murphy & Nixon 1997).

Growth hormone is thought to exert direct and or indirect effects on target tissues, the later most likely achieved via stimulation of somatomedins (ie IGF-I). Stimulatory effects on fibroblast replication and synthesis of collagen and proteoglycans have been reported. The development of equine recombinant growth hormone (Equine somatotropin eST®, Bresagen, Adelaide, South Australia) has occurred in more recent times providing the opportunity to assess its use in a variety of physiological responses including repair of musculoskeletal injury.

There were three objectives to this study. The first was to characterise the biomechanical properties of the metacarpal region of normal equine SDFT and establish a reliable and repeatable experimental procedure. The second was to assess the effects of eST on the biomechanical behaviour of normal tendons obtained from mature horses. It was hypothesised that eST may modify the biomechanical properties of normal SDFT and therefore provide a method of preventing or reversing degenerative changes associated with maturation. Given the reported positive effects of growth hormone on collagenous tissues, it was proposed that eST might also modify tendon healing. Using a collagenase model of tendonitis, objective measurements of tendon healing were obtained using an established in vitro biomechanical testing procedure.
Methodology

Experiment 1: Biomechanical properties of normal superficial digital flexor tendons.

1.1. Horses
The forelimb superficial digital flexor tendons were harvested from 18 horses of mixed age, breed and sex that were euthanased for reasons other than tendonitis. All horses were over 2 years of age.

1.2. Tendon harvesting
Tendons were transected just proximal to the proximal limits of the manica flexora and distal to the corresponding level of the base of the accessory carpal bone to give an overall length of approximately 15cm. Subcutaneous fascia and paratendon were resected from all specimens. Pilot studies demonstrated that excess fascia and paratendon increased the chance of tendon slippage within the clamps. Additional tissue thickness may have resulted in overestimation of cross-sectional area measurements and biomechanical properties of the tendons as well as adversely affecting tendon freezing in the clamps.

1.3. Tendon storage
Harvested tendons were wrapped in saline soaked gauze swabs and stored in physiological saline at $-70^\circ$C until biomechanical testing was performed. The tendons were thawed at room temperature and any remaining fascial attachments removed. Previous investigators have demonstrated that freezing of tendons prior to testing has a small yet significant effect on the modulus of elasticity on the specimen, but no effect on the ultimate tensile stress when compared to fresh tendons (Smith et al 1996).

1.4. Cross-sectional area measurements
The cross-sectional area of the tendon specimen was determined once the tendon was loaded within the clamps immediately prior to freezing using a digital micrometer (Mitutoyo Absolute Digimatic. Mitutoyo Corp. Japan). The tendon width and thickness were measured and multiplied to provide an estimate of cross-sectional area. The results of a previous pilot study comparing tendon cross-sectional areas measured using the digital micrometer and via ultrasonography (Ausonics, 7.5 MHz sector scanner) showed that the measurements had a coefficient of variation of 5.50 % and 8.31 % respectively. These results indicated that the micrometer was a more repeatable method of determining cross-sectional area than ultrasonography. Micrometer measurements also offered a more convenient and rapid method of measuring tendon CSA.

1.5. Tendon mounting
The tendons were mounted in specially designed clamps adapted from Wilson & Goodship (1990) such that at least 5.5cm from either tendon end was gripped by the clamps leaving 4cm between the clamps as the initial length. The tendon segments were mounted exactly the same way for each test. The tendon was aligned such that the
proximal end was always gripped by the top clamp. To prevent tendon slippage within the clamps, the clamps were initially tightened so as to firmly engage the tendon and conform it to the inscribed grooves within the clamps without causing obvious damage to the tendon substance.

1.6. Tendon freezing
Liquid CO₂ cylinders (BOC Gases, AUS.) were connected to the tendon clamps and the tendon ends were frozen by conduction as CO₂ was circulated through a recessed chamber adjacent to the tendon ends within the clamp. The temperature of the tendon at the tendon clamp interface (Tempₐ) prior to testing, and within the tendon mid-substance at the completion of testing (Tempₖ) was monitored using a Physitemp Bat 10 Type T Thermocouple Thermometer and Type MT-23/3 Hypodermic Needle Microprobe (Physitemp Instruments Inc. Clifton NJ 070013 USA, purchased from SDR Clinical Technology Middle Cove NSW 2068 AUS.). The temperature range of the thermocouple was –100 to +199 °C with a resolution of 0.1°C. Mean duration of tendon freeze for all normal tendons was less than that applied to injured tendons. Collagenase injured tendons were subject to a longer freeze time due to their larger cross-sectional area. The duration of tendon freeze and tendon Tempₐ were determined as being optimal following previous pilot studies. It was found that longer periods of CO₂ application resulted in progressive freezing of the tendon between the clamps and hence alteration to the initial specimen length and concomitant effects on the biomechanical behaviour of the specimen. To help prevent excessive freezing of the tendon substance between the clamps, gauze swabs soaked in physiological saline were wrapped around the tendon. Inadequate tendon freezing resulted in slippage of the tendon within the clamps. Following freezing of the tendon the clamps were tightened to further engage the tendon ends, as frequently the clamps had loosened as the tendon substance contracted with freezing.

1.7. Biomechanical testing
Biomechanical testing was performed only on the mid-metacarpal regions of the tendons. Biomechanical testing was performed using a MTS Bionex 858 materials testing apparatus (MTS Systems, Minneapolis, Minnesota, USA), with a 25 000 N load cell. Immediately after freezing the following static testing protocol was applied to all tendon specimens. The Bionex MTS was programmed using Os/2 software to displace at an elongation rate of 10 mm.s⁻¹ until failure occurred. Data acquisition was achieved at a rate of 1000 data points.s⁻¹. Data was subsequently saved on Zip disc (Iomega Corp. USA) and imported into the computer software program, Microsoft Excel Office ‘97 (© Microsoft Corp. USA) and converted into a graphical format from which the following information could be obtained.

1.8. Biomechanical variables
Data from the MTS is expressed as time, force and elongation. Measured variables included maximal load at tendon failure (N), ultimate tensile stress (MPa), ultimate tensile strain (% elongation) and tendon stiffness (N.mm⁻¹). Maximal load was calculated using Microsoft Excel software from the data file and from the graphical format of load (N) versus displacement (mm). Ultimate tensile stress is a function of maximal load (N) divided by specimen CSA (mm²) expressed as Megapascals (MPa). Ultimate tensile
strain was determined by calculating the specimen displacement (mm) at maximal load and expressing this as a percentage of the initial length of the tendon specimen. In this case all specimens had an initial length of 40 mm. Tendon stiffness was calculated from the linear region of the load versus displacement curve. Previous investigators have used video-dimensional analysis to capture biomechanical data, which can be expressed graphically as stress versus strain. From the stress versus strain curve a direct measurement of the specimen’s Modulus of elasticity (E_{max}) can be calculated. In the present experiments the data is expressed graphically as load versus displacement and as such the direct measurement of E_{max} is difficult. For these reasons an estimate of tendon elasticity, termed tendon stiffness, was calculated.

Linear regression analysis was performed within the linear region where the curve was steepest. Care was taken to exclude the toe region or the first 3% strain, which has been reported to correspond to crimp elongation. Normal tendons and injured tendons will respond differently and therefore the same region of the linear deformation curve cannot be assessed in all tendon groups. However, wherever possible a consistent region along the linear curve was analysed.

1.9. Statistical analysis
Results from the normal tendons are expressed as descriptive statistics only. A compatible computer software program (Statistica for Windows. © Microsoft Corp. USA) was used to calculate mean ± sem, and minimum and maximum values. A Pearson correlation coefficient was calculated for maximal load versus cross-sectional area. p <0.05 was considered significant.

Experiment 2: The effect of eST on normal superficial digital flexor tendons.

2.1. Horses
Nine Standardbred horses (2 females, 7 males), with a mean weight of 503.7 ± 36.2 kg, and aged between 6-9 years were randomly divided into 2 groups. All horses had complete physical and lameness examinations (Pasquini et al 1995) and only those that were clinically normal and free from lameness were utilised in the experiment. Prior to commencing the experiment all horses were subjected to routine husbandry procedures. Approval for the experiment was obtained from the Animal Care and Ethics Committee (ACEC # N07/7 – 97/1/2595).

2.2. Treatment groups
The first group consisted of 3 horses that served as control animals and received no eST. The second group consisted of 6 horses that received eST. Group 2 horses, the treatment group, were weighed and received daily intramuscular injections of equine somatotropin (eST) (Equine somatotropin eST® Bresagen, Adelaide, South Australia). Each phial contained 10 mg of eST that was reconstituted with 4 mls of sterile water for injection prior to administration. EST was commenced at an initial dose rate of 10 \( \mu \)g.kg\(^{-1}\).day\(^{-1}\) for one week increasing to 20 \( \mu \)g.kg\(^{-1}\).day\(^{-1}\) by intramuscular injection for an additional five weeks. All injections were administered at the same time each day (4pm) using 21G, 3.5cm needles.
(Terumo Medical Corp. USA). Injections were administered into either gluteal, cervical or pectoral musculature in a rotating pattern. Both groups of horses were weighed weekly and the dose of eST adjusted accordingly.

2.3. Tendon harvesting
At the end of the six week period all horses were sedated with xylazine hydrochloride (Ilium Xylazil-100, Troy Laboratories, AUS) at 0.4 mg.kg\(^{-1}\) intravenously and subsequently euthanased via an overdose of pentobarbitone sodium (Valabarb\(^{®}\), Jurox AUS) administered intravenously. The SDFT from both forelimbs were harvested and stored as previously described.

2.4. Statistical analysis
To account for within horse variance, results were analysed using a General linear model of analysis of variance and a compatible computer software program (Minitab for Windows. © Microsoft Corp. USA). Results are expressed as mean ± sem., p ≤ 0.05 was considered significant.

Experiment 3: The effect of eST on tendon healing.

3.1. Horses
Twelve Standardbred horses (11 females, 1 male), with a mean weight of 370 ± 9.7 kg, aged between 3-7 years were used in this experiment. All horses had complete physical and lameness examinations (Pasquini et al 1995) and only those that were clinically normal and free from lameness were utilised in the experiment. Standardised ultrasonographic evaluation (Genovese et al 1986) of both forelimb SDFTs were performed to ensure no abnormalities existed prior to commencing the experiment. Prior to commencing the experiment all horses were subjected to routine husbandry procedures. Approval for the experiment was obtained from the Animal Care and Ethics Committee (ACEC # N07/7 – 97/1/2595).

3.2. Collagenase injection
All horses were sedated using detomidine hydrochloride (Dormosedan\(^{®}\), Randwick Veterinary Laboratories, AUS) at 0.01 mg.kg\(^{-1}\) intravenously and pre-medicated with phenylbutazone (Butasyl Injection, Heriot Agvet, AUS) at 4.4 mg.kg\(^{-1}\) intravenously. The palmar metacarpal region of each forelimb was clipped using number 40 clipper blades and subsequently shaved. The palmar metacarpal regions of the limbs were aseptically prepared using Povidone Iodine scrub (E-Z Scrub\(^{®}\) 205, Becton Dickinson Acute Care, USA) and 70 % alcohol using a protocol accepted at the University Veterinary Centre for achieving surgical asepsis. SDFT injury was induced using collagenase (Sigma-Aldrich, AUS) 4000 IU.ml\(^{-1}\). Histologically and morphologically, collagenase induced tendonitis has been reported to closely resemble naturally occurring tendonitis. The severity of the injury is related to the concentration and volume of collagenase injected into the tendon (Williams et al 1984). In our experiment a total of 2000 IU was injected into both forelimb SDFT of each horse. The total dose was divided into three equal volumes of 0.15ml injected at 1cm intervals beginning at 6cm distal to the accessory carpal bone. Care was
taken not to inadvertently inject into the carpal tendon sheath, the palmar-metacarpal extension of which was determined by ultrasonographic examination. Each forelimb was elevated and held flexed at the carpus. The SDFT was palpated between the thumb and forefinger and elevated from the DDFT and held relatively immobile whilst the needle was introduced into the tendon substance. The needle was consistently introduced through the medial aspect of the tendon which has a larger, more rounded surface to reduce the risk of inadvertent injection outside of the tendon substance. The injections were performed using 1ml syringes and 27G, 1.25cm needles (Terumo Medical Corp, USA). The small dose volumes were chosen based on studies using methylene blue in cadaver limbs where it was found that volumes of 0.3 ml as used by other investigators (Henninger et al 1992) resulted in excessive leakage of dye from within the paratendon into the subcutaneous space. In pilot studies conducted at our facility larger volumes of collagenase resulted in excessive subcutaneous inflammation and were associated with an increase in the degree of lameness in treated animals. We were able to consistently reproduce an adequate tendon lesion based on ultrasonographic and post mortem examination using a smaller injection volume and total dose of 2000 IU collagenase per tendon. The operator could easily assess the correct placement of the needle within the SDFT through an increased injection pressure required to inject the collagenase into the tendon substance compared to the peri-tendonous tissue.

Following collagenase injections all horses were monitored for lameness daily and received phenylbutazone (Oralject P-Butazone, Vetsearch International, AUS) at 2.2 mg.kg⁻¹ per os q12 h for 5 days. Ultrasonographic examinations were performed at 48 hr intervals until tendon lesions reached a maximum at 7 days post injection (based on pilot studies).

3.3. Treatment groups
One group consisted of 6 horses that served as control animals and received no eST. The other group consisted of 6 horses that received eST commencing 7 days post collagenase injections. Equine somatotropin was administered as previously described.

3.4. Tendon harvesting
At the conclusion of the six week treatment period all horses were euthanased and tendons harvested as previously described. In this experiment however, one tendon from each horse was used to assess biomechanical variables while the other was used to measure various biochemical properties. The tendons were chosen at random to undergo either biomechanical or biochemical testing. Biomechanical testing was performed on the same day of harvesting for practical reasons and in an attempt to eliminate potential alterations in mechanical properties associated with freezing.

3.5. Statistical analysis
Results are reported as mean ± sem., p ≤ 0.05 was considered significant. Comparison of variables was performed using unpaired Student’s t-tests and a compatible computer software program (Statistica for Windows. © Microsoft Corp. USA).
Results

Experiment 1: Biomechanical properties of normal superficial digital flexor tendons.

Introduction

The *in vitro* biomechanical properties of normal equine SDFT have been reported elsewhere (Wilson & Goodship 1990; Crevier *et al* 1996). However, due to differences in experimental design and technique direct comparisons between results are not always possible. The purpose of this experiment was to become familiar with the experimental techniques required to characterise the *in vitro* biomechanical properties of normal forelimb SDFT. In addition to this it was important to obtain reproducible results such that the techniques could be employed in objective assessment of potential therapeutic agents in the treatment and prevention of tendonitis.

Results

Normal tendons had a mean CSA of 83.9 ± 4.4 mm² (Figure 3.1), a mean maximal load of 6931 ± 292 N (Figure 3.2), mean ultimate tensile stress of 84.1 ± 2.9 MPa (Figure 3.3), a mean ultimate tensile strain of 22.2 ± 1.1 %, a mean stiffness of 861.2 ± 62.9 N.mm⁻¹. Mean duration of tendon freeze was 4.6 min, mean Tempᵃ was –10.9°C (range –4.0 to –17.3°C), mean Tempᵇ was 7.9°C (range 2.4 to 13.8°C). Mean lower displacement value for calculation of tendon stiffness was 2.9 ± 0.3 mm and the mean upper value was 6.1 ± 0.4 mm. These correspond to 32 % and 69 % of mean maximal displacement respectively. Mean maximal displacement for both groups was 8.9 ± 0.5 mm. There was a significant correlation between maximal load and CSA ($r^2=0.57$) (p<0.05) (Figure 3.4).
Figure 1.1 Histogram of normal SDFT cross-sectional area values.

Figure 1.2 Histogram of normal SDFT maximal load values.
Figure 1.3. Histogram of normal SDFT ultimate tensile stress values.

Figure 1.4. Linear regression of load versus cross-sectional area.
Discussion
The purpose of this experiment was to collect data from a diverse group of horses and to become familiar with the experimental procedures. Horses ranged in size from ponies through to heavy breeds. The mean CSA for this group was $83.9 \pm 4.4 \text{ mm}^2$, the maximum and minimum values were 51.6 mm$^2$ and 118.8 mm$^2$ respectively reflecting the wide range in tendon size. The mean maximal load was $6391 \pm 292 \text{ N}$, and the minimum and maximum values were 4447 N and 9067 N respectively. Consequently a wide range in tendon stress was recorded, with minimum and maximum values of 63.1 MPa and 111.9 MPa respectively. Measurement of ultimate tensile strain yielded maximum and minimum values of 12.8 % and 29.1 % respectively. Similarly a wide range of values for tendon stiffness were calculated ranging from 424.1 N.mm$^{-1}$ to 1481.0 N.mm$^{-1}$.

SDFT CSA has been shown to vary along the tendon length and between horses (Gillis et al 1995b). Therefore the differences observed in tendon CSA and measurements derived from CSA can be explained by between horse variability. Previous investigators have recorded mean values for maximal load of 13600 N (Wilson & Goodship 1990), and 12400 N (Crevier et al 1996), which although greater than mean values obtained in this experiment, may be explained by differences in population sampling. A significant positive correlation between maximal load and specimen CSA was demonstrated (Figure 3.4). Therefore, it would seem that the mechanical strength of a tissue is proportional to the specimen’s CSA. Previous investigators have determined that tendon CSA is inversely proportional to its collagen content, dry weight of collagen and percentage of collagen fibres (Riemersma & De Bruyn 1986). This may explain regional differences in the biomechanical properties of SDFT. In our experiment it would appear that larger tendons experienced higher maximal loads suggesting these tendons had a higher proportion of load bearing collagen fibres within the region tested.

Mean ultimate tensile strain values have been reported as 12.3 % (Riemersma et al 1985), 21 % (Wilson & Goodship 1990), and 12.5 % (Crevier et al 1996). Our mean ultimate tensile strain was $22.2 \pm 1.1 \%$ which is in agreement with that obtained by Wilson & Goodship (1990). Differences in results may be explained by experimental methods. We were only testing a segment of mid-metacarpal SDFT where as Riemersma et al (1985) used entire hindlimb SDFT while Crevier et al (1996) used entire forelimb SDFT. In previous experiments authors used video-dimensional analysis (VDA) equipment to calculate strain values. VDA enables direct measurement of the change in tendon length, in comparison we calculated strain by measuring the final specimen length as recorded from the raw data, and expressing it as a percentage of the initial length which was kept at a constant 40mm for all specimens. Crevier et al (1996) tested whole SDFT, however divided each tendon into 40mm segments in order to calculate site specific properties. Using VDA equipment the error associated with determination of segment length was reported as being 0.4 mm or 1 %. The source of such error may be related to video focusing, the resolution of the video image, conversion of image pixels to centimetres and parallax error. In our experiment the specimen length was measured using a digital micrometer with a resolution of $\pm 0.005 \text{ mm}$. Measurements of specimen length and
width, and specimen elongation were recorded to 4 decimal places. However, results for CSA are expressed to the nearest one decimal place.

All tendons were tested under the same conditions of a static load to failure. A constant elongation rate of 10 mm.s\(^{-1}\) was applied which corresponds to a strain rate of approximately 0.25s\(^{-1}\). Previous investigators have used elongation rates of 1mm.s\(^{-1}\) which are reported to correspond to strain rates of 0.02s\(^{-1}\) (Crevier et al 1996). Strain rates experienced at the gallop are estimated to be in the order of 200s\(^{-1}\), well in excess of those generated during in vitro testing (Stephens et al 1989). To approximate in vivo strain rates, we would have had to use and elongation rate of 1m.s\(^{-1}\).

Tendons are viscoelastic structures therefore their response is rate dependent. Many different strain rates have been used in previous studies making direct comparisons of tendon stiffness or E\(_{\text{max}}\) difficult. In our experiment data was expressed as load versus displacement. In comparison VDA equipment provides data in the form of stress versus strain. This allows a calculation of E\(_{\text{max}}\) to be made as the derivative of the third degree polynomial curve that best fits the stress versus strain curve (Crevier et al 1996). In comparison we were unable to make a direct calculation of E\(_{\text{max}}\) and as such an estimate of tendon stiffness was calculated by performing linear regression analysis within the linear region of the curve where the curve was steepest. Care was taken to avoid the toe region or the first 3 % strain, which has been reported to correspond to crimp elongation. None the less estimation of tendon elasticity in this manner is still relatively subjective. Freezing of specimens prior to testing has been shown to result in a small yet significant reduction in the E\(_{\text{max}}\), yet have no effect on ultimate tensile stress (Smith et al 1996). While no direct comparisons are possible with data obtained by other investigators it is likely therefore, that tendon stiffness has been underestimated in this experiment.

It has been reported that in tendons tested under experimental conditions similar to those used here, rupture occurred through mid-substance failure with characteristic central fibrillar failure (Goodship & Birch 1996). In our experiment 11 tendons failed at the tendon-clamp interface (Figure 3.5), 2 tendons failed mid-substance (Figure 3.6) and 5 failed through inter-fibrillar failure (Figure 3.7). The larger proportion of tendons failing at the tendon-clamp interface may be explained by over tightening of the clamps resulting in compressional damage to the fibres causing a weakening of the tendon which subsequently failed. In an attempt to avoid this the grips were not tightened until after freezing, however fibrillar damage may have still occurred.
Tendon freezing is an important part of the testing protocol. Inadequate freezing results in tendon slippage within the clamps (Figure 3.8), while over freezing results in excessive freezing of the tendon between the clamps therefore reducing the length of the specimen being tested. In previous investigations there was no discussion on preventing excessive freezing of the tendon specimen between the clamps. In this experiment tendon freeze was monitored with the use of a needle thermocouple, therefore the initial length of the tendon specimen being tested was maintained without compromising tendon freeze.
Optimal freezing appeared to be associated with rapid freeze time and monitoring of the temperature of the tendon at the tendon-clamp interface.

Due to clamp design, leakage of CO$_2$ was inevitable and as such excess CO$_2$ would spray onto the tendon mounted within the clamps and freeze the specimen unless it was protected through the use of saline soaked cotton gauze swabs. The saline soaked gauze swabs not only prevented excessive tendon freeze but also maintained the tendon in a moist state. Alterations in specimen temperature and specimen drying have been reported to affect the biomechanical properties of the tendon being tested (Woo 1986; Woo et al 1987). Wrapping the tendons in saline soaked gauze may have prevented specimen drying and provided some insulation from excessive cooling. Monitoring of tendon core temperature at the completion of the test demonstrated that reduction in tendon temperature occurred. Mean Temp$^b$ was 7.9$^\circ$C (range 2.4 to 13.8$^\circ$C). The effect of this fall in tendon core temperature on the biomechanical response of the tendon is unknown.

Despite the problems associated with in vitro biomechanical testing, the methodology was shown to be repeatable, and, to provide objective data on the biomechanical properties of tendons in response to various treatments.
Experiment 2: Effect of equine somatotropin on the biomechanical properties of normal adult equine superficial flexor tendons.

Introduction
The purpose of this experiment was to determine if equine somatotropin had an effect on the *in vitro* biomechanical properties of normal adult SDFT. Recent investigations have suggested that maturation of equine SDFT occurs by approximately 2-3 years of age with stabilisation of collagen fibril mass average diameter (MAD), mature cross-link formation and collagen crimp morphology (Patterson-Kane *et al* 1997a; Patterson-Kane *et al* 1997b). Similarly tendons from horses >2 years of age are stiffer with significantly higher values for $E_{\text{max}}$ (Gillis *et al* 1995). Exercise and maturation induced alteration in the non-collagenous matrix has also been observed (Smith *et al* 1997a; Smith *et al* 1997b; Smith *et al* 1998a). Histopathology changes such as hypocellularity, matrix fibrillation and chondroid metaplasia become more evident in tendons from horses $\geq$3 years of age (Pool, 1996). Progressive hypocellularity may have adverse effects on collagen maintenance and synthesis resulting in tendon weakness (Fackelman 1973). Pharmacological manipulation of tendon homeostasis through eST administration may prevent tendonitis through improvements in the biomechanical properties of normal SDFT.

Results
Control tendons had a mean CSA of 115.8 ± 12.6 mm$^2$, a mean maximal load of 7553 ± 881 N, a mean ultimate tensile stress of 65.0 ± 4.1 MPa, a mean ultimate tensile strain of 17.3 ± 1.2 %, and mean stiffness of 1075.6 ± 92.8 N.mm$^{-1}$. Tendons from eST treated horses had a mean CSA of 117.9 ± 8.8 mm$^2$ (Figure 4.1), a mean maximal load of 7589 ± 624 N (Figure 4.2), a mean ultimate tensile stress of 64.9 ± 2.9 MPa (Figure 4.3), a mean ultimate tensile strain of 17.6 ± 0.9 % (Figure 4.4), and a mean stiffness of 1016.0 ± 65.6 N.mm$^{-1}$ (Figure 4.5). Mean duration of tendon freeze for both groups was 2.2 min, mean Temp$^\circ$ was −13.4°C (range −6.0 to −26.6°C), mean Temp$^\circ$ was 5.6°C (range 1.0 to 14.0°C). Mean lower displacement value for calculation of tendon stiffness was 2.4 ± 0.2 mm and the mean upper value was 5.2 ± 0.3 mm. These correspond to 34 % and 74 % of mean maximal displacement respectively. Mean maximal displacement for both groups was 6.9 ± 0.2 mm. There were no statistically significant differences between the control and eST treated tendons.
Figure 2.1. CSA values for control SDFT and tendons from eST treated horses.

Figure 2.2. Maximal load values for control SDFT and tendons from eST treated horses.
Figure 2.3. Ultimate tensile stress values for control SDFT and tendons from eST treated horses.

Figure 2.4. Ultimate tensile strain values for control SDFT and tendons from eST treated horses.
Figure 2.5. Stiffness values for control SDFT and tendons from eST treated horses.
Discussion

Recent investigations examining equine flexor tendons suggested that the equine SDFT attain maturity at 2-3 years of age. Collagen mass average diameter (MAD), mature collagen cross links, and crimp morphology had stabilised by 2 years of age (Patterson-Kane et al 1997a; Patterson-Kane et al, 1997b). All of the horses used in our study were 6 years of age or over. The lack of detectable effect of eST on normal tendons may be related to a diminished ability of mature tendons to respond to an eST stimulus.

The effects of ageing on the musculoskeletal system of humans have been extensively investigated (Buckwalter et al 1993). The mechanisms of age related alterations in the composition and behaviour of musculoskeletal tissues are diverse and complex, however the role of declining circulating concentrations of GH and IGF-I have been incriminated (Buckwalter et al 1993; Rudman et al 1990). Intrinsic tenocyte metabolism may be limited, or, occurring at a very slow rate in mature horses. Alternatively tenocyte receptors for eST or its mediators may not exist or respond to exogenous eST. The distribution of tenocytes has been shown to vary with age, between tendons and within tendons. Webbon (1978) demonstrated a predominance of Type II cells interspersed between fibres in foetal tendon compared with increasing numbers of Type I cells in horses 2 years of age and older. Cell numbers also changed with maturity such that in older horses a relative acellularity exists within the central core region of the mid-metacarpal forelimb SDFT. In contrast the region of SDFT enclosed within the carpal and digital synovial sheaths remains relatively cellular with increasing age. It is postulated that Type II & III cells have higher metabolic activities and are primarily involved in extracellular matrix maintenance (Smith & Webbon 1996). Recent investigations examining mRNA expression of bovine tenocytes for collagen types and proteoglycans have demonstrated both age related and regional differences between tendons and provided some information on tenocyte functions (Perez-Castro & Vogel 1998). High metabolic cellular activity was demonstrated within compressional and tensional regions of foetal and neonatal DDFT, however, only the compressional region of yearling DDFT showed evidence of mRNA expression. The lack of mRNA expression within the tensional region of tendon suggests a relative metabolic inactivity, which may have some relevance on tendon response to stimuli and development of injury. For the purposes of this study we were only interested in examining the biomechanical properties of the mid-metacarpal or tensional region of the SDFT and therefore it is possible that differences may have been detected within the compressional regions given its apparent higher cellular metabolic activity.

It is also possible that the dose rates used and duration of eST administration in this study were inadequate. The recommended dose rate for eST is 10µg.kg⁻¹.day⁻¹ administered by intramuscular injection for 7 days, increasing to 20µg.kg⁻¹.day⁻¹ for up to 7 weeks (Malinowski et al 1997). A study investigating the short term clinical, haematological, and plasma biochemical effects of high doses of eST, has suggested that higher dose rates may be safely tolerated (Dart et al 1998). Investigations into the biomechanical properties of fracture healing in rabbits have indicated that dose rates of growth hormone as high as 150µg.kg⁻¹ may not have any demonstrable effect (Carpenter et al 1992).
An additional factor that could not be accounted for in this experiment was the previous exercise history of the horses in the study. Exercise has been shown to accelerate age-related changes in collagen morphology and COMP concentrations within equine flexor tendons (Patterson-Kane et al 1997a; Patterson-Kane et al 1997c; Patterson-Kane et al 1997d; Smith et al 1998a). Consequently these horses may have experienced similar exercise induced alterations in tendon physiological function such that a potentially positive effect of eST may have been insufficient to reverse these changes.

Further investigations into tendon responses to eST at different dose rates, and treatment periods may be indicated.

**Experiment 3: Effect of equine somatotropin on the biomechanical properties of healing superficial digital flexor tendons.**

**Introduction**

Somatotropin is thought to have direct and or indirect effects on fibroblast replication and synthesis of collagen and proteoglycans. Somatotropin administration has been reported to positively influence the biomechanical properties of rat colonic anastomoses, and intact rat skin (Christensen & Flyvbjerg 1992; Christensen & Oxlund 1994; Jørgensen et al 1989). Similarly, positive effects on skin flap survival and fibroplasia of surgically created wounds have been reported (Walls et al 1995; Creis et al 1998, unpublished data). Given the supporting evidence it was postulated that somatotropin administration might positively influence the biomechanical properties of healing tendons injured previously using a collagenase model.

**Results**

The control tendons had a mean CSA of 371.8 ± 37.8 mm², a mean maximal load of 7265 ± 641 N, a mean ultimate tensile stress of 20.9 ± 3.1 MPa, a mean ultimate tensile strain of 34.9 ± 4.7 %, and a mean stiffness of 680.7 ± 51.7 N.mm⁻¹. The eST treated tendons had a mean CSA of 502.9 ± 41.8 mm², a mean maximal load of 6178 ± 413 N, a mean ultimate tensile stress of 12.9 ± 1.8 MPa, a mean ultimate tensile strain of 33.4 ± 4.3 %, and a mean stiffness of 477.0 ± 67.7 N.mm⁻¹. The mean lower displacement value for calculation of tendon stiffness was 4.8 ± 0.2 mm and the mean upper value was 9.2 ± 0.5 mm. These correspond to 35 % and 67 % of mean maximal displacement respectively. Mean maximal displacement for both groups was 13.6 ± 1.9 mm.

Mean duration of tendon freeze for both groups was 6.6 min, mean Tempᵃ was −11.3°C (range −5.0 to −15.8°C), mean Tempᵇ was 3.9°C (range 1.6 to 5.5°C).

Tendons from the eST treated group had a larger CSA (502.9 ± 41.8 mm²) compared with the control group (371.8 ± 37.8 mm²) (p<0.05) (Figure 5.1). Tendons treated with eST had a lower ultimate tensile stress when compared to untreated tendons (12.9 ± 1.8 MPa versus 20.9 ± 3.1 MPa) (p=0.05) (Figure 5.2). Control tendons were biomechanically stiffer than eST treated tendons (680.7 ± 51.7 N.mm⁻¹ versus 477.0 ±
67.7 N.mm\(^{-1}\) (p<0.05) (Figure 5.3). There were no other significant differences between treatment groups.

![Figure 3.1. CSA of control SDFT and eST treated SDFT.](image)

![Figure 3.2. Ultimate tensile stress of control SDFT and eST treated SDFT.](image)
Discussion

Somatotropin is known to have a stimulatory effect on endogenous IGF-I secretion (Chen et al. 1996; Hindmarsh 1997). IGF-I supplementation has been demonstrated to enhance collagen synthesis, preferential synthesis of Type I collagen, and tenocyte proliferation in SDFT explants (Murphy & Nixon 1997). In addition intra-tendonous IGF-1 resulted in enhanced ultrasonographic properties and Type I collagen expression in experimental tendonitis (Dahlgren et al., 1998). As well as acting via intermediaries, somatotropin is thought to exert direct stimulatory effects on many collagenous structures. Somatotropin administration has been reported to positively influence the biomechanical properties of rat colonic anastomoses, and intact rat skin (Christensen & Flyvbjerg 1992; Christensen & Oxlund 1994; Jørgensen et al. 1989).

Recent investigations into the biomechanical properties of SDFT with naturally occurring tendonitis have demonstrated significant increases in CSA, and reduction in ultimate tensile stress when compared to clinically normal tendons (Crevier-Denoix et al 1997). Similar results were obtained in tendons with collagenase induced injury in the present study. Significant differences existed between the CSA (greater) and ultimate tensile stress (lower) of eST treated horses compared to controls. The increase in CSA may be due to a positive effect of eST on fibroplasia, mediated via a direct effect or more likely through increased IGF-I secretion. Equine somatotropin has been demonstrated to induce a significant increase in fibroplasia and granulation tissue production in a wound-healing model (Creis et al. 1998, unpublished data). If indeed the increased in CSA in eST treated horses was due to increased fibroplasia, then the inferior biomechanical properties of eST treated tendons may be related to the presence of an immature or disorganised
collagenous matrix. It is possible that the observed differences in tendon stiffness may be related to this such that the eST treated tendons were more elastic. In contrast, intra-tendonous IGF-I reportedly had no significant effects on SDFT elasticity, ultimate stress or CSA in a collagenase model of tendonitis. These findings occurred despite apparent increased expression for Type I collagen in IGF-I treated tendons (Dahlgren et al., 1998). These apparently contrasting results may be due to differences in experimental technique, or actions of IGF-I and eST.

An additional component of this experiment was biochemical analysis of healing tendons such the contralateral tendon was used for proteoglycan quantification, histological and immuno-histochemical analyses. As such the duration of treatment with eST and the time at which horses were euthanased was influenced by the need to collect this information. A longer treatment period may have resulted in different biomechanical results as collagen maturation progressed. Therefore in terms of assessing the biomechanical properties in response to eST treatment, we may have tested the tendons too early in the healing phase.

Early investigations into the pathogenesis of tendonitis following collagenase induced injury reported that at 2 months post injury there was still evidence of an increased cellular reaction with prominent vascularity and little evidence of fibrous organisation along tension lines (Williams et al 1984). Admittedly these investigators injected larger volumes and concentrations of collagenase than were used in this experiment, consequently the lesion would be expected to be more severe. None the less one could expect similar findings in our tendons given the horses were euthanased at 7 weeks post injury.

The biomechanical results support diminished structural properties in the eST treated healing tendons. This may be due, in part, to an immature or biomechanically inferior, disorganized collagenous matrix. Future histological and immuno-histochemical analysis will help to determine the exact effect of eST on collagen synthesis and ultrastructural organisation.
General Discussion

Measurement of the in vitro biomechanical variables of equine superficial digital flexor tendons has been demonstrated to be repeatable and reliable. Its use in evaluating the efficacy of treatment regimens for tendonitis should not be neglected. It is apparent that there is much variation in the mechanical properties of SDFT from the normal equine population, however, in general a positive correlation exists between tendon cross-sectional area and maximal load. Therefore, it would seem that the mechanical strength of a tissue is proportional to the specimen’s CSA. Previous investigators have determined that tendon CSA is inversely proportional to its collagen content, dry weight of collagen and percentage of collagen fibres (Riemersma & De Bruyn 1986). This may explain regional differences in the biomechanical properties of SDFT. From the results obtained in our experiment it would appear that larger metacarpal specimens experienced higher maximal loads suggesting that these regions contained a higher proportion of load bearing collagen fibres compared to those with lower CSA measurements. The implication of this may be that horses with larger tendons might be able to tolerate higher maximal loads prior to tendon injury. However, in addition to biomechanical forces, other factors are likely to influence this somewhat simplistic assumption. Exercise has been shown to result in an increase in tendon CSA, however this may be related to subclinical injury, and alterations in collagen fibre diameter, morphology, and non-collagenous matrix (Gillis et al 1993; Patterson-Kane et al 1997c; Patterson-Kane et al 1997d; Patterson-Kane et al 1998; Smith et al 1998a).

Riemersma and Schwardt (1985) concluded that E_max varied along the SDFT such that it was inversely proportional to cross-sectional area and proportional to collagen content. However, despite this they determined load-strain characteristics to be independent of tendon site and as such a loaded SDFT tends to strain homogeneously. Furthermore if strain is independent of tendon site, and cross-sectional area varies along the tendon, then because stress is inversely proportional to cross-sectional area the stress-strain characteristics will be site specific. As such different experimental data may arise due to inconsistent selection of the test site. Stephens et al (1989) using Hall-effect transducers in vivo proposed that the magnitude of the weight bearing strains recorded at the gallop support the idea that tendon strain is site specific and largest in the metacarpal region. By concluding that strain was not homogenous they suggested that variation in tendon morphology and composition was responsible for the strain variation. Further to this Crevier et al (1996) and Crevier-Denoix & Pourcelot (1997) examined the segmental variations of SDFT mechanical properties, and suggested non-homogenous straining occurred along the SDFT. By grouping the SDFT segments into 3 regions, a proximo-middle-metacarpal (pmM), metacarpo-sesamoidean (MS), and a sesamoido-digital (SD) region, the authors were able to conclude that the pmM region was stiffer, with a lower yield strain compared to the SD region. It was suggested that the increased stiffness and a smaller CSA of the metacarpal region might partly explain the increased incidence of injury within this region.

The lack of detectable effect of eST on the biomechanical properties of normal, mature SDFT may be related to a number of factors. The equine SDFT has been shown to reach
maturity at approximately 2 to 3 years of age. It is possible that mature tendons have a reduced capacity to respond to an eST stimulus given the cellular changes that are apparent with maturity and within the metacarpal region of SDFT (Webbon 1978). In addition previous exercise history and eST dose rate may have influenced biomechanical response of the tendons. Further investigations into tendon responses to eST at different dose regimens, in immature animals, and under controlled exercise programs may be indicated.

The mechanical properties of naturally occurring tendonitis have been evaluated (Crevier-Denoix et al 1997). These authors reported that mean maximal loads tended to be higher in the injured group (14.5 ± 3.6 kN) when compared to the normal group (12.4 ± 1.3 kN), however the differences were not significant. The investigators found significant increases in tendon CSA of injured tendons (mean 282 ± 131 mm²) compared with a group of normal tendons (mean 117 ± 16 mm²). Consequently injured tendons had lower ultimate tensile stresses (mean 63 ± 29 MPa) when compared to normal tendons (mean 107 ± 11 MPa). Injured tendons tended to have lower mean ultimate tensile strains (10.8 ± 2%) when compared to normal tendons (12.4 ± 1.7%). Interpretation of these findings lead the authors to conclude that injured tendons undergo a compensatory increase in CSA resulting in a reduced stress, lower stiffness and lower strain. The authors also noted a tendency for tendon failure to occur adjacent to the junction of normal tendon and abnormal tendon, suggesting a relative overstress and or overstrain within these regions. It was suggested that the areas of tendon adjacent to the initial injury should be monitored closely as they may be predisposed to a recurrence of tendonitis.

Similar observations were made in the healing tendons in the present study. Tendons from horses treated with eST had significantly larger mean CSA, lower mean values for ultimate tensile stress, and lower mean values for stiffness when compared with control tendons. As suggested previously the increase in CSA observed in injured tendons is thought to be a protective mechanism against repeat tendon failure. Treatment with eST may be associated with increased fibroplasia which in itself may be beneficial in the long term provided healing progresses in an ordered manor with appropriate realignment of collagen fibrils according to biomechanical forces. Within the time frame of this experiment it was apparent that the eST induced fibroblastic response resulted in inferior mechanical properties within the tendons. The reasons for this may be illuminated with subsequent histopathological and immuno-histochemical analysis of tendon specimens.

Despite the apparently negative findings of this study, the pharmacological basis behind the use of eST in healing of collagenous tissue injuries, including tendonitis is relatively sound. Further investigations may be warranted using the described techniques, however a longer treatment period prior to biomechanical testing may yield more favourable results.
References


Appendix

DNA and Proteoglycan Composition and Biosynthesis of the Mid-metacarpal and Metacarpophalangeal Regions of the Superficial and Deep Digital Flexor Tendons of Standardbred Horses.

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SIDE ABSTRACT

The proteoglycans (PGs) and DNA content and their biosynthesis were determined in cultures of explants obtained from the mid-metacarpal (MMC) and metacarpophalangeal (MCP) regions of normal superficial and deep digital flexor tendons of five standard bred horses (2 - 5 years old). It was shown that the MMC region of the superficial and deep flexor tendon contained less PGs than the MCP regions. Moreover, the biosynthesis of PGs was enhanced in the MCP explants and the size of these molecules was larger than made by cells of the MMC region. These data confirm the hypothesis that those tendon regions subjected to compressive and tensional loading synthesised more aggrecan-type PGs than regions where compressional loading is low.

keywords: Equine, proteoglycans, biosynthesis
SUMMARY

Proteoglycan (PG) levels and their respective biosynthesis were determined in explant cultures taken from the mid-metacarpal (MMC) and metacarpophalangeal (MCP) regions of normal equine superficial and deep digital flexor tendons. Five standard-bred horses (2-5 years old) were used and explants dissected from the MMC and MCP regions of the superficial and deep digital flexor tendons. The explants (3mm²) were cultured in Ham's F12 media supplemented with 1% FBS. After 24 hours, Na₂S³⁵SO₄ was added and the culture continued for a further 48 hours. The media was removed and the explants were lyophilized and weighed. The explants and media were then subject to papain digestion and the DNA and sulphated glycosaminoglycans (S-GAG) concentrations and incorporation of [³⁵S] sulphate into PGs determined by established methods. The MMC regions of the superficial and deep digital flexor tendons contained less PGs than the MCP regions and these were predominantly small. The MCP regions, not only contained higher concentrations of PGs, but these were of larger hydrodynamic size. The incorporation of [³⁵S] sulphate into PGs was also higher in explants from the MCP region. These data indicate that in those regions of equine flexor tendon, subjected to both, compression and tension, PG content and biosynthesis is higher than in regions which only transmit tensional loads. These findings are consistent with the adaptive role of tenocytes to the nature and level of the mechanical stresses imposed upon them.


INTRODUCTION

Tendons are dense bands of connective tissue involved in the transfer of active biomechanical loads from muscle to bone (1). They are composed of cells and extracellular matrix. The extracellular matrix consists of four main groups of macromolecules: collagen, proteoglycans (PGs), elastin and glycoproteins (2). These four macromolecules are present along the entire length of the tendon. However, the proportions vary depending on the location within the tissue. Studies on tendons from a number of species, including rabbits, cattle, dogs and humans, have identified two distinct regions - a tendinous region and a fibrocartilaginous region (3-11). The former tissue type is present where the tendon is subject to tension. The latter is evident where the tendon passes over bony prominences and where cells are subject to compressional forces in addition to tension.

The regions of tendon subject to tension consist of elongated tenocytes embedded within dense collagen bundles, while regions of tendon subject to tension and compression consist of more rounded cells surrounded by an irregular, loose arrangement of collagen.
(3, 4). Other differences include a higher cell density, water and PG content than regions of tendon subject to tension only (6, 8, 10).

Not only does the PG content differ, but the ratio of small to large PGs also varies between the two regions (3, 5, 8, 10, 11). The regions of tendon subject to tension only have been reported to contain low concentrations of predominantly small PGs, while regions of tendon undergoing compression and tension have higher concentrations of PGs and there is a more equal distribution of small and large PGs.

At present, horses that sustain tendon injuries have a guarded prognosis in terms of a successful athletic career. Numerous treatments are available for tendon injuries. However, none has been shown to be completely effective. The damaged region of the tendon is replaced with scar tissue. This scar tissue may have high tensile strength but it does not have the biomechanical properties of normal tendon. Consequently, regions of normal tendon, adjacent to the scarred tendon, are subject to much higher stresses. Re-injury to healed tendons therefore tends to occur at those sites adjacent to the scarred region. The ideal treatment would be one that, as well as accelerating the healing process, also results in the formation of new tissue which produces minimal disturbance in the biomechanical properties of the tendon as a whole.

Proteoglycans, although only a small component of tendon, are considered to play important roles in collagen fibrillogenesis and organization during growth, development and healing (10-13), particularly in regions of tendon subject to both compression and tension stresses (5-11). Consequently, manipulation of the types and concentrations of PGs, which are synthesized by equine tendon cells, may provide the key to understanding the healing process, as has been suggested by Robbins et al. (14) using transforming growth factor-β (TGF-β) (14). On the basis of these observations, we undertook the present study as part of a long-term program to evaluate new therapeutic modalities which may influence PG synthesis in equine tendons.
MATERIALS and METHODS

Sample population: Five Standardbred horses, aged two to five years, untrained for two to eight weeks, were used for this study. Prior to euthanasia, an ultrasound examination was performed using the standard procedure to ensure that macroscopic tendon pathology was absent in the superficial and deep digital flexor tendons. The absence of macroscopic tendon pathology was confirmed when the tendons were dissected and examined at necropsy. Any tendons with evidence of pathology were excluded from the study. The horses were euthanatised (see O.E.D.) by an intravenous overdose of pentobarbitone and their forelimbs removed just above the carpus. The limbs were immediately transferred, on ice, to the laboratory.

Preparation of explants: Blocks of tendon, 4 cm long were dissected under sterile conditions from two regions of the superficial and deep digital flexor tendons (Fig. 1). These sites were 10 and 24 centimetres distal to the accessory carpal bone, coinciding with the mid-metacarpal and metacarpophalangeal regions respectively. Each block of tendon was then cut into smaller explants (3 mm³). Eight explants from each site were cultured. Six of these explants were subsequently used in order to determine the DNA S-GAG and newly synthesized PG concentrations, using a method described previously (15). The remaining two explants were used for SDS-PAGE and Western blot analysis.

The explants were placed into individual wells of a 48 well culture plate and covered with 1.0 ml of Ham's F12 culture medium supplemented with 76 mM NaHCO₃, 20 mM HEPES, 50 g/ml gentamicin sulphate and heat deactivated 1% foetal bovine serum. The explants were cultured at 37°C in an atmosphere of 5% (v/v) CO₂ in air with 98% humidity. After 24 hours the culture media was removed and replaced with fresh media to which 20 μCi/ml of [35S] sulphate had been added. Culture was continued for a further 48 hours. The media and explants were then separated. Media samples were frozen at -20°C until analyzed. The explants were lyophilized and weighed. DNA concentration: The DNA concentrations of papain-digested (16) explants were determined in triplicate using the fluorescent bisbenzimidazole dye Hoechst 33258d as described by Kim et al. (17). Calf thymus DNA was used as a standard.

Fluorescence was measured using excitation and emission wavelengths of 350 nm and 450 nm respectively and slit widths of 10 nm and 15 nm. Sulphated glycosaminoglycan (S-GAG) concentration: The S-GAG concentrations of papain digested explants and media were determined in triplicate using the 1,9 dimethylmethylene blueed assay as described by Farndale et al. (18). The absorbances of the samples were read in a microplate reader. Bovine tracheal chondroitin sulphated was used as a standard. The concentration of S-GAGs was used as an index of the total PG content of the sample.

Incorporation of [35S] sulphate into the proteoglycans: The in vitro synthesis of PGs was determined by measuring the incorporation of [35S] sulphate into the PGs over 48 hours incubation. Following papain digestion, the method of Collier and Ghosh(16) was used to determine in triplicate the incorporation of [35S] sulphate into the PGs. Radioactivity of the samples was determined in triplicate by liquid scintillation photometry.
Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Electrophoretic separation of PGs was performed under reducing conditions as previously described by Laemmli (19). Undigested explant samples were dissociatively extracted with 4.0 M Guanidine hydrochloride (Gu HCl), as described previously (15). Aliquots of the extracted sample were mixed with SDS-PAGE sample buffer (0.0625 M Tris-HCl, 10% glycerol, 2% SDS, 0.0025% bromophenol blue, 1% _— mercaptoethanol, pH 6.8) at a ratio of 1:1 and placed in an incubator at 100°C for five minutes. Electrophoresis (electrophoresis unit and power supply) was undertaken using 4-12% polyacrylamide gradient Tris - glycine gels at 120 V and a Tris- glycine buffer system (0.025M Tris, 0.192M glycine and 0.1% w/v SDS, pH 8.3), as described previously (15).

At the completion of electrophoresis, the gels were fixed and stained with Coomassie R250 (0.002%) and Alcian blue (0.001%) as described by Little et al. (15). Dried gels were placed in a phosphor screen cassette for 14 days and an image of the distribution of [35S] sulphate-labelled PGs obtained using phosphorimager.

Western blot analysis of the small PGs: Electrophoretic transfer of PGs to nitrocellulose sheets was performed essentially as described by Towbin et al. (20). The primary antibody used in this study to detect biglycan was MAb 69830916 raised against bovine biglycan core-protein (a gift from Professor Dick Heinegard, University of Lund, Sweden), and 6-B-6, a mouse anti-decorin antibody. The secondary antibodies used were alkaline phosphatase conjugated goat anti-rabbit IgG and a goat anti-mouse IgG and IgM. The membranes were blocked and digested overnight in Tris buffered saline (0.01 M Tris acetate, 0.15M NaCl, 0.05% NaN3, pH 7.25) containing 0.05% Tween 20, 0.02% BSA and 0.025 units/ml chondroitinase in a 37°C incubator. The membranes were washed (3 x 5 minutes) in Tris buffered saline and 0.05% Tween 20 (TBST)and then incubated with primary antibody for one hour at room temperature. The primary antibodies were used at a dilution of 1:1000 in TBST. They were washed again (5 x 2 minutes) in TBST and then incubated for one hour with the secondary antibody (dilution of 1:1000 in TBST). The membranes were washed once more (5 x 2 minutes) in TBST and then placed in an alkaline phosphatase development buffer which was supplemented with nitroblue tetrazolium and 5-bromo-chloro-3-indolyl phosphate. They were then left in this solution until the colour development was complete (approximately 15 minutes), and then washed (5 x 2 minutes) in double distilled water, air-dried and photographed.

Statistical Analysis: Analysis of variance (ANOVA) was used to compare values obtained from the four tendon regions sampled. The results were considered significant when p<0.05. If a significant 'p' value was obtained, comparisons, using Fisher's analysis of least significant difference, were made to determine where the significant differences occurred. Data that were not normally distributed were analyzed using the Kruskal-Wallis test.
RESULTS

DNA concentration: Significant differences (p<0.001) were detected between the four sites (Fig. 2). The SDFt region was found to have the highest concentration of DNA per milligram of dry weight then compared with the other three sites. The DDFc region had the lowest concentration of DNA per milligram dry weight. The SDFt and DDFt differed significantly (p<0.01), as did the SDFt and the DDFc (p<0.001) and the SDFc and DDFc regions (p<0.01). Thus the tendinous mid-metacarpal regions had higher concentrations of DNA than the fibrocartilaginous metacarpophalangeal regions.

S-GAG concentration: The concentrations of S-GAGs, as determined with respect to DNA (Fig. 3A) or dry weight of tissue (Fig. 3B), differed significantly between the four sites, with the exception of the mid-metacarpal regions of the superficial and deep digital flexor tendons (Fig. 3). The SDFt and DDFt both had lower concentrations of S-GAGs than the SDFc (p<0.05) and the DDFc (p<0.05). Although the S-GAG concentration in the SDFc region was greater than for the mid-metacarpal regions, it was found to have a lower S-GAG concentration than the DDFc region (p<0.001). Incorporation of [35S] sulphate into the PGs: The incorporation of [35S] sulphate into the PGs was expressed as the number of disintegrations per minute (DPM) per microgram of DNA (Fig. 4). Non-parametric analysis of the data revealed significant differences between the sites (p<0.01). Follow-up comparisons were made in order to determine which sites were significantly different. The metacarpophalangeal regions of both tendons incorporated more [35S] sulphate into PGs than the mid- metacarpal regions.

SDS-PAGE: Electrophoretic analysis of radio-labelled PGs from samples obtained from the mid-metacarpal and metacarpophalangeal regions demonstrated differences between these two regions, in terms of the ratio of small to large PGs. The gels of both regions revealed three areas of staining: 1) within the stacking gel (large PGs), 2) high in the separating gel, and 3) lower in the separating gel. The two latter regions represented different populations of small PGs and these were subsequently identified by Western blot analysis to be decorin and biglycan. Phosphor screen autoradiography of the dried gels revealed a ratio of small to large proteoglycans of 83 : 17 in the mid-metacarpal regions of both tendons and a ratio of 52 : 48 in the metacarpophalangeal regions.

Western blot analysis of the small PGs: Decorin and biglycan, two small PGs, were identified in the tendon explants by Western blot analysis using anti-decorin and anti-biglycan antibodies, as described in Materials and Methods. As is evident (Fig. 5), both decorin and biglycan were present in all of the four tendon regions sampled. Decorin migrated further along the gel (98 kDa) than biglycan (250 kDa). The bands observed were consistent with the published molecular weights of decorin and biglycan (90 to 140 kDa and 150 to 240 kDa respectively) (15, 21). In addition, narrow bands with molecular weights of approximately 36 kDa were also noted and assigned to the free core proteins of decorin and biglycan (21).
DISCUSSION

Studies of tendon from a number of species, including: rabbits (3, 4), cattle (5-7), dogs (8), humans (9) and horses (22), have demonstrated that the composition varies between regions that are subject to different mechanical stresses. In the horse, the proximal region of the tendon, between the carpus and fetlock, is subject to tension. By comparison, the distal region of the tendon, as it passes over the sesamoid bones of the metacarpophalangeal joint, is subject to both compression and tension (23).

In the study reported herein, the specimens were obtained from the flexor tendons in the mid-metacarpal and the metacarpophalangeal regions, areas subject to tension, and compression and tension, respectively. The concentration of PGs, as assessed from the S-GAG content, and the ratio of small to large PGs, determined by SDS-PAGE and phosphor screen radiography, at these two sites were shown to differ significantly. In addition, the synthesis of sulphated PGs in the high compression region, DDFc, was significantly higher than in regions of pure tension (SDFt and DDFt). These findings are similar to those reported for rabbit (3), bovine (5-7) and human tendons (9).

To date the function of the various PGs in tendon have not been conclusively determined. However, it has been suggested that the distribution and types of PGs present reflect their structural interactions with other macromolecules particularly collagen, the major fibrous protein of tendon. The small dermatan sulphate containing PG, decorin, is considered to play a role in regulating collagen fibrillogenesis and organization in the matrix (12-14, 24).

The predominant force acting on tendon is tension (1, 23). To achieve the maximum tensile strength possible, and thus to protect the tendon from rupture, the collagen fibres are arranged longitudinally in the direction of the strain (1, 2, 8, 10). This arrangement produces a strong tendon that is capable of withstanding the tensional forces to which the tendon is subject, not only during normal activity but, more importantly, during athletic endeavours when the forces transmitted through the tendons are high. Interruptions in this highly organized collagen framework will predispose the tendon to injury. As the assembly and organization of collagen is regulated in part by decorin, this PG plays an important role in the maintenance of the tensile strength in regions of tendon subject to tension.

It is well established that the large PGs, entrapped within the collagen network, provide resistance to compressional loading (5, 8, 10, 11, 15). The carboxylate and sulphate groups of the glycosaminoglycan chains projecting from the protein cores of the large PGs are negatively charged. The high density of fixed negative charge is maintained by the relatively inextensible collagen matrix which restricts the PG domain to 20% of that attained in solution (15). Therefore, the PGs exert a swelling pressure on the collagen matrix and provide osmotic resistance to compressive loading. The localization of these PGs in the metacarpophalangeal region of the equine tendon, which is required to distribute compressional stress, is consistent with the hypothesized biomechanical role of these PGs as well as with previous studies using tendons of other species (3, 5, 8, 10,
Gillard et al. (4) investigated the influence of mechanical forces on the glycosaminoglycan content of rabbit deep digital flexor tendons. They reported that removal of pressure, from the region of tendon normally subject to compression and tension, resulted in a loss of greater than 60% of the glycosaminoglycans within eight days. The chondroitin sulphate concentration decreased the most, such that dermatan sulphate became the predominant glycosaminoglycan. When pressure was restored, the composition of the tendon returned to normal.

Translocation of the tendon in the region subject to tensional forces only, resulted in an increase in the glycosaminoglycan content, mainly due to an increase in dermatan sulphate. They concluded that the tenocytes of tendon were able to respond to their new mechanical environment and adapt such that the matrix synthesised was capable of accommodating the new functional demands placed upon it. As well as differences in the PG concentration between regions of tendon subject to different mechanical forces, the rate of synthesis of PGs also varies (3,4, 6, 8, 24,25). Gillard et al. (8) suggested that there was a relationship between the tensile forces acting on the collagen fibres and the availability of fixed positive charges on the surface of collagen fibres. Their hypothesis was that the differences in charge distribution on the collagen molecule was part of a homeostatic feedback mechanism that controlled the type and amount of PG synthesized by adjacent cells (26).

Okuda et al. (8) reported that the cell densities of the fibrocartilaginous regions of the canine flexor tendon was twofold higher than purely tendinous regions. However, the results obtained in the present study indicated that the tendinous regions had slightly higher DNA concentrations than the fibrocartilaginous regions. The reason for this discrepancy is presently unknown but could be due to species difference. However, because the cell density in the metacarpophalangeal region of the equine tendon was low, while the concentration of PGs and their rate of PG synthesis was high, it is clear that the metabolic activity of cells in this region was also high. In contrast, the PG concentration and the rate of PG synthesis in the mid-metacarpal region was low, despite the high cell density and on similar reasoning would suggest that the metabolic activity of cells in this region was much lower.
REFERENCES


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LEGENDS TO FIGURES

Figure 1 - Schematic representation of the equine superficial and deep digital flexor tendons showing the regions sampled for biochemical studies. Black circles represent 3mm³ explants. Explants were obtained from the mid-metacarpal and metacarpophalangeal regions of the superficial and deep digital flexor tendons, areas subject to tension and tension and compression respectively.

Figure 2 - The DNA content (Mean ± SEM) of explants (n = 3 per region) from the left and right mid-metacarpal and metacarpophalangeal regions of the superficial and deep digital flexor tendons of five horses. Bars that have different superscripts are significantly different from each other (p<0.01).

Figure 3A - The sulphated glycosaminoglycan (S-GAG) content per microgram of DNA (Mean ± SEM) of explants (n = 3 per region) from the left and right mid-metacarpal and metacarpophalangeal regions of the superficial and deep digital flexor tendons of five horses. Bars that have different superscripts are significantly different (p<0.05).

Figure 3B - The S-GAG content per unit dry weight (Mean ± SEM) of the same explants, as examined in 3A. Bars that have different superscript are significantly different from each other (p<0.01).

Figure 4 - Incorporation of [35S] sulphate into proteoglycans (Mean ± SEM) into explants (n = 3 per region) from the left and right mid-metacarpal and metacarpophalangeal regions of the superficial and deep digital flexor tendons of five horses. Bars that have different superscripts are significantly different from each other (p<0.01).

Figure 5 - Western blot analysis of the small PGs of equine tendon. Decorin and biglycan were identified as the two major populations of small PGs in equine tendon. Decorin migrated further down the gel than biglycan as would be expected due to their differences in molecular weights (90 to 140 kDa and 150 to 240 kDa respectively). Both decorin and biglycan were present in all regions sampled. The core proteins of decorin and biglycan exhibited molecular weights of approximately 36 kDa (see text for details).

SonoAce 3200, Medison Co. Ltd., Korea; Excelray Aust. Pty. Ltd. Trace BioSciences, Australia. ICN, Australia Sigma, Australia Perkin Elmer LS 50 Luminescence Spectrometer, England Molecular Devices, USA Canberra Packard (Australia) Model 1500 Scintillation Analyzer Novex, Sydney, Australia Molecular Dynamics, Sydney, Australia Seikagaku Co., Tokyo Japan BioRad, Sydney, Australia Kirkegaard and Perry Laboratories, USA.