From collagen to tenocyte

How
the equine superficial digital flexor tendon
responds to
physiologic challenges and physical therapy

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How
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Van collageen tot tenocyt
hoe reageert de oppervlakkige buig pees van het paard op
fysiologische belasting en stimulatie middels fysische apparatuur?

(met een samenvatting in het Nederlands)

Proefschrift

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獻給雙親

aan mijn ouders

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Chapter I

General Introduction

The real Achilles tendon of the horse

When Achilles' mother Thetis made her son invulnerable by submerging him in the Styx, the river separating the living world from the underworld, she held the newborn baby by the tendon bundle running from the gastronomies and soleus muscles to the calcaneus. As a consequence, this was the only site of the boy's body that did not come into contact with the magic and protecting waters of the river and it would be the tragic and inevitable fate of the great hero of the Trojan war that he would eventually be killed by an arrow hitting him at that specific spot (Homer approx. 800 BC).

From this famous story in Homer's Iliad stems the name Achilles tendon for the tendon bundles and the metaphoric use of Achilles tendon or Achilles heel for any vulnerable site. When looking at the anatomy, composition and function as an elastic, energy-saving structure, there are striking similarities between the human Achilles tendon and the equine superficial digital flexor tendon (SDFT) (Batson et al., 2003; Birch et al., 2001). However, more striking even is the similarity in a metaphoric sense. In fact, the equine SDFT can be considered as the most vulnerable structure of the equine body and there is no doubt that whoever submerged the horse in the protecting waters of the Styx, he or she did it by holding the SDFT and not the anatomical homologue of the human Achilles tendon.

In a recent study on injuries and fatalities on British racecourses 82% of clinical incidences were found to be due to musculoskeletal problems and 46% of forelimb injuries involved the flexor tendons or suspensory ligaments (Williams et al., 2001). In an earlier study Rossdale *et al.* (1985) reported that 10% of all diagnosed lameness cases were caused by tendon injuries, but the incidence of underlying, often sub-clinical problems may be much higher. Apart from the high incidence, the problem is also important because of the prolonged recovery time and the considerable risk of recurrent injury (Goodship, 1993). In Thoroughbreds 20-60% of affected horse returned to racing successfully, but more than 80% of these sustain some form of re-injury (Sawdon et al., 1996; Silver et al., 1983). For the Warmblood horse no epidemiological data are available, but clinical data suggest that the re-injury rate is exceptionally high in these breeds too, especially in dressage horses and show jumpers performing at top level.

Notwithstanding many recent studies, our understanding of some aspects of basic tendon biology is still limited and there is no doubt that the management of tendon injuries poses a major challenge to the equine clinician. In recognition of the fact that both basic and applied research are badly needed to improve our understanding and management of tendon injuries, this thesis focuses on both these aspects of the problem. The first part of the thesis goes back to the basics and studies the biochemical composition and developments therein of the SDFT in young, growing horses and in mature horses. The first category of animals is of importance as there is growing awareness that not only the bony components of the musculoskeletal system are molded by the mechanical loads exerted on them (Wolff, 1892), but also the soft tissues such as cartilage and, presumably, tendon (Helminen et al., 2000; Van Weeren et al., 2000; Brama et al., 2002). On a more practical and applied track, the second part of the thesis looks at non-invasive physical therapies as possible ways to enhance tendon metabolism. Non-invasive physical therapies, although attractive from a practical viewpoint as an owner or groom can apply some of them safely during the

long recovery periods of tendon injuries, have received little attention thus far until the recent introduction of extracorporeal shock wave therapy (ESWT).

Tendon structure and function

Gross anatomy of the equine SDFT

The superficial flexor muscle originates from the medial epicondyle of the humorous and then runs distally along the palmar side of the radius (fig. 1). The transition from muscle to tendon is just proximal to the carpus. From that point, the SDFT continues in distal direction along the metacarpus, palmar to the deep digital flexor tendon (DDFT). After crossing the sesamoidean area at the palmar side of the fetlock, the SDFT splits and forms a tunnel through which the DDFT passes to follow its way down to the solar surface of the third phalanx. The SDFT itself inserts more proximally, mainly on the medial and lateral tubercles of the second phalanx, but a minor branch attaches to the latero-distal aspect of the first phalanx (Ellenberger and Baum, 1937).

SDFT: superficial digital flexor tendon
DDFT: deep digital flexor tendon
CDET: common digital extensor tendon
SL: suspensory ligament
AL-DDFT: accessory ligament of the deep digital flexor tendon

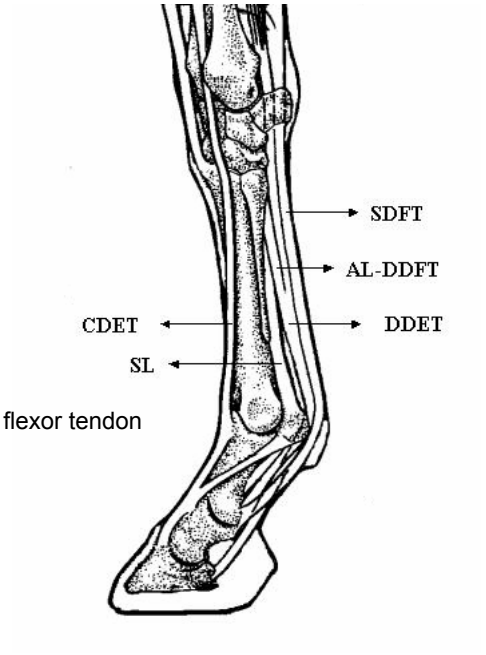

Figure 1: Schematic drawing of the medial aspect of the equine forelimb
(modified from Adams' Lameness in Horses)

Macroscopic, microscopic and molecular structure of the SDFT

The equine SDFT is a longitudinal structure that is composed of similarly oriented and to a large extent comparable substructures at macroscopic, microscopic and molecular levels. Although the terminology used is not always consistent, the configuration itself has been well established (Kastelic et al., 1978).

The entire tendon is surrounded by the *paratenon*, a loose areolar connective tissue that allows the movement of the tendons in relation to the surrounding tissues. The tendon itself is encapsulated in a fine connective tissue sheath called *epitenon* that contains the vascular, lymphatic and nerve supplies. This layer in fact penetrates the tendon, forming the *endotenon* that forms the separation between the tendon fascicles and subfascicular units, also called tertiary, secondary and primary tendon bundles (fig. 2). The subfascicular units consist of fibres, fibrils and microfibrils that are composed of the tropocollagen molecules. This essentially modular composition of tendons ensures that minor damage does not necessarily spread to the entire tendon and also provides a high total structural strength.

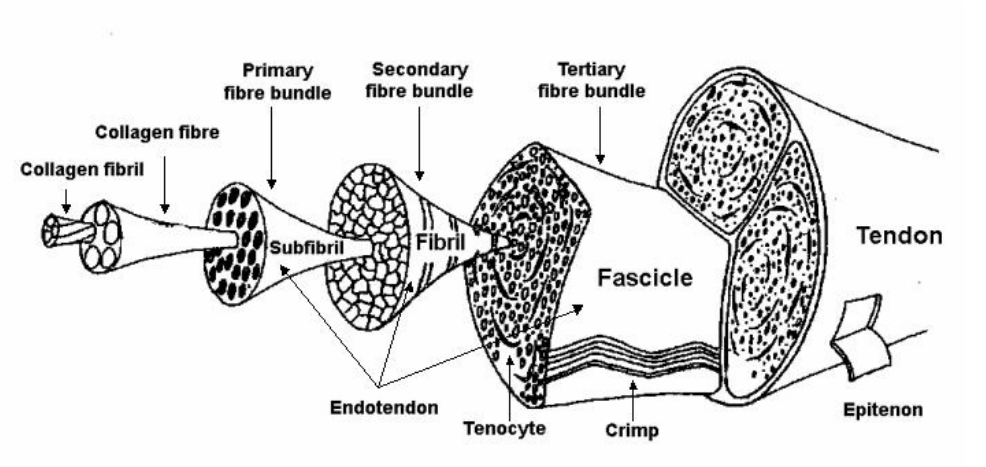

Figure 2. Schematic diagram representing the architectural hierarchy of the tendon (modified from Kastelic et al, 1978)

Biochemical composition of tendons

The basic components of the extracellular matrix (ECM) of tendons are collagen and a proteoglycan-rich viscous substance interspersed between the large collagen bundles.

-Collagen

Of the adult tendon dry weight, 60~85% is collagen with collagen type I as the predominant type (~60%). Further, there are small quantities of collagens II, III, IV, XII and XIV (Felisbino and Carvalho, 1999; Kannus, 2000). High levels of type III collagen are found in immature tendon (Birk and Mayne, 1997) and injured tendon (Maffulli et al., 2000). The physical properties of tendon collagen are largely dependent on intra- and intermolecular cross-linking (Parry, 1988), which is essential for the stability of collagen fibrils (Tsuzaki et al., 1993). Collagen biosynthesis involves several unique post-translational modifications. After translation of the procollagen, specific proline and lysine residues are hydroxylated by prolyl and lysyl hydroxylase, respectively. After fibril formation, intermolecular pyridinoline cross-links are formed: hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) cross-links. Hydroxyproline is needed for the formation and stabilization of the triple helix by hydrogen bonds with proline residues. Therefore HP is thought to be largely responsible for the increase in the mechanical strength of tendons as they mature. In the immature tendon, there are highly reducible crosslinks, such as hydroxylysinonorleucine and dihydroxylysinonorleucine. During the process of maturation these reducible crosslinks decrease rapidly to a negligible level (Patterson-Kane et al., 1997b). A second mechanism of intermolecular cross-linking of collagen is via a non-enzymatic reaction with glucose, also called non-enzymatic glycation (NEG). A well-identified NEG-product is pentosidine, which is derived from lysine, arginine and ribose moieties. Pentosidine has been used to assess the remodeling rate of the collagen network because of its slow turnover and the linear, time-related increase in tissue (Bank et al., 1999).

-Non-collagenous constituants

The major non-collagenous constituents of tendons are water (that accounts for up to 70% of wet weight) and proteoglycans (PGs, approximately 1% of dry weight) (Robbins and Vogel, 1994). Proteoglycans are composed of a protein core to which one or more glycosaminoglycans (GAGs) are covalently bound. Basically, these are a variety of highly anionic polysulphated glycosaminoglycans (PSGAGs) and the non-sulphated glycosaminoglycan hyaluronic acid (HA). The high negative charge has a marked effect on the physical-chemical properties of these molecules. PGs form bridges and bonds between the collagen fibrils in longitudinal as well as transverse directions (Cribb and Scott, 1995). PGs are known to influence fibrillogenesis and

matrix architecture (Iozzo, 1998), thus altering ultimate tensile strength (Garg et al., 1989). Tendon regions subjected largely to tensional forces contain mainly small proteoglycans, such as decorin and lumican, whereas segments of tendons that are subjected to compression contain larger proteoglycans, such as aggrecan and biglycan (Vogel and Koob, 1989). The larger proteoglycans are space-filling molecules with a pressure resistant function, and can therefore resist greater compressive forces. Not only collagen, but also PGs and water influence mechanical behavior of tendons with dehydration negatively influencing biomechanical properties (Thornton et al., 2001).

Other prominent non-collagenous components of tendons include Cartilage Oligomeric Matrix Protein (COMP) and elastin. COMP is a glycoprotein that was first described in cartilage, but is abundantly present in tendon as well (Smith et al., 1997). Its function is still not fully understood, but it is highly expressed in tissues that have to withstand high compressive forces, such as articular cartilage and certain areas of tendons, and may be involved in fibril formation (Smith et al., 2002). Elastin fibres feature a central core of this protein, with loosely arranged microfibrils surrounding it (Parry et al., 1978b). It plays a role in the elasticity of the tendon and an age-related decrease has been described in Achilles tendons (Kannus and Jozsa, 1991), but the exact mechanism has not been elucidated.

-*Cellular components*

Within the extracellular matrix network, tenoblasts and tenocytes constitute about 90~95% of the cellular elements of tendons. Tenoblasts are immature tendon cells. They are spindle-shaped and have numerous cytoplasmic organelles, reflecting their high metabolic activity. As they mature, tenoblasts become elongated and transform into tenocytes. Tenocytes have a lower nucleus-to-cytoplasm ratio than tenoblasts, with decreased metabolic activity. The remaining 5~10% of the cellular elements of tendons consists of chondrocytes at the insertion sites and synovial cells of the tendon sheath. Also, in some areas that are subjected to strong compression forces, such as the sesamoidean area of the SDFT fibrocartilage may develop in which chondrocytes can be found (Crevier-Denoix et al., 1998). Cells within the tendon are responsible for the production, deposition, organization and maintenance of the surrounding extracellular matrix (Birch et al., 1997a). When an injury occurs, tendon cells are stimulated to divide by activated endogenous growth factors (Banes et al., 1995) and play an important role in initiating regenerative responses following injury or degeneration.

The biomechanical function of tendons

Tendons connect muscle to bone. They are passive structures that, unlike muscles, do not actively produce motion. During muscular activity the force generated by the muscle is transmitted to the tendon and then to the bone, thereby initiating movement. Energy-storing tendons, to which category the SDFT belongs, not only transmit muscle-generated tensile forces to bone in order to move joints, or sustain compressive forces when wrapped around bone, but they additionally act like springs to store elastic energy, which vastly enhances the efficiency of locomotion (Alexander, 1984). Flexor tendons flex the digit during the swing phase, while during the stance phase they are loaded under tensile stress with loads up to two times body weight (Schryver et al., 1978). In the second half of the stance phase, the tendon recoils and releases the stored energy (Goodship and Birch, 1996), thus propelling the body forward. Therefore, retaining of mechanical properties such as stiffness and strength is essential for tendons to maintain their functionality.

Developmental aspects of tendons

The foal is a precocious animal. A healthy foal is on its feet within 55 minutes after birth (Koterba, 1990) and will be capable of following the herd a few hours later. This means that in the horse at birth the structures of the musculoskeletal system, that have been unloaded thus far or were only marginally loaded through intra-uterine movements of the foetus, become suddenly and fully loaded. In articular cartilage it has been shown that this sudden transition profoundly affects tissue composition. The originally homogeneous biochemical composition of the ECM of articular cartilage changes to a heterogeneous composition in which the topographical variations match the topographical variations in biomechanical loading (Brama et al., 2000b; 2001). Although some work has been done in tendons, especially with respect to collagen fibril diameter analysis, studies into the changes related to maturation have been limited so far.

At birth the equine SDFT is highly cellular with a so-called unimodal distribution of small collagen fibrils and a high proportion of type III collagen (Birk and Mayne, 1997; Parry et al., 1978b). With maturation, fibril distribution changes into a bimodal one (Parry et al., 1978a) and the collagen type changes to a clear predominance of type I. Also, biomechanical characteristics of the SDFT change. There is an increase in elastic modulus (Gillis et al., 1995) a decrease in the waveform that can be seen as alternating dark and light bands under polarized light and is commonly referred to as “crimp” (Patterson-Kane et al., 1997a), an increase in collagen fibril diameter (Patterson-Kane et al., 1997c) and replacement of immature cross-links by mature ones (Patterson-Kane et al., 1997b), resulting in an overall increase of tendon stiffness and strength.

Most of these studies have focused on changes in yearlings and young-adult horses, but recently the insight has gained ground that dramatic changes may occur directly after birth (Batson et al., 2001). For instance, the SDFT and the common digital extensor tendon (CDET) have been shown to have a similar matrix composition and mechanical properties in the non-weight bearing foal, but diverge rapidly (within one week) after birth to develop into functionally, biochemically and biomechanically different tendons (Batson, 2002). Knowledge of the processes of change in tendon ECM composition during the juvenile period and understanding of the mechanisms that incite these would be of great help in the management and the prevention of tendon lesions.

Tendinopathy

In energy storing tendons such as the SDFT, in vivo strains during strenuous exercise and ultimate failure lay very close. In an in vitro study Riemersma and Schamhardt (1985) determined the failure strain of the SDFT at 10.9-12.8%, whereas in vivo strains of 9% and more during galloping exercise have been measured (Dimery et al., 1986; Herrick et al., 1978; Stephens et al., 1989). These figures indicate clearly the very narrow safety margin of the SDFT during peak physiological activity. For this reason, simple overloading lies most at hand as the main cause for tendon injuries, but it seems that serial minor overloads with ensuing degeneration are more frequent causes of tendinopathy than single major events (Kannus and Jozsa, 1991). In the horse, localized degenerative changes and damage to the core region of the mid-metacarpal region have been demonstrated already in the late 1960s (Stromberg and Tufvesson, 1969). At a molecular level, there are changes in collagen fibres (disarrangement, increase in number and decrease in mean diameter), and an alteration in the GAG component of injured SDFTs (Kobayashi et al., 1999) and it is clear that there are both degenerative and inflammatory changes. Apart from the changes in the ECM, there is increasing interest for the role of the tenocytes in tendinopathy. Exercise-induced hyperthermia has been suggested as a possible cause of degeneration in the equine SDFT (Wilson and Goodship, 1994). However, in a later in vitro experiment it was shown that the core temperatures of 45ºC that had been measured did not induce cell death after one hour of exposure (Birch et al., 1997b). It has recently been shown that high strain mechanical loading rapidly induces tendon apoptosis (Scott et al., 2005), probably through the activation of intracellular stress activated protein kinases (Arnoczky et al., 2002). In inflamed equine SDFTs apoptotic tenocytes and a significant increase in activated caspase-3 protein, an apoptotic mediator, have been demonstrated (Hosaka et al., 2005). An increase in apoptosis rate

can be presumed to affect collagen synthesis and repair (Yuan et al., 2002; Hosaka et al., 2005), and hence result in a weakened collagen matrix.

The scar tissue that is induced (Hildebrand and Frank, 1998) is of inferior quality compared to the original tissue due to alterations in biochemical composition and structural organization, compromising the functional restoration of the pre-injured structure (Watkins, 1999). This leads to high re-injury rates when athletic activity is resumed (Williams et al., 2001).

Micro-current Electrical Tissue Stimulation (METS) and Extracorporeal Shock Wave Therapy (ESWT) as potential treatments for tendinopathies

It has been tried in many ways to improve tendon healing and to minimize the formation of scar tissue. Treatments that have been used (and are, to a varying extent, still in use today) range from the age-old practice of firing (Silver et al., 1983) and invasive therapies like tendon splitting (Henninger et al., 1992) to the intra-lesional application of drugs such as hyaluronic acid (Foland et al., 1992), polysulphated glycosaminoglycans (Dow et al., 1996), beta-aminopropionitrile fumarate (Reef et al., 1996), and the more advanced growth factor insulin-like growth factor-I (IGF-I) (Dahlgren et al., 2002), to stem cell therapy (Smith et al., 2003).

Treatment modalities that have received relatively little attention until recently, when ESWT was introduced in equine medicine are non-invasive physical treatments that, at least theoretically, have great potential. In human sports medicine, the use of physical therapy devices in sports medicine is widespread; in the horse work in this area is sparse, although some studies have been performed in the fields of ultrasound therapy (Morcos and Aswad, 1978; Keg, 1989), magnetic field therapy (Watkins et al., 1985), and laser therapy (Kaneps et al., 1984).

Micro-current Electrical Tissue Stimulation (METS)

Electric currents have been used in physical therapy and medicine since more than a century (Stainbrook E, 1948). The introduction of micro-current electrical therapy is of a more recent date. Micro-current is a physical therapy modality that is a form of transcutaneous electrical nerve stimulation (TENS) with the output in the micro-ampere (μA) range, *i.e.* at a level similar to the currents generated during physiologic processes such as the depolarization of cell membranes (Hodgkin and Huxley, 1952). A traditional TENS device will produce currents that are 1000 times stronger. Electrical therapy by METS has been reported to alleviate pain, probably by altered levels of β-endorphin (Gabis et al., 2003), and speed up wound healing by increasing cell proliferation (Goldman and Pollack, 1996).

Applications of electric currents in tendons have been limited so far, but there are some promising preliminary results. Achilles tendons in rat treated with anodal current withstood significantly greater loads (Owoeye et al., 1987). Further, direct current applied in low amperage to rabbit tendons *in vitro* suppressed adhesion-causing synovial proliferation in the epitenon and promoted active collagen synthesis by the tenocytes (Fujita et al., 1992). METS might thus have a beneficial effect on tendon healing, but there is no doubt that basic research into the effects of these micro-currents on equine tenocytes is needed before clinical trials could be undertaken.

Extracorporeal Shock Wave Therapy (ESWT)

Shock wave therapy originally was introduced in human medicine as lithotripsy to break up and disrupt calcified deposits within the body, more specifically stones within the renal, biliary, and salivary gland tracts. Since the early 1980s clinical application of shock wave therapy has received attention for various soft-tissue pathologies as well, including lateral humeral epicondylitis, Achilles tendonitis, rotator cuff calcifying tendinopathy, and plantar fascitis (Furia, 2005; Gerdesmeyer et al., 2003; Rompe et al., 1997). Outcomes have been favorable in general, but the exact mechanism that induces accelerated soft tissue healing has remained unclear.

Shock waves generate high positive pressures, up to 100 MPa (500 bar) within less than 10 nanoseconds, followed with a negative pressure of 5~10 MPa, thus causing a cavitation effect. Biological effects occur principally at sites where there is a change in impedance (Sturtevant, 1996), such as the bone-soft tissue interface. The mechanism is thought to act via stimulation of new bone and tissue formation, following the damage provoked by the cavitation effect (Suhr et al., 1991; Delius et al., 1990). Shock waves are also used to relieve pain, by so-called hyper-stimulation analgesia, which elevates the pain threshold. Recent studies in dogs have demonstrated that shock waves induce revascularization at the tendon-bone junction that, in turn, relieves pain and improves tissue regeneration and repair (Wang et al., 2002). Research in equine palmar digital nerves demonstrated post-treatment analgesia that may potentially impair perception of peripheral pain (Bolt et al., 2004).This analgesic effect is likely independent of any other potential beneficial effects on tissue healing and is observed rapidly after treatment.

Shock waves have been used in horses for the treatment of various musculoskeletal disorders, such as proximal suspensory desmitis (Caminoto et al., 2005; McClure et al., 2004), navicular syndrome (Revenaugh, 2001), and tendon disorders (Kreling, 2001). Clinical outcome seems to be favorable in most cases, but there is still very little known about the basic mechanisms behind it.

Scope of the thesis

When, after a siege of more than 10 years, the Greek had still not taken the city of Troy, they had apparently become so fatigued that they gave up and sailed off. Doing this, they left a strange construction, a large wooden horse, on their now deserted campsite. The Trojans were overcome with joy, dragged the horse inside the city and celebrated the end of the siege dancing and drinking around it. When the night fell and the city was asleep, the strange structure opened its belly to let a group of elite Greek warriors out, who quickly occupied the defenseless city and opened the gates for their fellow countrymen, who had landed again after their fake departure (Homer, approx. 800 BC).

The metaphor of the Trojan horse was born and has stayed alive during the past 3 millennia: do not trust appearances as long as you do not know what is really going on inside.

This thesis tries to focus on what is going on inside the tendon and not to lend credibility to new but unproved therapies, whether they are en vogue or trendy, or not. To achieve this in a more general sense, fundamental knowledge on the normal composition and development of tendons is necessary. If specific therapies have to be evaluated, the effect of these on the metabolism and proliferation of the tenocytes, as the only cellular component of the tendon that influences composition and hence functionality, should be studied.

The basics

Different areas of the superficial digital tendon are subjected to different biomechanical challenges, which, given the line of thought outlined above, can be presumed to influence biochemical characteristics. Most age-related studies of the SDFT so far had focused on histological and biomechanical aspects, and the knowledge with respect to the biochemical composition was far less than with respect to articular cartilage. The first step to be taken was thus filling this gap in knowledge. In analogy to earlier work on articular cartilage (Brama, 1999), differently loaded sites of the SDFT were to be examined for their biochemical make-up with respect to the major constituents of the ECM (collagen with its most important post-translational modifications, proteoglycans, hyaluronic acid, DNA). This was done in an age series that started with neonatal foals and continued until 36 months to identify changes related to development (Chapter II), and in a series of samples from mature horses (4-23 years), to evaluate the relationship of site (with its specific loading characteristics) and biochemical composition (Chapter III). As sites of interest the mid-metacarpal region and the sesamoidean region were taken, as sites that are challenged by merely tensile loads and combined tensile and compressive loads respectively.

New therapies under scrutiny

An increase in fundamental knowledge, as pursued in Chapters II and III, is essential for any area in science to progress, but does not give a direct answer to the everyday challenges of the equine practitioner who faces with the unrewarding task of treating tendon lesions in the horse. New therapies do, but should be put to the test critically. The second half of this thesis, Chapters IV to VI, focus on the critical evaluation of two of those therapies by trying to assess some basic effects of them, or by analyzing fundamental problems associated with their application.

Micro-current Electrical Tissue Stimulation (METS) has recently been applied with clinical success in different areas and has been advocated to have a positive effect on tissue regeneration as well. To assess the validity of this claim, the effect of METS on the metabolic activity in equine tenocytes cultured from explants of equine SDFTs was studied as a first step in the assessment of METS for possible use in equine tendon repair (Chapter IV). Given the fact that the specific resistance or bio-impedance of different tissues in the body is not identical and that bio-impedance can be expected to change according to fibre direction, the second step was the determination of the effect of electrode placement on actual field strength in the target tissue (Chapter V).

Another potentially attractive treatment modality used in equine musculoskeletal disorders is extracorporeal shock wave therapy (ESWT). Initial clinical investigations show promising results. However, very little is known about the effects of this relatively new therapy at tissue level. Chapter VI evaluates in a preliminary study whether the acute (3 hours) and long-term (6 weeks) effects of *in vivo* ESWT application on the metabolism of tenocytes can be demonstrated using *ex vivo* cultured explants harvested immediately and 6 weeks after shock wave therapy.

Where basics and clinics meet

The final, concluding chapter (Chapter VII) summarizes the main findings of the preceding chapters and tries to define their relevance for the practical management of SDFT lesions in the horse, which includes aspects of prevention and treatment. It tries to put the work presented in this thesis in the perspective of the overall research effort on equine tendon physiology and pathology and to indicate some of the most promising directions equine tendon research could take.

Chapter II

Functional adaptation through changes in regional biochemical characteristics during maturation of equine superficial digital flexor tendons

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Abstract

Objective

To quantify and compare biochemical characteristics of the extracellular matrix (ECM) of specimens harvested from tensional and compressive regions of the superficial digital flexor tendon (SDFT) of horses in age classes that include neonates to mature horses.

Sample Population

Tendon specimens were collected on postmortem examination from 40 juvenile horses (0, 5, 12, and 36 months old) without macroscopically visible signs of tendonitis.

Procedure

Central core specimens of the SDFT were obtained with a 4-mm-diameter biopsy punch from 2 loaded sites, the central part of the mid-metacarpal region and the central part of the mid-sesamoid region. Biochemical characteristics of the collagenous ECM content (i.e., collagen, hydroxylysylpyridinoline crosslink, and pentosidine crosslink concentrations and percentage of degraded collagen) and noncollagenous ECM content (percentage of water and glycosaminoglycans, DNA, and hyaluronic acid concentrations) were measured.

Results

The biochemical composition of equine SDFT was not homogeneous at birth with respect to DNA, glycosaminoglycans, and pentosidine concentrations. For most biochemical variables, the amounts present at birth were dissimilar to those found in mature horses. Fast and substantial changes in all components of the matrix occurred in the period of growth and development after birth.

Conclusions and Clinical Relevance

Unlike cartilage, tendon tissue is not biochemically blank (i.e., homogeneous) at birth. However, a process of functional adaptation occurs during maturation that changes the composition of equine SDFT from birth to maturity. Understanding of the maturation process of the juvenile equine SDFT may be useful in developing exercise programs that minimize tendon injuries later in life that result from overuse.

Introduction

Horses are precocious animals and the typical foal stands 55 minutes after birth (Koterba, 1990). Therefore in horses, birth signifies a dramatic physiologic change not only for the respiratory and cardiovascular systems, but for the musculoskeletal system as well. Immediately after birth the musculoskeletal system is faced with new postural, locomotive, and gravitational forces. Tissues that make up the musculoskeletal system are in a phase of rapid growth and adaptation and are able to readily adapt to these changes (Van Weeren et al., 2000). The study of these adaptive processes in various tissues will yield valuable information that may have implications for the maximum exercise load of (young) horses.

In articular cartilage it has been shown that a loading-driven process of functional adaptation exists at an early age, shaping the biochemical make-up of the tissues (Brama et al., 2000a). In this process a change occurs from a homogeneous blank joint to a distinct topographic heterogeneity with respect to all constituents (collagenous and noncollagenous) of the extracellular matrix (ECM) (Brama et al., 2002; Little and Ghosh, 1997). This heterogeneity in biochemical characteristics matches the biomechanical challenge, and seems to be formed under the influence of early postnatal biomechanical loading in a so-called process of functional adaptation (Brama et al., 2001; 2000a). This is not unlike the adaptation of bone to loading as already described by Julius Wolff in the late 1800s (Wolff, 1892).

Tendon tissue and articular cartilage have several features in common. They have a large ECM to cell ratio, and consist of basically the same following components: collagen fibrils, proteoglycan aggregates, and water. As in cartilage, the biochemical composition varies considerably between regions, apparently reflecting differences in loading (Lin et al., 2005). These similarities may lead to the presumption that in tendons the same concept of gradual functional adaptation applies.

Research into the development of tissue characteristics of tendons in juvenile horses has been limited. Work has mainly focused on ultrastructure and biomechanical characteristics. From this work a picture of rapid and profound changes emerges. At birth the equine superficial digital flexor tendon (SDFT) is highly cellular with a so-called unimodal distribution of small collagen fibrils and a high proportion of type III collagen (Birk and Mayne, 1997; Parry et al., 1978b). During postnatal development, fibril distribution changes into a bimodal one (Parry et al., 1978a) and the collagen type changes to a clear predominance of type I. With maturation, biomechanical characteristics of the SDFT, such as elastic modulus (Gillis et al., 1995), crimp angle and crimp length (Patterson-Kane et al., 1997a), collagen fibril mass-average diameter,

and collagen fibril index (Patterson-Kane et al., 1997c) change gradually, resulting in an overall increase of tendon stiffness and strength.[a] The purpose of the study reported here was to quantify and compare biochemical characteristics of the ECM of biopsy specimens harvested from tensional and compressive regions of the SDFT of horses in age classes that included neonates to mature horses. We hypothesized that the biochemical composition of the tendon from the metacarpal and sesamoid regions would be identical at birth, but would become distinctly different as horses matured.

Materials and methods

Specimen collection

Tendon specimens were collected on postmortem examination from 40 juvenile horses, age 0 (n = 8), 5 (8), 12 (8), and 36 (8) months and mature horses (n = 60; mean ± SD age, 12.2 ± 4.6 years; range, 4 to 23 years). The foals aged 0 months were stillborn animals or animals that died at delivery, the juvenile horses aged 5 to 36 months were euthanatized for other reasons than orthopaedic ailments using an overdose of barbiturates[b] after sedation with detomidine[c] 0.1 mL /100kg, the mature specimens came from slaughter horses. All horses were warmbloods, and tendons had no visible signs of any tendinopathy. Specimens were obtained with a 4-mm-diameter biopsy punch from the central part of the mid-metacarpal region of the SDFT in all horses and from the central part of the mid-sesamoid region in all horses except for the 5-month-old horses. The specimens were split in half (in dorsopalmar direction) to be used for the analysis of glycosaminoglycans (GAG), DNA, and hyaluronic acid (HA) concentrations, and for collagen network analysis, respectively. After recording wet weight immediately after specimen collection, specimens were lyophilized for 24 hours and dry weight was recorded. Specimens were frozen and stored at -80°C until further analysis.

Determination of water, DNA, GAG, and HA content

The percentage of water was calculated by use of the following equation: [(wet weight − dry weight)/wet weight] × 100%. Without preprocessing, tendon specimens (< 20 μg) for DNA, GAG, and HA analysis were digested by papain (1 U/mL)[d] in 400 μL of 50 mM of phosphate buffer (pH 6.5), containing 2.0 mM Na_2EDTA and 2mM cysteine for approximately 18 hours at 65°C (Cherdchutham et al., 1999). The DNA analysis was according to Kim et al (1988). Briefly, the fluorescent dye Hoechst 33258 was added to papain-digested tendon and fluorescence was measured immediately after mixing by use of a fluorimeter,[e] with excitation at 366 nm and emission at 442 nm. Calf thymus DNA[d] was used as a reference. Results were expressed as μg of DNA per mg

dry weight. The GAG concentration was analyzed by use of the modified 1,9-dimethylmethylene blue dye[d] binding assay as described by Farndale et al (1982) and measured by a microtiter plate reader[f] with an absorbency of 525 nm. Shark chondroitin sulfate[d] was used as a standard. Results were expressed as μg of GAG per mg dry weight. The HA concentration was measured by use of the microtiter plate assay as described by van den Hoogen et al (1998). Briefly, HA was measured after removing sulfated GAGs in a papain digest of tendons by adding an equal volume of a suspension of diethylaminoethyl Sepharose[g] in 1M NaCl. The uronic acid residues in the supernatant were assumed to originate from HA and were quantified by a microtiter plate reader[g] at an absorbency of 525 nm. The change in absorbance reflects uronic acid and values were standardized with galacturonic acid.

Determination of degraded collagen

An assay that is based on the observation that α-chymotrypsin digests denatured collagen, but not the intact triple helix of collagen type I, was used (Bank et al., 1997b). In brief, after treatment with α-chymotrypsin, the supernatant (containing the digested collagen) was separated from the remaining insoluble matrix (containing the intact collagen) and both were hydrolysed in 6M HCl. The amount of the collagen-specific amino acid hydroxyproline (hydroxyproline) released by α-chymotrypsin was determined after reaction with chloramine T and dimethylaminobenzaldehyde by absorption at 570 nm as described previously (Creemers et al., 1997).[h] The percentage of degraded collagen was calculated from the amount of hydroxyproline by the following equation: [(hydroxyproline concentration in the supernatant α-chymotrypsin digest)/(hydroxyproline concentration in the supernatant α-chymotrypsin digest + hydroxyproline concentration of digested tendon plug)] $\times$ 100%.

Determination of total collagen concentration and cross-link analysis

Papain digested tendon specimens were hydrolyzed in 6N HCl (110°C, 20 hours). After drying, the hydrolysates were dissolved in internal standard solution (10 mM pyridoxine[d] and 2.4 mM homoarginine[d] in water). For crosslink analysis, (hydroxylysylpyridinoline [HP] and pentosidine), samples were diluted 5-fold in 10% acetonitrile and 1% heptafluorobutyric acid solution[i] and crosslinks were quantified by reversed phase-high pressure liquid chromatography (Bank et al., 1997a). For amino acid composition, these diluted samples were additionally diluted 50-fold with 0.1M borate buffer (pH 11.4) and amino acids were derivatized by use of 9-fluorenylmethyl chloroformate.[i] The labelled amino acids were separated and quantified by reverse phase-high pressure liquid chromatography as described previously (Bank et al., 1996). Concentrations of cross-links were expressed per collagen triple helix (mol/mol of

collagen) (Bank et al., 1998) to normalize for variations in total collagen concentration. In all instances, 1 collagen molecule (triple helix) is assumed to contain 300 hydroxyproline residues. Collagen concentration was expressed as mg of collagen per mg of dry weight tendon (mg/mg of dry weight).

Data presentation and analysis

All measured biochemical variables were expressed as mean ± SD. Biochemical data were analyzed by use of a software program.[j] Normality was assessed by use of the Kolmogorov-Smirnov method and data were found to be normally distributed, allowing the use of parametric tests. Differences between 2 regions and among age groups (different time points) were tested by use of a 2-way ANOVA. The Fisher least significant difference multiple comparisons test of the means was applied as a post-hoc test. The level of significance was set at values of $P < 0.05$. Data from mature horses were obtained from a previous study (Lin et al., 2005).

Results

Neonatal horses

In neonatal horses, no regional differences in the amount of water, HA, degraded collagen, and HP cross-links were found (Figs 1 to 4). However, regional differences in DNA, GAG, and pentosidine concentrations were found that were significantly higher in the sesamoid region, compared with the metacarpal region (Figs 5 to 7). A regional difference in total collagen concentration was fount that was significantly higher in the metacarpal region, compared with the sesamoid region (Fig 8).

Development during maturation

All biochemical variables significantly changed during the development from neonate to adult. Most biochemical variables had significant site differences at 1 or more stages during development, but the patterns along which these differences developed varied among variables.

Noncollagenous biochemical variables

The percentage of water was approximately 80% in both regions at birth and then decreased gradually. This decrease was similar in both regions until 36 months of age, when mean percentage of water was 70%. From 36 months to maturity a significant site difference developed with a higher percentage of water in the metacarpal region (63%) than in the sesamoid region (57%; Fig 1). The DNA concentration decreased after birth in both regions and thereafter stayed at the same concentration (sesamoid region), or increased again (metacarpal region). The DNA

concentration was significantly higher in the sesamoid region at birth and at 12 months, compared with the metacarpal region. In mature horses DNA concentrations were higher in the metacarpal region, compared with the sesamoid region (Fig 5). Total GAG concentration increased steadily but slowly in the sesamoid region throughout maturation. In the metacarpal region a steep increase in GAG concentration was found during the first 5 months, followed by a sharp decrease thereafter. The GAG concentration was significantly higher in the sesamoid region at birth and in mature horses, compared with the metacarpal region. The GAG concentration was significantly higher in the metacarpal region at the age of 12 months, compared with the sesamoid region (Fig 6). The HA concentrations increased rapidly and significantly from birth until 5 months in both regions. In the metacarpal region the HA concentrations decreased afterwards. In the sesamoid region the increase continued until 12 months of age and then HA concentrations started to decrease until 36 months of age, after which a small increase was observed again. Concentrations were higher in the sesamoid region in the 12-month-olds and in mature horses, compared with the metacarpal region (Fig 2).

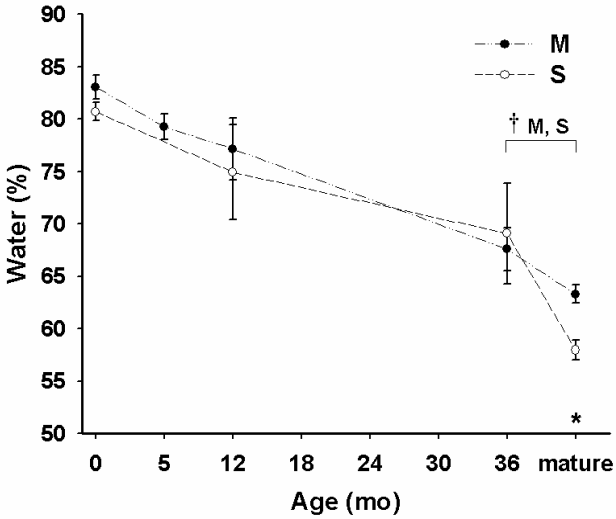

Figure 1. Mean ± SD percentage of water, as a noncollagenous extracellular matrix component, versus age in normal equine superficial digital flexor tendon (SDFT) tissues from 2 differently loaded sites.

M = Tissue biopsy specimens from the central core of the metacarpal region of SDFT.

S = Tissue biopsy specimens from the central core of the sesamoid region of SDFT.

*Significant ($P < 0.05$) difference between regions.

†Significant ($P < 0.05$) difference between age groups in either the M or S region.

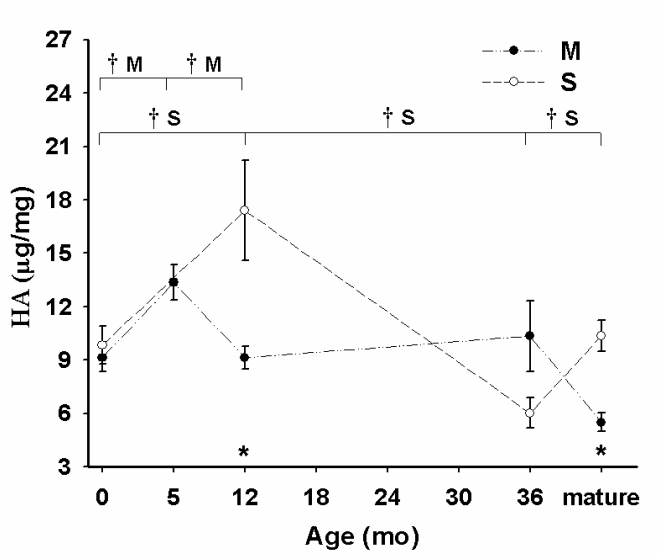

Figure 2. Mean ± SD concentration of hyaluronic acid (HA) on a dry weight basis, as a noncollagenous extracellular matrix component, versus age in normal equine SDFT tissues from 2 differently loaded sites. *See* Figure 1 for remainder of key.

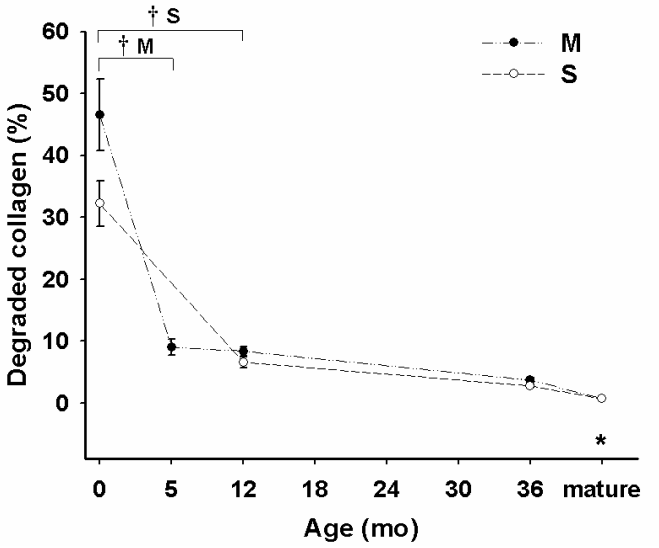

Figure 3. Mean ± SD percentage of degraded collagen, as a component of the collagen network, versus age in normal equine SDFT tissues from 2 differently loaded sites. *See* Figure 1 for remainder of key.

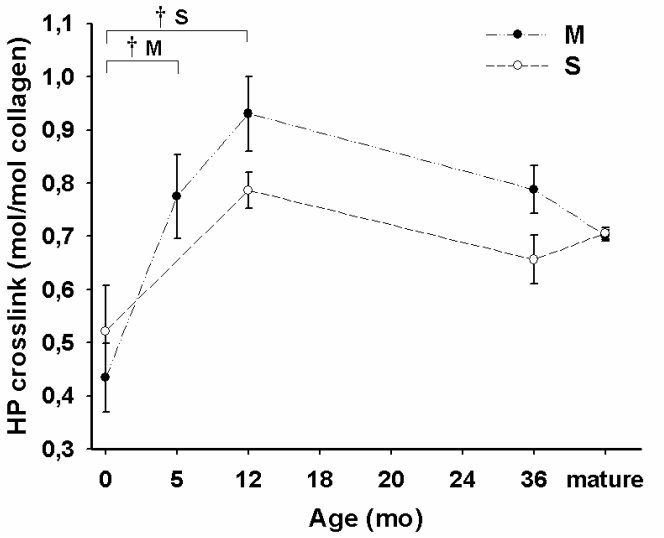

Figure 4. Mean ± SD concentration of hydroxylysylpyridinoline (HP) crosslinks, as a component of the collagen network, versus age in normal equine SDFT tissues from 2 differently loaded sites. *See* Figure 1 for remainder of key.

Collagen biochemical variables

Collagen concentration decreased from birth to 5 months of age in the metacarpal region. After this the collagen concentration in the metacarpal region remained constant until 36 months of age, followed by an increase. In the sesamoid region, collagen concentrations slowly decreased throughout the entire period. Collagen concentration was significantly higher in the metacarpal region at birth and in mature horses (Fig 8). The percentage of degraded collagen was similar between the 2 sites. A sharp decrease after birth occurred until approximately 5 months of age, and then a steady state was reached. The relative amount of degraded collagen in mature horses was approximately 3% (Fig 3). The HP cross-link concentrations increased significantly during the first 12 months of life, and then reached a steady state. No significant difference in HP cross-link concentration was found between the 2 sites (Fig 4). Pentosidine cross-link concentrations were higher in the sesamoid region than in the metacarpal region at birth. After that, a gradual increase was found in both regions and at 36 months of age pentosidine concentrations were significantly higher in the metacarpal region, compared with the sesamoid region (Fig 7). In an earlier study (Lin et al., 2005), pentosidine concentrations in the mature horses continued to increase in the sesamoid region (but not in the metacarpal region). Therefore mean pentosidine concentrations were not given for mature horses.

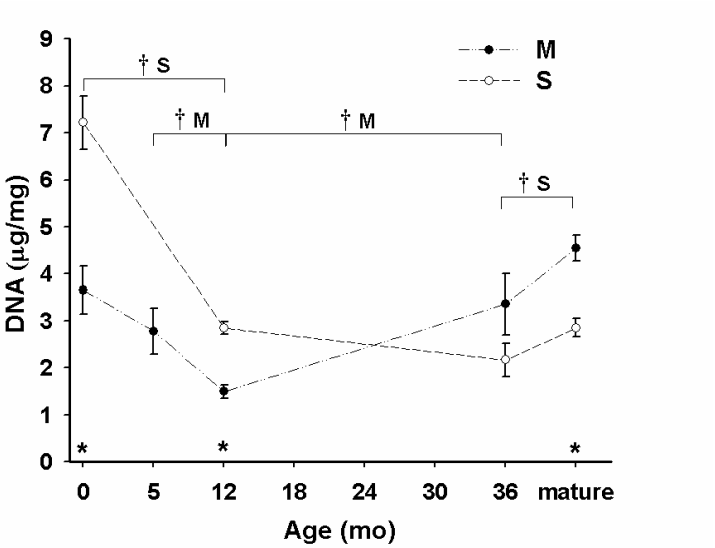

Figure 5. Mean ± SD concentration of DNA on a dry weight basis, as a noncollagenous extracellular matrix component, versus age in normal equine SDFT tissues from 2 differently loaded sites. *See* Figure 1 for remainder of key.

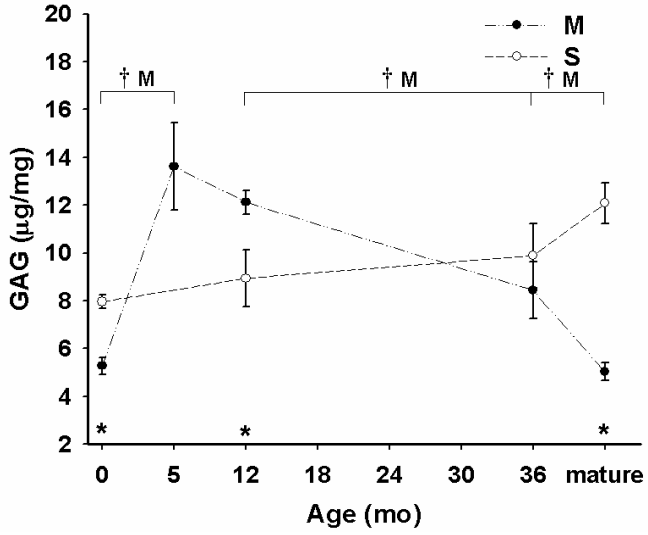

Figure 6. Mean ± SD concentration of glycosaminoglycan (GAG) on a dry weight basis, as a noncollagenous extracellular matrix component, versus age in normal equine SDFT tissues from 2 differently loaded sites. *See* Figure 1 for remainder of key.

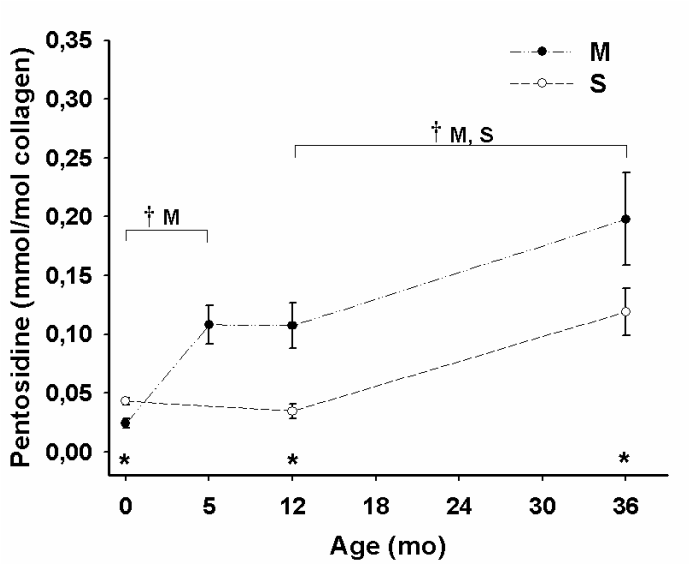

Figure 7. Mean ± SD concentration of pentosidine crosslinks, as a component of the collagen network, versus age in normal equine SDFT tissues from 2 differently loaded sites. *See* Figure 1 for remainder of key.

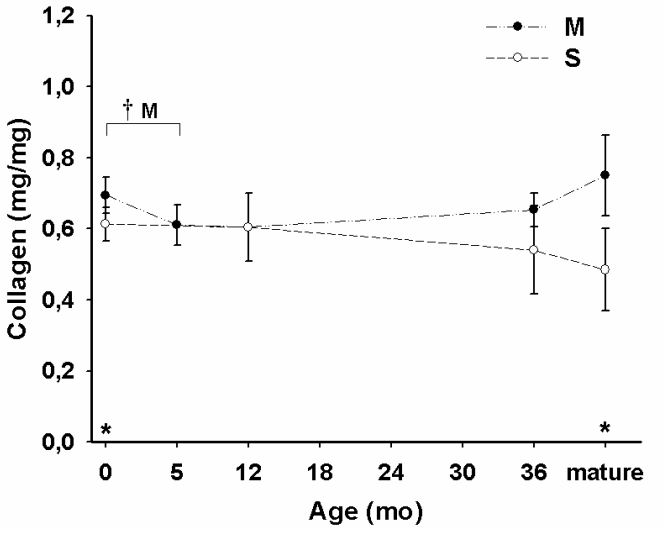

Figure 8. Mean ± SD concentration of total collagen on a dry weight basis, as a component of the collagen network, versus age in normal equine SDFT tissues from 2 differently loaded sites. *See* Figure 1 for remainder of key.

Discussion

In newborn horses the composition of the ECM at the 2 differently loaded sites was dissimilar with respect to some measured biochemical variables. Therefore, unlike in articular cartilage, already some topographic heterogeneity exists at birth in tendon tissue and foals are hence not born with a blank (homogeneous) tendon. Although other factors cannot be excluded, movements of the fetus in utero, which have been determined to be a prerequisite for the correct development of functional tendons (Beckham et al., 1977), may be a cause. Fetal movements influence the expression in tendon tissue of vascular endothelial growth factor, which is 1 of the most important angiogenic factors during embryo genesis (Carmeliet et al., 1996; Petersen et al., 2002). Articular cartilage may be considered as unloaded during fetal development, but movements of the fetus in utero will exert some forces on the developing tissue of the flexor tendons. These forces will be principally tensional; substantial compressive forces will not be exerted until the foal is on its feet and subjected to gravitational forces. However, the pulley-like conformation of the SDFT in the sesamoid region will lead to different force patterns at that site than in the metacarpal region where the force vector is exactly in line with the long axis of the tendon.

The connective tissues of the musculoskeletal system develop from a relatively simple mesenchymal ECM in the embryo (Hay, 1991). In mature horses this original tissue has differentiated into various specialized tissue types that are adapted to withstand various forms of mechanical stress such as compression, tension, and shear. This extensive remodeling is thought to be effected through the mechanical stimulation of integrins (or associated proteins), which trigger adaptive cellular responses (Shyy and Chien, 1997; Galbraith and Sheetz, 1998).

Biomechanical challenges to the equine flexor tendons change during the development from fetus to mature horse. Various periods can be discerned. During the period in utero movements take place, but without the influence of gravitational forces. An abrupt change occurs after birth when the structures are first subject to gravity and concomitant weight bearing. In the immediate postnatal period, rapid growth occurs and thus a rapid increase in weight bearing. The onset of locomotive activities will further increase the mechanical challenge of the tissues in the period immediately after birth. After these first months, growth slows down and locomotion will remain approximately the same. Around 3 years of age, the typical warmblood horse will be taken into training, and hence athletic challenge will increase. Once in full athletic activity, the mechanical environment will not change much during the rest of the horse's functional life. It can be assumed that these variations in mechanical stimulation will have their effect on the connective tissues of the musculoskeletal system.

To a limited extent in our study, interference by batch differences in the biochemical analyses may have occurred, as it was not possible to analyze the large number of samples in a single batch. This is a potential weakness of our study. However, standards were added in each batch for analysis of DNA, GAG, and HA and the samples were run in triplicate. For the collagen biochemical variables, internal standards were always used for each run by use of high-pressure liquid chromatography. During analyses, cross-reference checks were performed that revealed that almost no difference occurred in the DNA, HA, and dimethylmethylene blue assays (inter-assay coefficient of variation was below 5% in all assays). The current routine laboratory procedures have been proven to yield repeatable results in earlier studies (Brama et al., 2000a; Cherdchutham et al., 1999; van der Harst et al., 2004), and it was felt that group comparisons were possible.

In the development of various biochemical components from birth to maturity some general patterns emerged. A general pattern was found in the percentages of water and degraded collagen and in HP cross-link concentration, where the biochemical variables start at similar amounts at both investigated sites followed by age-related change in these variables that are similar at both sites. To a large extent, the same applies to pentosidine concentrations, although a small, but significant, difference occurs in pentosidine concentrations between the 2 sites in neonatal horses.

The percentage of water decreased from 80% at birth to approximately 65% at age 3 years in an almost linear fashion, indicating that the desiccation of the tissue is a time-related process that is apparently not affected by the biomechanical environment. The high percentage of water in the metacarpal region in mature horses has been linked to the accumulation of microdamage (Lin et al., 2005) and may hence be degenerative in character.

The relative amount of degraded collagen decreases rapidly during the first 5 months of life, and levels out afterwards. The assay cannot discriminate between degraded collagen generated by physiologic turnover, or by pathologic processes. However, this quick decrease in the percentage of degraded collagen during the first months of life in healthy foals seems indicative of the decrease in remodeling rate of structural elements of the tendon tissue, rather than pathologic degradation. Remodeling processes known to be active in young animals are for instance the changes from a high proportion of type III collagen to type I collagen, and from a unimodal fibril diameter distribution to a bimodal one (Parry et al., 1978a).

The concentration of HP cross-links increased during the first year and remained stable thereafter. This finding is in line with earlier observations in articular cartilage

(Brama et al., 2002), where changes in HP cross-link concentrations continue for a longer time than for most other collagen biochemical variables.

Pentosidine cross-links are products of the (time-related) process of nonenzymatic glycation and have been used to assess the remodeling rate of the collagen network (Bank et al., 1999). A linear time-related increase in pentosidine concentration in tissue is indicative of a low metabolic rate. Pentosidine concentrations were stable in the sesamoid region during the first year of life and started to increase afterwards. In the metacarpal region pentosidine concentrations increased earlier and reached a plateau at 5 months of age, which was the same concentration of pentosidine as in the sesamoid region at 36 months of age. These differences may be related to the dissimilar influence of birth and the relatively sharp increase in weight during the first year on the metacarpal and sesamoid regions. The sudden change to weight bearing may have influenced the sesamoid region, which until birth did not experience compressive forces, more than the metacarpal region in which tensional forces were present from the early embryonic stage. This may have induced higher metabolic activity, and hence less accumulation of pentosidine directly after birth in the sesamoid region, compared with the metacarpal region, explaining the initial plateau in the curve.

For GAG and collagen concentrations, another pattern was found. Here, significant differences were found between the 2 investigated sites at birth that either resolved or became reversed during the juvenile period, but returned in mature horses. These changing patterns may have to do with the different biomechanical environments in utero, during the phase of rapid growth, and in mature horses.

Concentrations of GAG are known to be associated with the determination of collagen fibril diameter (Parry et al., 1982). The immediate increase in tensile forces, that is most evident in the metacarpal region, is known to result in a strong increase in tendon fibril diameter during tendon matrix maturation (Parry et al., 1978a; Scott and Parry, 1992; Cherdchutham et al., 2001a), which may thus be related to the rapid increase in GAG concentrations in the first 5 months in the metacarpal region.

In collagen, the decrease in concentration in the metacarpal region after birth may represent the rapid restructuring of the collagen fibrils, especially in this portion of the tendon. The gradual increase after 5 months of age may reflect the functional response to principally tensional demand. In the sesamoid region the gradual decrease of collagen concentrations after 12 months of age probably reflects a certain down-regulation for collagen in favor of the production of fibrocartilage components at sites that face compression (Benjamin and Ralphs, 1998).

The DNA concentrations decreased after birth in both the metacarpal and sesamoid regions. This may be related to the overall decrease in metabolic activity and to the increase in importance of the ECM, as functional demand increases with age. It is not clear why cellularity is high in the sesamoid region in neonates. The high cellularity in the metacarpal region in mature horses is likely related to the frequent occurrence of microdamage in this region (Birch et al., 2002).

Hyaluronic acid concentrations seem to react quite rapidly to loading. The substantial increase in the sesamoid region in the early juvenile period and the smaller increase in the metacarpal region may reflect cell migration and proliferation for the accomplishment of structural changes (Burd et al., 1991) and thus differences in metabolic status of those regions. Not only GAG concentrations, but HA concentrations are also associated with the determination of collagen fibril diameter (Parry et al., 1982). While the primary stage of fibril growth is occurring, the transverse or circumferential growth of collagen fibrils is limited by the HA rich matrix, so that only small fibrils in diameter are formed (Parry et al., 1982). The decrease of HA concentrations until 3 years of age, which is the age most horses are taken into training, might be related to the lateral growth of fibrils, which is a response of the cells to a changing mechanical or microelectrical environment (Flint et al., 1980).

It is concluded that, in contrast to the situation in cartilage, the tendon does not have a homogeneous biochemical composition at birth. The idea of a blank tendon does therefore not apply, which is most probably caused by movements in utero, but the more general concept of functional adaptation (Brama et al., 2000a; Helminen et al., 2000) seems to hold. After birth, the foal passes through various phases characterized by different biomechanical challenges that seem to influence tissue composition. The transitions between these phases are either sudden or gradual, which probably influences the speed of adaptation. Adaptation can go quickly, especially in young foals. It has been shown that within 1 week after birth the energy-storing flexor tendon increased substantially in size in the neonate, with concomitant increased strength and stiffness.[a] Knowledge of these processes, which take place before a steady state is reached in mature horses, is crucial. Understanding of the processes that determines the ultimate quality of 1 of the most vulnerable structures in horses may help in the development of optimal exercise regimens to prepare young horses in the best possible way for future athletic activity.

Footnote

a. Batson EL. Tendons with dissimilar functions have different structural and material properties. PhD dissertation, The Royal Veterinary College, University of London, United Kingdom, 2002.
b. Euthesate, Apharmo BV, Arnhem, the Netherlands.
c. Domosedan, Orion Corp., Espoo, Finland.
d. Sigma Chemical Co, St Louis, Mo.
e. LS 2B Fluorimeter, Perkin-Elmer Corp, Norwalk, Conn.
f. Microplate reader, Bio-Rad Laboratories, Hercules, Calif.
g. CL-6B DEAE Sepharose, Amersham Pharmacia Biotech, Uppsala, Sweden.
h. Multiskan MCC/340, Titertek Instruments, Huntsville, AL.
i. Fluka Chemie AG, Buchs, Switzerland.
j. SPSS, version 10.0 for Windows, SPSS Inc, Chicago, Ill.

Chapter III

Extracellular matrix composition of the equine superficial digital flexor tendon: relationship with age and anatomical site

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Summary

The objectives of the present study were to test the hypotheses that: 1) the composition of the extracellular matrix of the equine Superficial Digital Flexor Tendon (SDFT) shows great functional similarities with articular cartilage, *i.e.* that significant differences exist in biochemical composition of differently loaded areas (which in the case of tendons may be more apparent as tendon shows more obvious differences than cartilage); and 2) that, as in articular cartilage, no substantial alterations in biochemical composition take place during ageing once adulthood has been attained. Tendon samples were taken from 60 adult slaughter horses from a central area at cross-section in the mid-metacarpal region and at the height of the proximal sesamoid bones (sesamoid region) of the SDFT. Contents of collagenous and non-collagenous components were determined. None of the parameters were correlated with age in either region, except for a significant increase in pentosidine crosslinks with age in the sesamoid region. Between the two anatomical regions, there were significant differences in all variables, except for hydroxylysylpyridinoline cross-links. It was concluded that in the equine SDFT, similar to articular cartilage, most molecular parameters are not influenced by age in mature horses, indicating a low remodeling rate. Tendon composition is clearly different between regions, apparently reflecting different specific modes of biomechanical loading at the points sampled.

Introduction

Musculoskeletal injury is the most common cause of wastage in horses. Injuries affecting either articular cartilage (Pool, 1996) or (flexor) tendons are by far most important in this category (Williams et al., 2001). Tendon consists, not unlike articular cartilage, principally of extracellular matrix (ECM) that is made up of collagen, water and proteoglycans (Goodship et al., 1994). The basic ultra-structural unit of the tendon is the collagen fibril, which consists mainly of collagen type I. The physical properties of tendon collagen are largely dependent on intra- and intermolecular cross-linking (Parry, 1988), which is essential for the stability of collagen fibrils (Tsuzaki et al., 1993). Hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) cross-links are two major non-reducible intermolecular cross-links of collagen that are found in tissues such as cartilage, bone, and tendon (Brama et al., 1999; Eyre et al., 1984). HP is the major crosslink found in tendon, whereas LP levels in tendon are low (Eyre et al., 1984). Pentosidine cross-links are products of the (time-related) process of non-enzymatic glycation (NEG) and have been used to assess the remodeling rate of the collagen network in a wide range of tissues, including dura mater, skin (Monnier et al., 1992), cartilage (Bank et al., 1998), and tendon (Bank et al., 1999). A linear, time-related

increase in pentosidine or other so-called advanced glycation end products in tissue is indicative of a low metabolic rate. Apart from collagen, the sulphated glycosaminoglycans, that are heavily negatively charged and hence highly hydrophilic, and hyaluronic acid, a non-sulphated glycosaminoglycan, are essential to resist compressive loads and to redistribute load to the collagen fibrils (Jackson et al., 1991; Prehm, 1984).

The superficial digital flexor tendon (SDFT) is the most heavily loaded tendon and the central zone of the mid-metacarpal region is particularly susceptible to exercise-related degeneration and subsequent injury (Genovese, 1993; Webbon, 1977). In contrast, the distal region of the tendon, where it passes over the sesamoid bones of the metacarpophalangeal joint, is less involved in pathology. These areas of the SDFT are subjected to basically different types of loading: the mid-metacarpal region is subjected to tensile forces only and the sesamoid region is subjected to both tensile and compressive forces. Recent research in equine articular cartilage has shown that there are significant differences in various biochemical components of the ECM between areas that undergo different biomechanical challenges (Brama et al., 2000b). These distinct differences appear to be formed in the juvenile period and will, once formed, not undergo significant alteration with increasing age during the entire life span of the animal (Brama et al., 1999; 2000a). The relationship of biochemical parameters with different anatomical sites in tendons has received little attention so far and investigations have not included extensive analyses of the collagen component (Birch et al., 2002; Micklethwaite et al., 1999). Most age-related studies of SDFT have focused on histological and biomechanical aspects (Patterson-Kane et al., 1997a; 1997c). Webbon (1978) noted variations in histological matrix composition related to age. Later, it was shown that cellularity decreases with age with a concomitant increase in the occurrence of diffuse chondroid metaplasia in horses older than 6 years (Crevier-Denoix et al., 1998). Crimp angle was significantly less in older horses than in younger ones, which was related to an increase in toe elastic strain, but not to a change in elastic modulus (Patterson-Kane et al., 1997a; Wilmink et al., 1992). In another study the elastic modulus was found to increase with time until age 15 years (Gillis et al., 1995). In a study comprising a group of “young” (3-6 years) and “older” (8-16 years) horses Birch et al. (1999) found a higher degree of collagen-linked fluorescence in the older age category as the only age influence in the SDFT.

In the present study the hypothesis is tested that, like in cartilage, significant regional differences exist in differently loaded areas and that no substantial alteration in biochemical composition takes place in the SDFT during aging of mature individuals. Central parts of the mid-metacarpal (tensile load) and sesamoid (tensile and compressive load) regions of the SDFT were chosen as typical examples of differently

loaded areas and the biochemical composition of samples from these areas were analyzed in an age range of mature horses. Given the vital role of the collagen network for tendon function we focused on collagen content and the post-translational modifications in collagen.

Materials and methods

Sample collection

Tendon samples were collected from 60 adult slaughter horses, age range 4-23 years (equally distributed), with no visible signs of tendonitis. Tendon specimens were obtained from the central core area on cross-section of the mid-metacarpal (M) region and the region at the height of the proximal sesamoid bones (sesamoid or S region) of the SDFT (Fig. 1). Samples were taken from the same location as the central core samples taken by Cherdchutham et al. (1999) in the middle and distal sections as described in that study. At each site a tendon sample was taken with a 4 mm diameter biopsy punch. Samples were split in half to be used for the analysis of glycosaminoglycans, DNA, and hyaluronic acid, and for collagen analysis, respectively. After recording wet weight immediately after sampling, samples were lyophilized for 24 hours and dry weight was recorded. Finally, samples were frozen and stored at –80°C until further analysis.

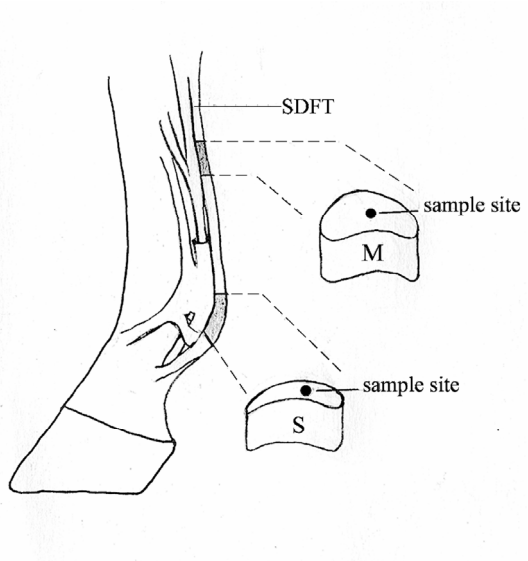

Figure 1. Schematic drawing of the equine lower limb. Sites where samples were taken and their respective locations in a transverse section through the tendon are indicated. SDFT: superficial digital flexor tendon; M: metacarpal region; S: sesamoid region

Determination of water content

Water content of the tendon samples was calculated by [(wet weight-dry weight)/wet weight] x 100%.

Papain digestion

Tendon samples were digested by 400 µl of papain solution, composed of 1U/ml papain (Sigma Chemical, St. Louis, USA) and 50mM of phosphate buffer (pH 6.5), containing 2.0 mM Na_2EDTA and 2mM cysteine for approximately 18 hours at 65°C.

Determination of DNA content

Cellularity of the tendon was determined by analyzing the DNA content according to Kim et al. (1988). The fluorescent dye Hoechst 33258 (Molecular Probes, Leiden, The Netherlands) was added to papain-digested tendon and fluorescence was measured immediately after mixing using a fluorimeter (LS 2b, Perkin Elmer, United Kingdom), with excitation at 366 nm and emission at 442 nm. Calf thymus DNA (Sigma Chemical, St. Louis, USA) was used as a reference. Results were expressed as µg DNA per mg dry weight.

Determination of Glycosaminoglycan (GAG) content

As a measure for the glycosaminoglycan content, the papain-digested tendon was analyzed for polysulphated glycosaminoglycan content using the modified 1.9-dimethylmethylene blue dye (DMMB) binding assay as described by Farndale et al. (1982). Briefly, to 10 µl of sample, 10 µl of 1% bovine serum albumin were added. Then, 200 µl of Farndale reagent was added and measured by Benchmark microtitre plate reader (Bio-Rad Laboratories, CA, USA) with an absorbency of 525 nm after 20 min at room temperature. Shark chondroitin sulfate (Sigma Chemical, St. Louis, USA) was used as a standard. Results were expressed as µg GAG per mg dry weight.

Determination of Hyaluronic acid (HA)

The concentration of HA was measured using a microtitre plate assay as described by van den Hoogen et al. (1998). Briefly, HA concentration was measured after removing sulfated GAGs in a papain digest of tendons by adding an equal volume of a suspension of DEAE Sepharose CL-6B (Amersham Biosciences, Sweden) in 1 M NaCl. The uronic acid residues in the supernatant were assumed to originate from hyaluronic acid and were quantified with help of a Benchmark microtitre plate reader (Bio-Rad Laboratories, CA, USA) at an absorbency of 525 nm.

Determination of degraded collagen

The assay is based on the observation that α-chymotrypsin (αCT) digests denatured collagen but not the intact triple helix of collagen type I (Bank et al., 1997). In brief, after treatment with αCT, the supernatant (containing the digested collagen) was separated from the remaining insoluble matrix (containing the intact collagen) and both were hydrolyzed in 6 M HCl. The amount of the collagen-specific amino acid hydroxyproline (Hyp) released by αCT was determined after reaction with chloramine T and dimethylaminobenzaldehyde by absorption at 570nm using the Titertek mulitscan MCC 340 (Titertek, USA) as described previously (Creemers et al., 1997). The percentage of degraded collagen was calculated from the amount of Hyp by [(Hyp content in the supernatant αCT digest) / (Hyp content in the supernatant αCT digest + Hyp content of digested tendon plug)] x 100%.

Determination of total collagen, amino acid content and crosslink analysis

The measurement was based on Bank et al. (1999), in brief, after hydrolysis in 6N HCl, for crosslink analysis samples were diluted with 0.5% heptafluorobutyric acid (HFBA) and were, together with 200 pmol of pyridoxine (internal standard), injected into the high-performance liquid chromatography (HPLC) system. Cross-links (HP, LP and pentosidine) were expressed per collagen triple helix. The same hydrolysates were used for amino acid analysis. Since hydroxyproline is specific for collagen, this amino acid was used to calculate the amount of collagen present in the tendons, assuming 300 hydroxyproline residues per collagen triple helix. The amount of lysyl hydroxylation was expressed as hydroxylysine residues (Hyl). Levels of amino acids and cross-links were expressed per collagen triple helix (mol/mol collagen), thus accounting for variations in total collagen content.

Data presentation and analysis

The biochemical data were analyzed statistically using SPSS 10.0 for Windows (SPSS Inc, Chicago, IL, USA). Differences between regions were compared using paired t-tests. The level of statistical significance was set at $p < 0.05$. First order linear regression analysis was used for all parameters to evaluate the relationship between biochemical parameters and age.

Results

Collagen network

There was no age-related change in most of the collagen network parameters (collagen content, degree of lysyl hydroxylation, or the enzymatic cross-links HP and LP) in either region. A significant positive correlation was observed between the non-enzymatic cross-link pentosidine versus age in the S region ($r = 0.67$, $p < 0.0001$) (Fig. 2a). However, this was not the case in the M region (Fig. 2b). There was no age-related change in the amount of degraded collagen in either of the sites.

There were various differences with respect to the collagen variables between the two anatomical regions (Table 1). Total collagen content and lysyl hydroxylation were significantly higher in the M region. HP cross-linking was the only variable that was similar in both regions, whereas LP cross-linking was twice as high in the S region, although absolute levels were low. There was a significantly higher proportion of degraded collagen in the M region than in the S region. In the M-region, degraded collagen accounted for 3.8% of total collagen, which is 30% more in comparison to the S region. Because pentosidine levels increased with age in the S region, but not in the M region, no comparison of mean levels could be made.

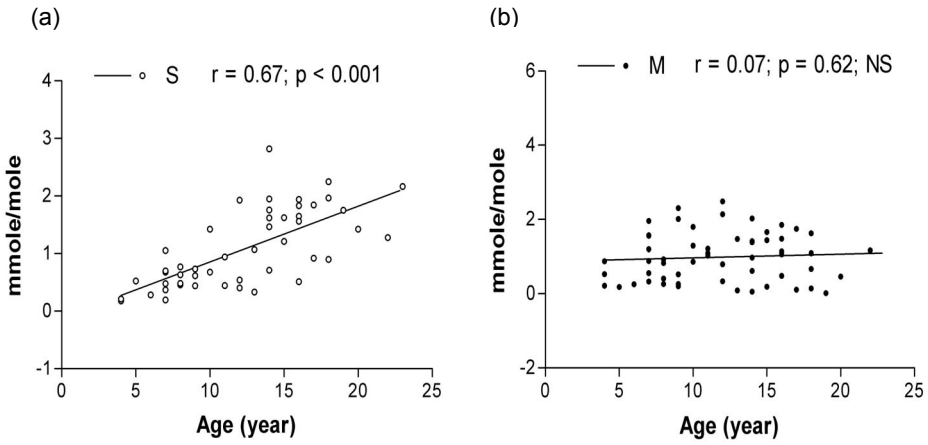

Figure 2 (a, b). Pentosidine cross-linking in the mid-metacarpal (M) and sesamoid (S) regions of the superficial digital flexor tendon in relation to age. A significant positive correlation was observed between the non-enzymatic cross-link pentosidine versus age in the S region ($r = 0.67$, $p < 0.0001$) (a), but not the case in the M region (b). NS: non-significant.

Table 1.
Biochemical parameters in the central part of the metacarpal and sesamoid regions of the equine SDFT

Parameter	Metacarpal region (mean ± SD)	Sesamoid region (mean ± SD)	p-value
Water (%)	63.29 ± 6.68	57.96 ± 6.73	$p < 0.001$
DNA (µg/mg dw)	4.55 ± 1.16	2.85 ± 0.43	$p < 0.001$
GAG (µg/mg dw)	4.95 ± 1.64	12.08 ± 3.14	$p < 0.001$
HA (µg/mg dw)	5.32 ± 1.10	10.18 ± 2.36	$p < 0.001$
Collagen (mg/mg dw)	0.87 ± 0.27	0.56 ± 0.11	$p < 0.001$
Degraded collagen (%)	3.70 ± 2.40	2.80 ± 1.97	$p < 0.05$
Hyl (mol/mol collagen)	45.26 ± 3.16	43.12 ± 3.41	$p < 0.001$
Lys (mol/mol collagen)	67.05 ± 4.35	78.84 ± 8.60	$p < 0.001$
HP (mol/mol collagen)	0.70 ± 0.06	0.71 ± 0.07	$p = 0.72$, NS
LP (mol/mol collagen)	0.02 ± 0.01	0.04 ± 0.02	$p < 0.001$

Non-collagenous matrix components

No significant age-related changes were found in water, DNA, GAG, and HA content in either region. GAG and HA showed a slight non-significant increase with age in the S region. However, there were significant differences for all these variables between the M region and the S region (Table 1). DNA levels, reflecting cellularity, were 40% lower in the S region; both GAG and HA were approximately twofold increased in the S region compared to the M region. Further, there was a significantly higher water level in the M region than in the S region, water content representing more than 60% of total tissue weight in the M region, but 10% less in the S region.

Discussion

Effect of aging

No significant changes with aging were encountered at either of the two sites in the biochemical characteristics of the collagenous and non-collagenous components of the extracellular matrix of mature individuals in the SDFT. This finding matches entirely the observations made in articular cartilage in two differently loaded sites (Brama et al., 1999; Vachon et al., 1990). Our findings corroborate those by Birch et al. (1999), who

compared centrally and peripherally located sites of the mid-metacarpal region of both the SDFT and the Deep Digital Flexor Tendon (DDFT), and showed that there were no differences in collagen content, water, GAGs, DNA, and HP cross-links in neither the central nor the peripheral area of the SDFT between two groups of horses with mean ages 4 and 16 years respectively. They included collagen type III in their study as a possible indicator of repair after the occurrence of micro-damage. Although they could not show significant differences between the age groups, collagen type III levels were highest in the central core of the SDFT, an area that is known to frequently feature focal degeneration (Birch et al., 1998; Webbon, 1977). Batson et al. (2003) found a weak, but significant decrease with age in DNA level of the SDFT. The discrepancy of that study with our study and with the findings by Birch et al. (1999) may possibly be explained by the wider age range (starting at 3 years and including horses up to 34 years). The lack of change in biochemical composition with ageing may indicate, as in articular cartilage, a very low metabolic level that precludes substantial remodeling of the ECM once this has been constructed. Alternatively, it may reflect an adequate basic activity of the tenocytes that under physiological conditions succeed in timely adaptation of the ECM, keeping the levels of the constituents constant. In this context it should be stressed that not only ECM composition, but also the structure of the ECM are determinants of biomechanical strength. A biochemical analysis as used in this study does not provide structural information.

An indication for a relatively low metabolic rate is the age-related linear increase in pentosidine cross-link levels as found in the sesamoid region in this study. Birch et al (1999) were not able to detect any pentosidine cross-links in their samples, which may have been due to a less sensitive analysis technique, but showed a significant higher level of collagen-linked fluorescence in their older age class, both in SDFT and DDFT. Collagen-linked fluorescence is a more general measure for non-enzymatic glycation (NEG) products, which include more than pentosidine alone. Here again there is a link with articular cartilage as in that tissue also a linear increase with age of pentosidine levels was found (Brama et al., 1999). However, in the SDFT there was an interesting difference between the two sites investigated, as there was no such increase in the M region. This may be due to the fact that the two regions investigated in this study sustain very different forces, a factor that is known to be able to actually change tissue composition (Flint et al., 1984), and in fact can be considered different tissues, be it that they are located in the same anatomical structure. However, it can also be speculated more audaciously that this difference is related to the incidence of injury. The S region rarely sustains injury, permitting the undisturbed accumulation of advanced glycation end products (AGEs). The lack of such an accumulation in the M region may indicate that more repetitive injuries are sustained in this region, which are subsequently

repaired by the deposition of newly synthesized collagen. The newly synthesized collagen lacks pentosidine and no age-related increase will be seen. These observations are entirely in line with those made in human rotator cuff syndrome (Bank et al., 1999). There, no increase in pentosidine cross-links is seen in the supraspinatus tendon (which is frequently involved in pathology) and a linear increase in the biceps brachii tendon (which is rarely, if ever, involved in pathology).

Regional effects

There were large and significant differences in various biochemical parameters of the collagen network between differently loaded regions. Collagen content was higher in the M region, corroborating earlier statements that tissues with greater tensile strengths are those with the highest percentages of collagen (Parry, 1988), and collagen cross-links (Bailey et al., 1980), that together determine the strength of the collagen fibril. Although the relative amount of hydroxylysylpyridinoline (HP) cross-links did not differ between the M and the S region, numbers per unit of tissue were obviously higher in the M region because of the higher collagen density in that region. LP cross-link levels were higher in the S region than in the M region. LP was originally thought to be present only in calcified tissues, like dentine and bone (Eyre et al., 1984). Fibrocartilage, that is known to be present in the sesamoid region (Crevier-Denoix et al., 1998), may account for the higher LP levels. Cross-linking occurs extracellularly, but the nature of the cross-link also depends on the previous intracellular post-translational modifications to the collagen molecule, in particular hydroxylation of lysine residues (Prockop and Kivirikko, 1995). During collagen synthesis, hydroxylation of proline and lysine residues results in the formation of hydroxyproline and hydroxylysine, respectively. Hydroxylysine levels were significantly higher in the M region than in the S region and lysine (Lys) was higher in the S region. The larger amount of hydroxylated lysine in the areas tendons subjected to higher tensile loads, suggest a role for stress in the regulation of lysine hydroxylation by lysyl hydroxylase (Gerriets et al., 1993).

In the non-collagenous matrix, the higher DNA level in the M region may exemplify the fact that tendon cells can divide and alter their matrices in response to external biomechanical influences (Lundborg and Rank, 1980). Tensional stress seems to be instrumental in this process. In rabbits, it was also shown that regions subject to compression were relatively avascular with lower levels of DNA synthesis compared to adjacent tension bearing regions (Abrahamsson, 1991). A significantly higher HA level was seen in the S region. HA content is increased in the event of cell migration and proliferation, and is essential for the accomplishment of structural changes (Prehm, 1984). High compressive loads (S-region) are related with high GAG-levels. This is in line with earlier findings in articular cartilage (Brama et al., 2000b), and also in tendons

of horses (Micklethwaite et al., 1999), cattle (Ehlers and Vogel, 1998), and humans (Vogel et al., 1993). The interaction of the hydrophilic GAGs with cations causes an osmotic pressure that allows the tissue to resist compressive forces (Jackson et al., 1991), which is important for the development and maintenance of tendon fibrocartilage (Gillard et al., 1979; Koob et al., 1992). Although GAG levels were higher in the S region, that sustains both compression and tension, and less in the tension-loaded M region, as was expected, water levels were significantly lower in the S region than in the M region. Given the hydrophylic nature of the GAGs, this finding may at first sight perhaps be unexpected, but the phenomenon may again be related to the occurrence of micro-trauma in the M region. The osmotic pressure by the hydrophylic GAGs is normally counter-balanced by the swelling-restraining capacity of the (intact) collagen network (Maroudas et al., 1985). If pathological processes affect the integrity of the collagen network, water content will rise and fewer GAGs may attract more water. The significantly higher levels of degraded collagen in the M region may therefore account for the approximately 10% higher water content in this region compared to the S region, notwithstanding the fact that the S region is richer in GAGs. This study thus gives support to the work of Patterson-Kane et al (1997c) who suggested that the central core of the SDFT at mid-metacarpal level may frequently sustain sub-clinical injury. The observation is also in line with the relatively high level of collagen type III in this area as reported earlier (Birch et al., 1999). Further support for a higher incidence of micro-trauma in this area comes from the observation that the percentage of degraded collagen was significantly higher in this area, compared to the sesamoid area. However, it should be realized that this could also indicate a physiological higher remodeling rate, as denatured collagen is not only a measure for mechanically induced damage, but may indicate turnover caused by physiological proteinase-mediated tissue remodelling as well.

It is concluded that there are great similarities in the general (patho)biological behavior of tendons and articular cartilage. In the equine SDFT, as in cartilage, there are significant differences in molecular composition of differently loaded areas. This is perhaps less surprising in tendons than in articular cartilage, which is recognizably the same across a joint whereas in tendons there are macroscopically detectable differences in the tissue. Another similarity, that is perhaps less obvious, is that, there are no physiological age-related changes in the molecular composition of the tissue once maturity has been attained. This is in agreement with the well-known refractoriness of both tendon tissue (Watkins, 1999) and articular cartilage (McIlwraith, 1996) in mature individuals to heal fully.

It lies at hand to speculate that, as in articular cartilage (Brama et al., 2000a), it is also function, thus loading conditions, which determine tendon properties during growth and development. The lack of change during ageing suggests that this is, as in articular cartilage (van Weeren and Brama, 2003) a once-in-a lifetime process and thus of paramount importance as it may be decisive for eventual injury resistance in the mature animal. To test this hypothesis, studies will have to be performed in animals in various developmental stages, probably including fetuses. It is recognized that biochemical studies are a part of the puzzle only and should be complemented by histological, ultra-structural and biomechanical studies. Once the puzzle is solved how equine tendons develop in a molecular and structural sense, and hence how resistance to loading develops, information is gained that will prove invaluable for the prevention of tendonitis, still one of the most important threats to the equine athlete.

Chapter IV

The effect of Micro-current Electrical Tissue Stimulation on cultured equine tenocytes

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Abstract

Objective

To determine effects of microcurrent electrical tissue stimulation (METS) on equine tenocytes cultured from the superficial digital flexor tendon (SDFT).

Sample population

SDFTs collected from 20 horses at slaughter.

Procedure

Tenocytes were isolated following outgrowth from explants and grown in 48-well plates. Four methods of delivering current to the tenocytes with an METS device were tested. Once the optimal method was selected, current consisting of 0 (negative control), 0.05, 0.1, 0.5, 1.0, or 1.5 mA was applied to cells (8 wells/current intensity) once daily for 8 minutes. Cells were treated for 1, 2, or 3 days. Cell proliferation, DNA content, protein content, and apoptosis rate were determined.

Results

Application of microcurrent of moderate intensity increased cell proliferation and DNA content, with greater increases with multiple versus single application. Application of microcurrent of moderate intensity once or twice increased protein content, but application 3 times decreased protein content. Application of current a single time did not significantly alter apoptosis rate; however, application twice or 3 times resulted in significant increases in apoptosis rate, and there were significant linear (second order) correlations between current intensity and apoptosis rate when current was applied twice or 3 times.

Conclusions and Clinical Relevance

Results of the present study indicate that microcurrent affects the behavior of equine tenocytes in culture, but that effects may be negative or positive depending on current intensity and number of applications. Therefore, results are far from conclusive with respect to the suitability of using METS to promote tendon healing in horses.

Introduction

The superficial digital flexor tendon (SDFT) is the supporting structure of the horse's limb that is most susceptible to injury (Webbon, 1977) and the mid-metacarpal region of the SDFT is particularly prone to stress-induced injuries (Fackelman, 1973). Tendon injuries heal slowly and imperfectly, resulting in repair tissue that is biomechanically inferior to normal tendon (Watkins, 1999; McCullagh et al., 1979). In undamaged tendons, there is no endotendinous cellular activity in the central part of the tendon (Abrahamsson et al., 1989), although tendons have an intrinsic capacity for repair (Mass and Tuel, 1991). Tendon repair is accomplished primarily by resident tendon fibroblasts or tenocytes, which are the principal cellular components of tendon tissue (Manske and Lesker, 1984). Tenocytes produce both the fibrillar and the non-fibrillar components of the extracellular matrix (Ross et al., 1989) and play an important role in initiating regenerative responses following injury or degeneration.

Electrical current is known to influence biological processes at the cellular level, and devices that deliver various types of currents to tissues with the aim of engendering a therapeutic effect have been developed since the end of the 19th century (Stainbrook E, 1948). Most recently, techniques that use currents in the microampere range, which are similar to the currents generated during physiologic processes such as the depolarization of cell membranes, have been introduced (Hodgkin and Huxley, 1952). Electrical current has been reported to alleviate pain, probably by altering β-endorphin concentrations (Gabis et al., 2003), and speed up wound healing by increasing cell proliferation (Goldman and Pollack, 1996). Reported improvements in fracture healing associated with electrical current (Wahlstrom, 1984) might reflect the effects of electrical stimulation on the sequential events of osteoblast differentiation (Hartig et al., 2000) and mineral formation (Wiesmann et al., 2001). Circulation can also be enhanced by increasing the concentration of the vasodilator nitrogen oxide by means of electrical stimulation (Knispel et al., 1992).

Depending on the condition being treated and the administered dose, the effects of electrical current may be positive or negative. Electrical current may stimulate cells to proliferate but inhibit proliferation after a certain threshold (Ross, 1990). High electrical currents will result in overt tissue damage and provoke cell necrosis, but lower currents may stimulate cells to go into apoptosis (Blumenthal et al., 1997). Although apoptosis does not cause the inflammation associated with necrosis (Kerr et al., 1972), it may still be an unwanted phenomenon during tissue healing.

The mechanism by which microcurrent affects tissues remains largely unclear, although activation of signal transducing pathways is a likely candidate. Effects on nitric

oxide synthase, which regulates nitric oxide production, may play a role, as nitric oxide is known to promote the healing process of tendons (Murrell et al., 1997). Microcurrent application increases concentrations of transforming growth factor-beta 1 and insulin-like growth factor II, which are associated with bone cell proliferation (Zhuang et al., 1997; Fitzsimmons et al., 1992). Electrical fields may play a role in extracellular communication, and stimulation of progenitor cells by microcurrent has been shown to speed up the process of endochondral ossification (Aaron and Ciombor, 1996). The phase of the cell cycle may be important to the effects of microcurrent, as cell cycle phase seems to influence the receptivity of the cell to extracellular signals. Generally, cells in the G_0/G_1 stage undergo division when they receive signals that instruct them to enter the active phases of the cell cycle (Morgan et al., 2002).

Changes in electrical potential of the tissue may also play a role in the effects of electrical current. Trauma affects the electrical potential of damaged cells, and an injured area has higher electrical resistance than the surrounding tissue, resulting in decreased electrical conductivity through the injured area and decreased cellular capacitance (Windsor et al., 1993). Exogenous microcurrent might augment the endogenous current flow and allow the traumatized area to regain its capacitance. Total resistance of the injured tissue would thereby be reduced, allowing physiologic amounts of bioelectricity to enter the area and re-establish homeostasis.

Noninvasive method for improve the quality of repair tissue or shortening the recovery time in horses with tendon lesions would be of great value in veterinary medicine. Potentially, microcurrent electrical devices would be good candidates. However, given the ambiguous effects of electrical currents on tissues, there are potential adverse effects as well. For instance, microcurrent treatment might be far from innocuous if it were to enhance apoptosis, which could be expected to adversely affect tendon healing. Studies of the effects of microcurrent on calf osteoblast-like cells (Curtze et al., 2004) and rat tendon fibroblasts (Blumenthal et al., 1997) have been reported, but relatively little is known about the effects at the cellular level in general, and no specific data exist on the effect in equine tenocytes. The purpose of the study reported here, therefore, was to determine the effects of microcurrent electrical stimulation on equine tenocytes in culture. We hypothesized that microcurrent application would promote cell proliferation and increase DNA and protein synthesis, but would also increase the apoptosis rate.

Materials and Methods

Tenocyte culture

Tenocytes were obtained by means of an explant technique, as described (Birch et al., 1997b; Evans and Trail, 1998). Briefly, SDFTs without macroscopic lesions were collected from 20 horses at the time of slaughter (mean ± SD age, 7 ± 2 years), and samples were taken from the central core of the mid-metacarpal region of each tendon. Samples from various horses were mixed, and tissue fragments of approximately 1 mm^3 were placed in tissue-culture-grade flasks (25 cm^2). Initially, 5 tissue fragments were added to each flask, but during subsequent studies, 10 fragments were added to each flask to increase cell production. Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and streptomycin-penicillin (50 U/mL) was used for culture medium. Cultures were incubated in a humidified incubator with 5% CO_2 at 37°C. Medium was refreshed twice weekly.

Explant-derived cells took approximately 4 days to emerge from the tissue fragments and another 2 weeks to become confluent. At confluence, the explants were removed, and the cells were transferred to larger flasks (75 cm^2). Cells between passages 2 to 5 were used for electrical current stimulation experiments. For these experiments, cells were trypsinized and seeded at a density of 3 X 10^4/mL in 48-well plates and grown until they were nearly confluent (9 x 10^4/mL). Cells were supplied with serum-free medium 24 hours before application of electrical current to synchronize them in the G_0/G_1 phase, as described (Liu et al., 2004).

METS device

A microcurrent electrical tissue stimulation (METS) device[a] that delivered a pulsating direct electric field was used in the study. The waveform consisted of a brief monophasic square pulse (duration, 0.8 ms) followed by exponential decay to base level. The pulse frequency was 150 Hz. The device delivered a constant current; that is, it automatically changed voltage as necessary in order to deliver the same current when peripheral resistance changed.

Development of method of electrical current application

A series of experiments was performed to identify the optimal experimental setup for application of electrical current to cultured tenocytes and to determine the most suitable cell density for assessing the effects of METS. Four methods for electrode placement were tested. With method 1, the electrodes were placed at the bottom of each well, in direct contact with the cells. With method 2, the electrodes were placed just under the surface of the medium in each well. With method 3, the electrodes were

placed in separate containers of PBS solution that were connected to the culture wells with agarose bridges to avoid electrolysis. Each bridge consisted of a 5-cm-long, 0.2-mm inner diameter capillary filled with sterile 0.1% agarose. With method 4, the electrodes were attached to paper strips[b] (0.5 x 3 cm^2) soaked in PBS solution that were placed in the culture wells.

Electrical current treatment

After selection of the optimal method for current application, electrical current consisting of 0 (negative control), 0.05, 0.1, 0.5, 1.0, or 1.5 mA was applied to cells in wells on 48-well plates (8 wells/current intensity) once daily for 8 minutes. Cells were treated for 1, 2, or 3 days. To assess the effect of cell density, cell concentrations of 4 X 10^4/mL (50% confluence) and 9 X 10^4 /mL (90% confluence) were used.

Assessment of tenocyte proliferation

To assess the effects of METS on tenocyte proliferation, viable cells were quantified by use of a non-radioactive, colorimetric cell proliferation kit.[c] This method is based on metabolism of tetrazolium salts to formazan dye, and the outcome relates directly to the number of viable, metabolically active cells (Roehm et al., 1991). Cell proliferation was measured 24 hours after the end of each specific current treatment. For assessment of proliferation, cells were incubated for 2.5 hours, and spectrophotometric absorbency was measured with a microtitration plate reader[d] at a wavelength of 450 nm. The reference wavelength was set at 650 nm.

Determination of DNA and protein contents

To evaluate the effect of METS on DNA and protein contents, cells were rinsed 2 times with PBS solution 24 hours after the end of each specific current treatment and scraped from the plates with a rubber spatula. Cells were lysed by freeze-thawing, placed in liquid nitrogen for a few seconds to destroy the cell membrane, and then stored at –20°C until further analysis. The DNA content was determined as described (Kim et al., 1988). Briefly, a fluorescent dye was added to the lysed cells, and fluorescence was measured with a fluorometer[e] immediately after mixing, with excitation at 366 nm and emission at 442 nm. Calf thymus DNA[f] was used as a reference. Results were expressed as µg of DNA per well.

Protein content was measured by use of a bicinchoninic acid protein assay reagent kit.[g] Briefly, 25 µL of lysed cells was added to 200 µL of kit reagent, and absorbency was measured with a microtitration plate reader[d] at an absorbency of 595 nm after 30 minutes of incubation at 37°C. Bovine serum albumin provided by the

manufacturer of the kit was used as a standard. All data were related to results for a negative control sample.

Determination of apoptosis rate

The apoptosis rate was used as a measure of possible cytotoxicity of METS. Apoptotic cells were detected on the basis of annexin V binding to translocated plasma membrane phosphatidylserine, as described (Vermes et al., 1995). Propidium iodide was used to test for loss of cell membrane integrity, which is indicative of necrotic and late apoptotic cells. Briefly, 24 hours after the end of each specific current treatment, culture medium was collected and tenocytes were rinsed briefly with PBS solution, trypsinized, and centrifuged at 2900 *g* for 5 minutes at 4°C. Apoptosis rate was determined by use of an annexin V and propidium iodide double staining kit,[h] following the manufacturer's instruction. The stained cells were then analyzed by means of flow cytometry.[i] The fluorescence intensity of the pulse area signal from 10,000 events was analyzed. Cells that had undergone no electrical treatment were used as negative controls, and cells directly stimulated with 3.5 mA of current were used as positive controls. Viable cells were negative for annexin V and propidium iodide, apoptotic cells were positive for annexin V but negative for propidium iodide, and necrotic cells were positive for annexin V and propidium iodide. Further analysis for cell sub-populations was performed with quadrant gates. In this analysis, the lower left quadrant represented the viable cells; the upper right quadrant represented non-viable, necrotic or late-stage apoptotic cells; and the lower right quadrant represented apoptotic cells. Relative cell death was calculated by counting apoptotic cells and dead cells together (i.e., upper and lower right quadrants), because the terminal phase of cell death in vitro represents a gradual shift from apoptosis to necrosis.

Statistical analyses

Measured values were standardized to mean values for control samples (i.e., no electrical stimulation) and expressed as mean ± SD. Effects of METS were tested by means of 2-way ANOVA, followed by the Bonferroni post hoc test. Correlations between DNA content, protein content, cell proliferation, and apoptosis rate were examined by calculation of the Pearson correlation coefficient. All statistical analyses were performed with standard software.[j] Values of $P < 0.05$ were considered significant.

Results

Optimal method of electrical current application

When electrodes were placed at the bottom of each well (method 1), apoptosis was detected immediately after current application, even when a low current (0.2 mA) was used. When electrodes were placed just under the surface of the medium in each well, so that they were not in direct contact with the cells (method 2), apoptosis was seen at low (0.2 mA) and high (3 mA) currents, although effects were dose-dependent. When electrodes were placed in separate containers connected to wells by agarose bridges (method 3), no apoptosis was seen, and it was nuclear whether current transmission was effective, as there were no differences between control cells (no current) and treated wells in regard to cell proliferation, DNA content, or protein content. Based on the disadvantages of the previous models, model 4 was generated and appeared to work well. For this reason, attachment of electrodes to paper strips that were placed in the culture wells (method 4) was selected as the optimal method for microcurrent application.

Cell density was associated with severity of detachment and apoptosis caused by microcurrent, with apoptosis rate being lower when cell density was higher. Therefore, a cell density of 9 X 10^4/mL (90% confluence) was used.

Effect of METS on cell proliferation, DNA content, and protein content

Application of microcurrent had a stimulatory effect on cell proliferation that was dependent on current intensity and number of current applications (Figure 1). Cell proliferation was significantly increased, compared with the negative control, after a single application of current at 0.5, 1, or 1.5 mA. However, when current was applied twice, cell proliferation was significantly increased by application of current as low as 0.1 mA, and when current was applied 3 times, cell proliferation was significantly increased by application of current as low as 0.05 mA. Application of current multiple times had a greater effect on cell proliferation than did application a single time. Cell proliferation was significantly greater after application of 0.5 mA of current twice than after application of the same current only once, and cell proliferation was significantly greater after application of 0.1, 0.5, 1, or 1.5 mA of current 3 times than after application only once.

Similar patterns were found for DNA content (Figure 2), except that application of current a single time did not have any significant effect on DNA content, compared with the negative control. However, application of 0.1, 0.5, or 1 mA of current twice or 0.05, 0.1, 0.5, 1, or 1.5 mA of current 3 times significantly increased DNA content, compared

with the negative control. Also, application of 0.05, 0.1, 0.5, or 1 mA of current 3 times resulted in significantly greater DNA content than did application only once.

Protein content was significantly increased, compared with the negative control, after application of 0.5 of 1 mA of current once and after application of 0.1, 0.5, or 1 mA of current twice (Figure 3). However, application of current 3 times resulted in a significant decrease in protein content, compared with application of current only once.

There was a significant (r = 0.928; P = 0.007) correlation between DNA content after application of current a single time and protein content after application of current a single time.

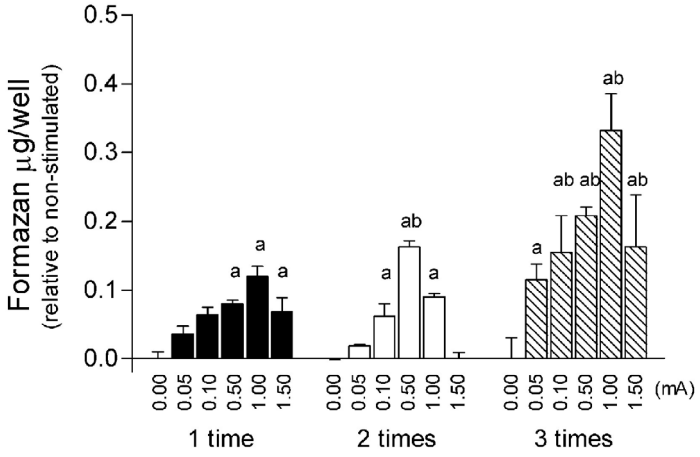

Figure 1. Effect of microcurrent electrical tissue stimulation (METS) on proliferation of equine tenocytes in culture. Tenocytes were grown in wells on 48-well plates, and electrical current consisting of 0 (negative control), 0.05, 0.1, 0.5, 1.0, or 1.5 mA was applied to cells (8 wells/current intensity) once daily for 8 minutes. Cells were treated for 1, 2, or 3 days. Cell proliferation was measured 24 hours after the end of each specific current treatment by use of a commercial kit that measures metabolism of tetrazolium salts to formazan dye Measurements were standardized by subtracting the mean value of the corresponding control group. Values are expressed as mean; error bars represent SD. a = Significantly (*P* < 0.05) different from value for negative control. b = Significantly (*P* < 0.05) different from value obtained after a single application of current at the same intensity.

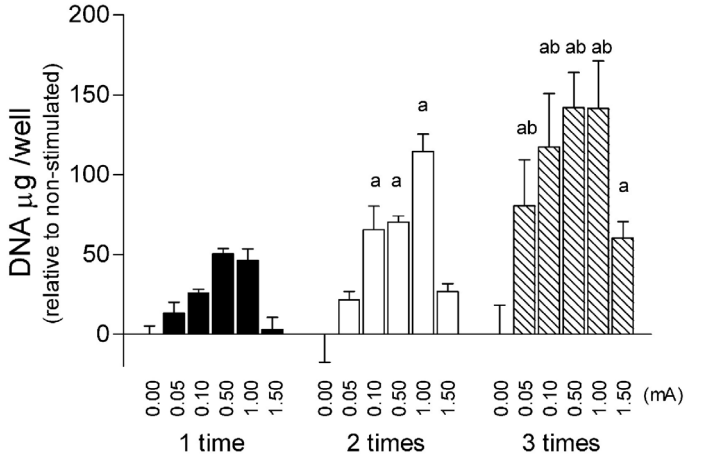

Figure 2. Effect of METS on DNA content of equine tenocytes in culture. Measurements were standardized by subtracting the mean value of the corresponding control group. *See* Figure 1 for remainder of key.

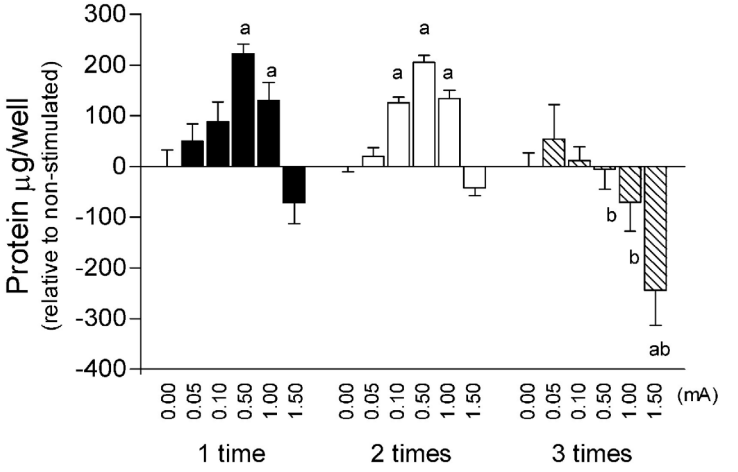

Figure 3. Effect of METS on protein content of equine tenocytes in culture. Measurements were standardized by subtracting the mean value of the corresponding control group. *See* Figure 1 for remainder of key.

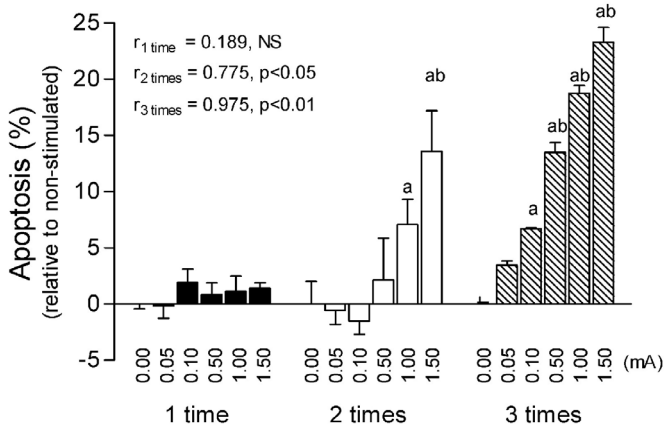

Figure 4. Effect of METS on apoptosis rate of equine tenocytes in culture. Measurements were standardized by subtracting the mean value of the corresponding control group. See Figure 1 for remainder of key.

Effect of METS on apoptosis rate

Apoptosis rate was dependent on current intensity and number of current applications (Figure 4). Within the range of current used, application of current a single time did not significantly alter apoptosis rate. However, application of current twice or 3 times resulted in significant increases in apoptosis rate, and there were significant linear (second order) correlations between current intensity and apoptosis rate when current was applied twice (r = 0.775; P < 0.05) or 3 times (r = 0.975; P < 0.01). Current intensity was not significantly correlated with apoptosis rate when applied only once (r = 0.189; P > 0.05). There was a significant negative correlation between apoptosis rate and protein content after application of current 3 times (r = –0.83; P = 0.04).

Discussion

In our attempts in the present study to identify the optimal method for applying microcurrent to tenocytes in culture, we found that allowing for direct contact between the electrodes and the culture medium or the cells themselves resulted in extensive cell death. This phenomenon could have been a result of electrolysis, whereby dissolved positive and negative ions in the medium were discharged at the cathodal and anodal electrodes, respectively, and was probably mediated through formation of H_2O_2 at the anodal electrode. The use of bridges to allow for indirect current transmission avoided this. However, only the paper bridges worked satisfactorily. The agarose bridges most

likely had an intrinsic resistance that was high enough to prevent sufficient current to generate a biological effect to pass to the cells.

In the present study, the apoptosis rate was lower when cells that were 90% confluent were used than when cells that were 50% confluent were used. This was not attributed to cells in the denser monolayers receiving less current, because the METS device that was used automatically increased the voltage if peripheral resistance changed. However, when cells were 50% confluent, they were fibroblast-like, with more evidence of mitotic and metabolic activity. When cells were 90% confluent, they were spindle-shaped and more closely apposed and appeared quiescent and less metabolically active. It is probable that cells in this less active state are less susceptible to stimulation by electric current, but there are other possible explanations for the influence of cell density on effects if microcurrent application. In particular, cell-to-cell contact and communication will be different in close-packed cell populations than in more sparse populations. Also, the greater amount of matrix with higher cell density (Blumenthal et al., 1997) might alter resistance and mitigate the direct effects of current on tenocytes, although it is arguable whether culturing time and conditions used in the present study were sufficient to allow for the formation of substantial differences in amount of extracellular matrix. The presumptive role of matrix in the dissipation of electrical energy may have an important influence on the in vivo effects of METS. Because tenocytes are embedded in a three-dimensional network of extracellular matrix components in vivo, it can be expected that higher currents or longer application times may be needed to have positive effects in vivo. Further study is needed to determine whether effective field strength can be obtained with a treatment regimen that is still practical; what effects intermediate structures, such as skin and fascia, might have on field strength in target tissues; and what effects transducer placement (i.e., dorsal-palmar vs. lateral-medial vs. proximal-distal) might have.

Results of the present study indicate that microcurrent affects the behavior of equine tenocytes in culture. As expected, alterations in cell proliferation, DNA content, and protein content could be detected, depending on current intensity and number of applications. This is in line with previous findings for fibroblasts (Bourguignon and Bourguignon, 1987), chondrocytes (Lee et al., 1982; Rodan et al., 1978), and osteoblast-like cells (Ozawa et al., 1989). On the basis of the positive effects on cell proliferation and DNA content, it seems that microcurrent can induce tenocytes to pass from the G_0/G_1 stage to the active phase of the cell cycle.

There was a clear effect of number of current applications on results of the present study. Application of current twice seemed to have an additional stimulating effect on cell proliferation and DNA content, at least at certain current intensities.

However, the overall effect was not much different from results of a single application. Three daily applications of current had a somewhat ambiguous effect. Cell proliferation and DNA content were higher, at least at intermediate current intensities, but protein content was negatively affected and apoptosis rate increased substantially.

Overall patterns of changes in cell proliferation and DNA content were similar, which was expected because DNA content is related to cell number. However, results were not identical, possibly because cells in the S and M2 phases have twice as much DNA as do cells in other phases of the cell cycle. Our results also showed that cell proliferation and apoptosis rate may increase at the same time. This may seem contradictory; however, it is conceivable that individual cells respond differently depending on the phase of the cell cycle that they are in at the time of current application.

It is evident from our results that the effects of electrical current on the function and metabolism of cells is not only positive and stimulating, but may have negative aspects as well. The effects appeared to depend on the intensity of the current that was applied, as shown earlier for fibroblasts from rat skin (Cheng et al., 1982). In particular, our findings suggest that the stimulatory effects on cell proliferation, DNA content, and protein content decrease with application of current at intensities > 1 mA. With a single application of current, the apoptosis rate remained the same throughout the range of current intensities used; however, when current was applied twice or 3 times, there was a significant linear increase in apoptosis rate with current intensity. This implies that cells are less susceptible to a single exposure to relatively high current than to repeated exposure to lower current.

The increase in apoptosis rate found in the present study should not be taken too lightly. Tendon is a tissue with a high ratio of extracellular matrix to cells, and the tenocytes that are present are solely responsible for the repair process. Loss of even a relatively minor quantity of these cells may severely impair the repair capacity of the tissue. Therefore, any substantial loss of cells as a result of an increase in the apoptosis rate associated with METS may render the whole procedure counter effective. The negative correlation between protein content and apoptosis rate may be indicative of this. Results of the present study do not yet permit a determination of a detailed scheme for application of METS in vivo. However, it seems that 3 consecutive applications is too intensive for cells to cope with. In addition, long-term effects of METS were not evaluated in the present study. Although there are no indications in the literature of delayed cytotoxic effects if the correct dose of electric stimulation is given, this is a possibility that must be investigated in more detail before any clinical application of METS can be recommended.

The mechanism by which microcurrent exerts its effects on cells is not known. There may be a direct effect through influences on transmembrane potential (Cheng et al., 1982), which may result in an increase in permeability of the plasma membrane (Stacey et al., 2003). This could also affect intracellular pathways (Borgens, 1988). In this regard, it has been suggested that cells are capable of detecting external electric fields when frequency and phase of the external current match with those of intracellular metabolic oscillators (Labat-Robert et al., 1990). Negative effects may be generated through the disruption of cellular membranes, which might be primarily due to large ion shifts. In particular, DNA is a heavily charged intracellular structure whose folded structures and proximity to the nuclear membrane make it susceptible to the effects of current. Damage to DNA will have consequences for cell survival and may result in cell cycle anomalies (Stacey et al., 2003). Protein content may be negatively affected by higher current intensity because of inhibitory effects on amino acid transport (Ozawa et al., 1989).

The hypothesis of this study was proven to be partially true, but results were not unambiguous. Microcurrent stimulation indeed enhanced cell proliferation and increased DNA and protein synthesis, but only at certain current intensities. At lower and higher intensities, no effects or negative effects were detected. The apoptosis rate also was affected by microcurrent application, but results depended on current intensity and number of applications. The current range used in the present study (0.05 to 1.5mA) did not have an irreversible effect, as apoptosis rate did not change after a single application of current in this range. However, additional applications of current within a short time apparently influenced the mechanisms of cellular response, as apoptosis rate increased. We conclude that microcurrent may have positive and negative effects on equine tenocytes in culture, depending on application. Although microcurrent is capable of enhancing cell metabolism and, therefore, may promote tissue regeneration (Borgens, 1988), results of the present study are far from conclusive with respect to the suitability of using METS to promote tendon healing in horses.

Footnote

a. APS device MK 1.1 Medeusa, BV Bilthoven, The Netherlands.
b. Whatman CHB chromatography paper, Fisher Emergo BV, Landsmeer, The Netherlands.
c. Cell proliferation kit II XTT, Roche Molecular Biochemicals, Basel, Switzerland.
d. Microplate reader, Bio-Rad Laboratories, Hercules, Calif.
e. LS 2B Fluorimeter, Perkin Elmer Corp, Norwalk, Conn.
f. Sigma Chemical, St Louis, Mo.
g. BCA protein assay reagent kit, Pierce Chemical Co, Rockford, Ill.
h. Annexin-V-Flous staining kit, Roche Molecular Biochemicals, Indianapolis, Ind.
i. Becton Dickinson, San Jose, Calif.
j. Prism, version 4.0, GraphPad Software Inc, San Diego, Calif.

Chapter V

The influence of electrode placement on effective field strength in the equine superficial digital flexor tendon: a preliminary study for the evaluation of micro-current as a potential therapy for tendon healing in the horse

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Abstract

Objective

To determine the relationship between the output of an electrical treatment device, that might potentially be useful to promote tendon healing, and the effective field strength in the target tissue. It was hypothesized that due to the influence of surrounding tissues and the spatial arrangement of tendon fibres effective field strength would be significantly affected by the direction of current application, *i.e.* by electrode placement.

Sample population

Horse forelimbs without visible defects (n=8) were obtained freshly from a slaughterhouse. Superficial digital flexor tendons (n=8) were dissected from the same limbs. For the *in vivo* study one pony under general anaesthesia was used.

Methods

Micro-currents were generated by a Micro-current Electrical Therapy (MET) device and applied in proximal-distal, dorsal-palmar and medial-lateral directions in the entire distal limbs, in the bare tendons and in the living pony with various machine output settings. Corresponding field strengths in the target tissue (SDFT) were measured using custom-made electrode sets.

Results

There was a linear relationship between current and field strength in all conditions and in all three directions ($p<0.001$), but there were significant differences between the conditions using bare tendons and the entire distal limb. In the bare tendon condition, there were significant differences between all three directions with highest field strength in the proximal-distal direction and lowest in the dorsal-palmar direction. In the distal limb condition the field strength in the proximal-distal direction was significantly lower than in medial-lateral direction. The results in the living pony were similar to those in the entire distal limbs.

Conclusions and clinical relevance

Electrode placement significantly affected field strength in the target tissue. Further, the presence of many surrounding structures reduces field strength in the target tissue considerably. These factors should be taken into account when establishing protocols for electrical current based therapeutic devices once it has been proven that these devices are clinically effective.

Introduction

Tendon disorders are a major problem in sports and occupational medicine. Equine flexor tendons are loaded close to their physiological limit under normal conditions (Goodship et al., 1994), making them prone to injury. Lesions of the equine superficial digital flexor tendon (SDFT) are common in performance horses and often lead to permanent disability, as tendon tissue is known to heal poorly through the formation of repair tissue that is functionally inferior to the original tissue (Watkins, 1999). For these reason tendon injuries account for significant losses to the equine industry and are a significant threat to equine welfare.

It has been tried in many ways to improve tendon healing and to minimize the formation of scar tissue. Apart from a limited number of reports on the effect of ultrasound therapy (Morcos and Aswad, 1978; Keg, 1989), non-invasive physical techniques have received little attention thus far, although from a practical viewpoint they are very attractive for use during the prolonged recovery periods that occur in equine tendon healing. Watkins *et al.* (1985) used a pulsing electromagnetic field to treat artificially created tendon lesions. They found a delay in maturation of the scar tissue, probably due to a delay in collagen type transformation. A delay in the maturation of the collagen component of the extracellular matrix, *i.e.* the formation of cross-linking, is the rationale behind the treatment of tendon lesions with beta-aminoproprionitrile fumarate (Cohen, 1985), which has enjoyed some popularity in equine medicine (Reef et al., 1996). Effects of electrical or electromagnetic therapy that have been reported include alleviation of pain, which might relate to the internalization of substance P receptors (Allen et al., 1999), increased cell proliferation in wounds (Goldman and Pollack, 1996) and a stimulation of bone fracture healing (Bassett et al., 1982; Wahlstrom, 1984). Electric treatment may further enhance local circulation by increasing the level of the vasodilator nitric oxide (Knispel et al., 1992). This may contribute to a modulation of inflammatory reactions and a reduction of swelling in the treated area. In tendons, electric currents have been reported to result in better collagen alignment and a greater reduction of inflammation in tendonitis (Lee et al., 1997). Direct current applied in low amperage to rabbit tendons in vitro suppressed adhesion-causing synovial proliferation in the epitenon and promoted active collagen synthesis by the tenocytes (Fujita et al., 1992). Cell orientation is sensitive to electric fields too (Song et al., 2002), with current apparently orienting new collagen formation (Reger et al., 1999). In a recent preliminary study by our research group we showed that the application of direct pulsating micro-current (150 Hz) to cultured equine tenocytes stimulated cell metabolism in terms of protein and DNA production at certain current levels and treatment frequencies.[a] It also became clear from that study that

relatively small changes in current level may change a beneficial effect at cell level into a deleterious effect, or the other way round.[a]

It is the current through the tissue that is responsible for the biological effect of electric therapy. This current (I) is determined by the specific resistance, or bio-impedance, of the tissue concerned (R) and the actual field strength (V) according to Ohm's law (I=V/R). The parts of the body to which electrodes are applied are multi-composite, heterogeneous structures and the specific resistance or bio-impedance of different tissues in the body is not identical (Kushner, 1992). Further, bio-impedance can be expected to change according to fibre direction. These considerations lead to the conclusion that field strengths in the tissues that constitute the body parts under treatment will not be identical. Therefore, knowledge of the effect of electrode placement on actual field strength in the target tissue is imperative, if at some stage clinical trials are to be performed. In the present study the hypothesis is tested that, due to the influence of surrounding tissues and the specific spatial arrangement of the tendon fibres, effective field strength will be affected significantly by electrode placement.

Material and methods

General set-up of the study

Various settings of a MET device were applied to fresh, isolated SDFT's, fresh intact distal limbs, and to a living, anaesthetized pony using three different, standardized electrode placements. Custom-made probes were used to measure effective field strength under these conditions, and the relationship between machine settings and field strength was established.

Specimens and animal

For the *ex vivo* study, horse forelimbs without visible defects or ailments (n=8) were obtained freshly (not older than 6 hours) from a slaughterhouse. For the condition in which the effect of direct current application to the tendon was tested, superficial digital flexor tendons (n=8) were dissected from the same limbs. The *in vivo* study was performed using a pony under general anaesthesia that afterwards was to be used for a regular practical training session for veterinary students, and that was scheduled to be euthanised at the end of this training.

The device

Micro-currents were provided by a Micro-current Electro Tissue Stimulation device[b] with a current setting of 0 to 3.5 mA and a fixed frequency of 150 Hz. The pulse wave form was a brief monophasic square pulse (duration 0.8 ms), followed by

exponential decay to base level. The device is current constant, *i.e.* it will adapt voltage if resistance of the target tissue changes. The current was delivered via flat, and self-adhering rectangular surface electrodes (area 5 x 1.5 cm^2).

Field strength measurement

Field strength was measured in three directions in the mid-metacarpal area of the SDFT: proximal-distal, medial-lateral and dorsal-palmar. Since the cross-section of the tendon measures only 6 to 8 mm in dorsal-palmar direction, but around 20 mm in medial-lateral direction, two types of probes were developed to measure the corresponding voltage. The probe for the proximal-distal direction was the same as the one used for the medial-lateral direction. Probes were constructed using a modified 20-gauge, stainless steel catheter.[c] The needles were 1.1 mm in diameter, 33 mm long, and coated with non-conducting epoxy, leaving 2.5 mm of the distal tip exposed. The cap and the plastic top were removed to expose the stainless steel top. One set was made by connecting two needles side by side, with 17 mm distance between the needle tips (type-A) (Fig. 1). The other set was made in a similar fashion, but the distance between the two needle tips was 4 mm (type-B) (Fig. 1). The stainless steel tops of both sets could be connected to a computer equipped with a TP 5008 A/D converter.[d] To measure the field strength the distal tips of the needle sets were inserted over 2 mm into the SDFT. The MET device was set at 0.02, 0.05, 0.2, 0.5, 0.8, 1.0, 1.3, 1.5, 2.0, 2.3, 2.5, 3.0 and 3.5 mA respectively. The corresponding voltage was recorded as baseline-to-peak value and was transformed to the field strength by expressing as voltage per centimetre, which accounts for the differences in distance between the needle tip for the two types of probes.

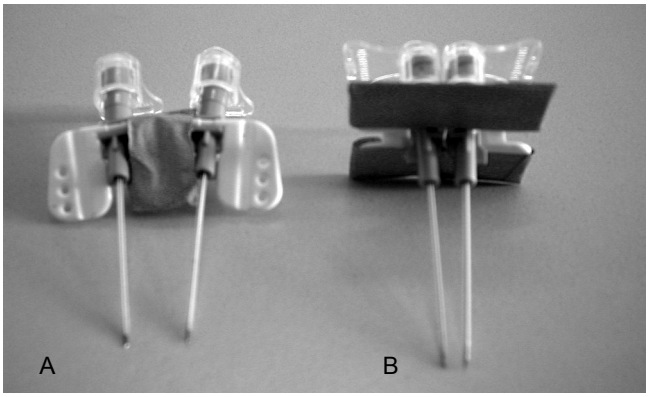

Figure 1. Two sets of probes constructed using stainless steel butterfly needles. Type A was used in proximal-distal and medial-lateral directions. Type B was used in dorsal-palmar direction.

Measurement conditions and electrode placement

Three different conditions were tested: *in vivo* the live pony, and *ex vivo* entire distal forelimbs and bare SDFT's. The setting was similar for the limbs from the slaughterhouses and for the live pony. The pony was measured under anaesthesia while lying on its right side. After shaving the metacarpal region, electrodes linked to the MET device were attached to the skin of the lower forelimbs. The electrodes were placed in three different directions: proximal-distal, medial–lateral and dorsal-palmar (the positive electrode being mentioned first, the actual flow of the electrons is thus in opposite direction). In the proximal-distal direction, electrodes were both placed on the palmar side, one just below the distal carpal joint, the other just above the fetlock joint (a distance of approximately 20 cm). A type-A probe was inserted through the skin into the tendon in proximal-distal direction (parallel to the axis of tendon) at the palmar side of the mid-metacarpal region of the SDFT for voltage measurement (Fig. 2a). For the medial-lateral direction, electrodes were placed medial and lateral to the mid-metacarpal region. A type-A probe was put perpendicular into the mid-metacarpal region of the SDFT in transverse direction (Fig. 2b). In the dorsal-palmar direction, one electrode was placed on the dorsal part of the mid-metacarpal region, and the other one was placed at the palmar aspect. A type-B probe was used in this direction (Fig. 2c). Probes were placed perpendicular to the limb from the medial side in the mid-metacarpal region. For the bare tendons, a similar set-up was used (Figs. 3a-c). Each direction was measured in triplicate under each condition. All experiments were performed at room temperature.

Statistical analysis

Statistical analysis was performed using the Prism statistical package from Graph Pad Software.[e] Differences among models and directions were tested by linear regression. Differences between regression lines were tested by comparing the sum of squares (SS) of the regression lines through the individual data sets, and by the SS of the regression line through the combined data set using F-statistics. For the live condition in which only a single individual was used, it was determined whether the values found fell within the range of one of the other conditions or not. Differences were considered significant when $p<0.05$.

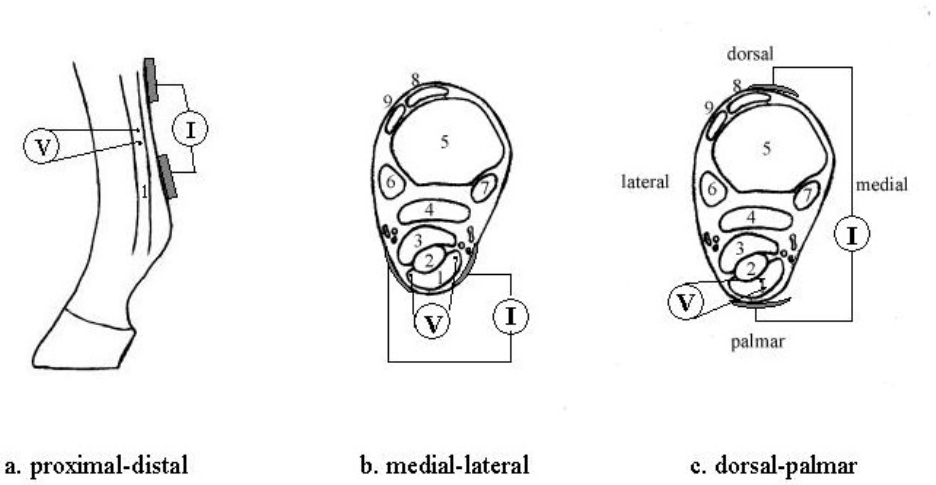

Figure 2. Electrodes placement in the *ex vivo* condition using the entire lower limbs and in the *in vivo* condition. Transverse sections demonstrate the positions of electrodes and the probes in the dorsal-palmar and the medial-lateral directions (a, b). The setting in the proximal-distal direction is shown along the longitudinal axis (c). I: current generated by MET device; V: voltage; 1: superficial digital flexor tendon; 2: deep digital flexor tendon; 3: accessory (inferior check) ligament; 4: suspensory ligament; 5: third metacarpal bone; 6: fourth metacarpal bone; 7: second metacarpal bone; 8: common digital extensor tendon; 9: lateral digital extensor tendon.

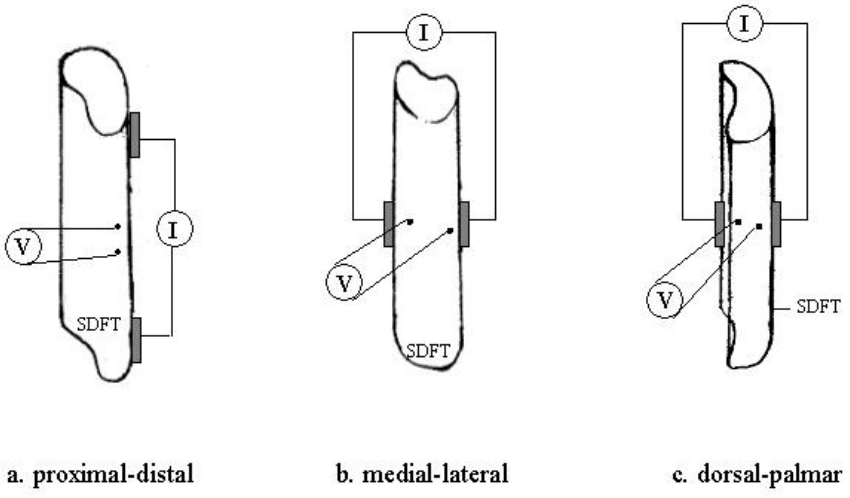

Figure 3. Electrode and probe placements in the *ex vivo* condition using bare tendons. SDFT: superficial digital flexor tendon; I: current generated by MET device; V: voltage.

Results

In the distal limb and live condition, total resistance was considerably more than in the bare tendon condition and current settings of 0.02 and 0.05 mA appeared to be too low to result in a measurable field strength. Apart from this observation, linear relationships between the applied current and the resulting field strength were found in all conditions and in all directions ($p < 0.001$) (Fig. 4). Since values found in the *in vivo* condition were comparable to those found in the condition using the entire distal limbs in all directions, this data was combined. In the *ex vivo* condition using the entire distal limb, the field strength corresponding to a specific machine setting was lowest when the current was applied in the proximal-distal direction and highest when applied in medial-lateral direction. However, only the difference between medial-lateral and proximal-distal direction was statistically significant (Fig. 4a). In the condition using the bare tendon the order was different. Here application in proximal-distal direction led to highest field strengths, followed by medial-lateral and dorsal-palmar applications. In this condition differences between all 3 application directions were significant.

Field strengths in the bare tendon condition are, for the application generating the highest field strength, about 5 times higher than in the entire distal limb condition. When comparing the slopes of the curves representing the relation of machine output current and the field strength (Figs. 4a,b), the slopes of the curves representing the same current directions were all significantly different in the bare tendon and distal limb conditions. In proximal-distal direction the slope has a coefficient of 1.07 in the bare tendon and of 0.13 in the entire distal limb ($p<0.005$). In medial-lateral direction, the slope was 0.76 in the tendon and 0.18 in the distal limb ($p<0.005$). In dorsal-palmar direction the slope was 0.26 in the tendon and 0.14 in the distal limb ($p<0.001$). These data indicate that an increase in output current will generate more effect in bare tendon than in an entire limb.

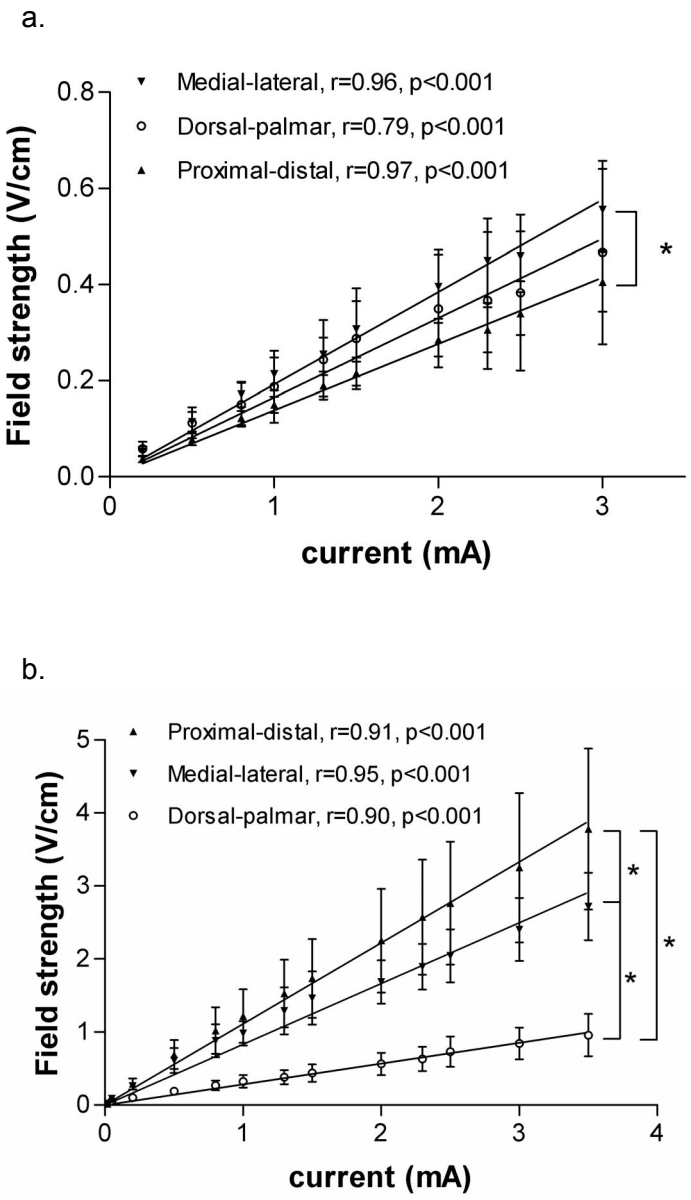

Figure 4. First order linear regression showing correlation between machine output and field strength in the tendon tissue. A. the condition using the entire distal limb; B. condition using the bare tendon. Values are expressed as mean ± SD. Linear regression was calculated by first order. V = voltage, A = current strength; r = correlation coefficient; p = significance level; * significant differences between directions of current application, p<0.01. Note the difference in scale of the Y-axes giving the false impression of a comparable relative increase in both conditions.

Discussion

The effect of the electrical stimulation of biological structures is related to the current flow through the target tissue. This study clearly demonstrates that the effective field strength in the target tissue may vary greatly with the plane in which the current is applied and with the tissues located between electrodes and target tissue. Main conclusion from this study is therefore that the current output of any therapeutic device has to be considered a very unreliable indicator of actual field strength in the target tissue, and hence of potential therapeutic effect. We did not vary the direction of the current, *i.e.* the positions of the negative and positive electrodes. To the authors' knowledge there is no data on possible semi-conductivity of biological tissues, but it is an item that might be addressed in future studies.

For the major part of the present study cadaver specimens were used. Previous research has shown that the electrical characteristics of dissected tissue gradually change over time with specific resistance rapidly decreasing within about two days after resection (Geddes and Baker, 1967). Taking this into consideration, the limbs and tendons used in this experiment were measured within 6 hours after death.

As more or less expected there were linear relationships between current and field strength in all conditions. This indicates that the bio-impedance of the tissue did not change with increasing applied current, at least within the relatively limited current range that was used in this study.

The current passed only through a single tissue type in the condition using the bare tendons. Since tendon tissue is homogeneous and has an almost uniform cross-sectional area, the slope of the curve is indicative of tendon resistance in different directions. The measured resistance was significantly higher in proximal-distal direction than in the other directions. This may have to do with the fact that the electric field generated within the tendon by the electrodes that were placed one above the other on the same side of the tendon was not homogeneous over the entire cross-section of the tendon with a higher field strength at the side of electrode placement. Further, due to this electrode configuration and in contrast to the other positions, the probes were not placed on a straight line between the electrodes. The resistance was least in dorsal-palmar direction. It can be speculated that this has to do with the packing density of the collagen fibres, which is not necessarily the same in dorsal-palmar and medial-lateral direction, or with differences in minor molecular components such as decorin that is bound to the collagen fibres and interacts with chains from neighbouring fibrils to form interfibrillar bridges (Scott, 1992; Vesentini et al., 2005).

The equine distal limb is not a homogeneous single structure, but is made up of skin, bone, tendons and a variety of connective tissue forms. The specific resistance of different tissues in the body is not constant (Kushner, 1992), therefore the distal limb cannot be viewed as a homogeneous conductor, and the overall resistance can be viewed as a circuit of in series and parallel connections of a number of shorter segments. In the proximal-distal and medial-lateral directions, most of the electric current will pass through skin and tendons. In dorsal-palmar direction, the third metacarpal bone is in the way. However, since the resistance of bone is rather high (Tzukert et al., 1983), the current might be bypassing the bone. The eventual effective field strengths were not significantly different for both applications in the transverse plane. In this context it should be noted that the cross-sectional shape of the SDFT is like a crescent and the real distance between the voltage probes in the medial-lateral direction is thus a little longer than the linear distance. As this presents a constant error independent of current strength, no correction was deemed necessary. However, real field strengths in the medial-lateral direction will be somewhat lower than indicated. The application in proximal-distal direction, *i.e.* along a line in the sagittal plane of the limb, resulted in lower corresponding field strengths, indicating a higher resistance in this direction.

In the voltage-current relationship no differences between a living pony and *ex vivo* limbs were found. The electrical impedance of a tissue is known to change with the load applied to that tissue (Suganuma and Nakamura, 2004), but in this case the pony was lying under anaesthesia and thus without loading the tendons. Blood flow could be supposed to have an effect in the living animal since MET increases local blood circulation. However, electrical conductivity of blood depends on flow volume and the relative contribution in tendons of blood to total volume of the limb is small, making that the effects of changes in blood flow volume probably can be neglected.

As expected, there were significant differences between the bare tendon condition and the entire distal limb condition in all directions. In the distal limb, the current has to pass through the skin and other tissues before reaching the target tissue. All structures have a certain resistance that can vary strongly per tissue type. For example, lipids of the stratum corneum provide the principal electrical resistance in the skin (Nicander et al., 1998). The sum of these smaller components makes up the total resistance. Depending on the direction of application chosen, this total resistance may be high enough to affect therapeutic application. According to our previous *in vitro* data,[a] 0.5 mA was found as a beneficial current strength to stimulate tenocytes without damaging them. It is recognized that there may be many reasons why a direct extrapolation of this data may be incorrect and it is acknowledged that additional research on the effectiveness of electric therapy must be performed before this data

can be used for clinical recommendations. However, this empirically found value may serve as an example. If applied to this experiment, field strength of 0.17 v/cm is required to generate 0.5 mA in dorsal-palmar direction in tendon, which means that 0.87 mA should be applied at the skin *in vivo*. For the medial-lateral application these values are 0.56 v/cm and 3.0 mA respectively. However, in the proximal-distal direction, the corresponding current for 0.63 v/cm in the leg was 4.7 mA, which already exceeds the maximum capacity of the machine. Therefore, the dorsal-palmar direction will be the most efficient in generating sufficient current to influence the tenocytes.

There is another point with respect to the direction of current application. Physiological electric fields can also control the direction of cell migration (Zhao et al., 2002). In general, cells orientated parallel to an electric field will retract, and reorient perpendicular to the electric field (Curtze et al., 2004). After injury tenocyte proliferation and collagen synthesis is randomly organized, which may result in the formation of inferior tissue. Creation of an electric field in dorsal-palmar direction, *i.e.* perpendicular to the longitudinal loading axis, might be helpful in tissue remodelling and realignment of cells and collagen fibres after tendon injury.

It is concluded that tissue composition and electrode placement of electrical therapeutic devices strongly affect the effective field strength in the target tissue. Knowledge about these effects is necessary for the further assessment of these devices with respect to their potential value for clinical use.

Footnote

a. Lin YL, Moolenaar H, van Weeren PR, et al. The effect of Micro-current Electrical Tissue Stimulation on cultured equine tenocytes. Am J Vet Res 2005; accepted.
b. APS MK 1.1, Medeusa, BV Bilthoven, The Netherlands
c. Vasocan, Braunüle, 20G 11/4", B. Braun Melsungen AG, Germany
d. TiePie engineering, Sneek, The Netherlands
e. Prism, version 4.0, GraphPad Software Inc, San Diego, CA.

Chapter VI

The use of tendon explants to assess the effect of *in vivo* applied extracorporeal shock wave therapy on equine tendons: a preliminary study

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Paper in preparation

Introduction

Tendon injuries are common in performance horses and account for significant losses to the equine industry. Apart from their frequent occurrence tendon lesions are notorious for their slow and imperfect healing (Watkins, 1999; McCullagh et al., 1979). Therefore, tendon lesions are the cause of untimely retirement of many horses and hence are a significant threat to equine welfare.

The poor healing tendency of tendons has been attributed to the relatively limited number of tenocytes compared to the extracellular matrix (ECM), as tenocytes are the sole cells responsible for the production, deposition, organization and maintenance of the surrounding extracellular matrix which is composed of mainly collagen and proteoglycans (Birch et al., 1997a). Following injury, tendons have an intrinsic capacity for repair through the ability of these cells to proliferate and produce matrix components. The initial reparative response is accomplished primarily by the resident tenocytes. Therefore, tenocyte metabolism is an important parameter in studies focusing on tendon healing.

Surprisingly, only little information on equine tenocytes is available. Studies thus far have focused mainly on morphology and distribution within the tendon (Webbon, 1977; Webbon, 1978; Goodship et al., 1994; Smith and Webbon, 1996; Crevier-Denoix et al., 1998). Reports on tenocyte metabolism are rare. Birch *et al.* (1997b) investigated the effect of hyperthermia on tenocyte survival after an earlier report that exercise-induced hyperthermia might be responsible for tendon degeneration (Wilson and Goodship, 1994). Murphy and Nixon (1997) showed that insulin-like growth factor-I (IGF-I) enhances collagen synthesis in a monolayer culture of normal equine flexor tenocytes. Equine tendon explants have been used earlier, but infrequently. Riley *et al.* (1996) were able to culture explants for 28 days, investigating which media would suit best for the culture of equine tendon explants. Dahlgren *et al.* (2001) used tendon explants to show that *in vitro* administered β-aminopropionitrile negatively affected matrix production by equine tenocytes. However, unlike in equine articular cartilage, where it was shown in explants that chondrocyte metabolism in explants harvested from OC-affected or exercised horses was different from that in explants from normal or unexercised horses (van den Hoogen et al., 1999a; 1999b), there are to the authors' knowledge no reports that tendon explants have been used to show differences induced by *in vivo* applied treatments.

Tendon tissue is known to respond to mechanical stimuli. Banes *et al.* (1999; 1995) showed that collagen synthesis increases with mechanical loading indicating that the biomechanical environment probably is an important contributor to tendon healing.

This is in line with reports in rabbits demonstrating the ability of tenocytes to alter morphology and matrix synthesis in response to mechanical load (Gillard et al., 1979; Malaviya et al., 2000). This responsiveness to mechanical stimuli may lie at the basis of the reported success of extracorporeal shock wave therapy (ESWT), which has become one of the most popular treatment modalities for equine musculoskeletal problems. Initial clinical application showed promising results in treating proximal suspensory desmitis (Caminoto et al., 2005; McClure et al., 2004) and tendon disorder of the SDFT (Kreling, 2001; Kersh et al., 2005). In experimental animals it has been shown that shock wave therapy increases the ultimate tension load of tendons and enhances hydroxyproline synthesis (Hsu et al., 2004; Orhan et al., 2001). Transforming growth factor-beta 1 (TGF-β1) and IGF-I, growth factors that are known to regulate tendon repair, were increased after shock wave application (Chen et al., 2004). These findings suggest that shock wave may bring tissue regeneration by triggering tendon metabolism, but there are thus far no data on this subject in the horse.

This study was designed as a preliminary study to evaluate the use of tendon explants for the assessment of ESWT that was applied *in vivo* to sound tendons. To this end we examined the rate of newly synthesized collagen and proteoglycans by measuring the incorporation rate of radioactively labelled proline and sulphate into *ex vivo* cultured tendon explants. Explants were harvested from various sites and various tendons and shock waves were applied using three different energy volumes. It was hypothesized that anatomical location and *in vivo* applied shock wave therapy would affect tenocyte metabolism as measured in explant culture.

Material and methods

Experimental animals

Six Shetland ponies (male, age 7 ± 3 years, average body weight 150 kg) were used. The animals were free of lameness and had ultrasonographically normal tendons and ligaments. The Ethical Committee of Utrecht University had approved the experiment.

Experimental design

The areas under investigation that were sampled after the experiment were:

1. the mid-region of the superficial digital flexor tendon (SDFT.m)
2. the origin of the suspensory ligament (SL.ins) at the third metacarpal bone and a site 1 cm distal to that origin (SL.p)
3. the middle part of the common digital extensor tendon (CDET.m), the insertion of the CDET (CDET.ins) on the extensor process of the 3rd phalanx coffin bone and a site 1 cm proximal to that insertion, or the distal CDET (CDET.d).

At day 1 of the experiment the ponies were sedated with detomidine 0.1 mg/100 kg (Domosedan®, Orion Corp., Espoo, Finland) and butorfanol tartrate 1 ml/100 kg (Torbugesic®, Fort Dodge, IA, USA) the SDFT.m regions of all 4 limbs, except for the left front limb that was left untreated, were shocked with the Equitron extracorporeal shock wave device (High Medical Technologies, Lengwil, Switzerland). Energy output was set at scale 6, energy flux density was 0.14 mJ/mm^2, and focal size of the shockwave in -6dB zone was 7.2 x 28.3 mm. A 5 mm probe was used. The number of shocks applied was 300 pulses in the left hind limbs (LH), 600 pulses in the right fore limbs (RF), and 1200 pulses in the right hind limbs (RH).

At day 42 the procedure was repeated, now shocking the CDET.m regions. Again, the left front limb was left untreated and the number of shocks was similar to the first session. Three hours after this last treatment the animal were humanely euthanised.

Sampling procedure and sample handling

Directly after euthanasia, the areas that had been treated were dissected free from surrounding tissues, taking care that sterility was maintained. Samples were surgically taken using a 11 scalpel blade and immediately placed in 15mL sterile tubes with pre-warmed culture medium for transportation to the laboratory. Each sample was cut into 3 pieces of approximately 2 mm^3 for culturing. The explants were cultured individually in 200 µl Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated foetal bovine serum (FBS), 0.85 mM ascorbic acid, and 50 U/ml streptomycin/penicillin medium in round bottomed 96-well polystyrene microtitre plates (NUNC, Roskilde, Denmark) for 1 day prior to metabolic labelling.

Determination of metabolic parameters

Labelling

One hour before labelling, the incubation medium was changed to a medium without serum. After that, 10µl DMEM containing 14.8 x 10^4 Bq $Na_2{}^{35}SO_4$ and 14.8 x 10^4 Bq L-[2,3-^{3}H]proline (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) was added. The labelling period was 4 hours. Then samples were rinsed 3 times for 15 minutes with PBS. Thereafter the samples were cultured for an additional 2 days in 10% serum in standard medium without label. After that, the culture media were collected and frozen at -20°C until further processing. After thawing, tendon samples were washed twice with 0.3 M NaCl to remove free label. Then tendon samples were lyophilized for 24 hours and dry weight was recorded. Specimens were frozen and stored at -80°C until further analysis.

Determination of DNA content

Thawed tendon explants were digested in 400 µl 0.2% collagenase in PBS, containing 0.4 mM phenylmethylsulphonylfluoride (PMSF) and 2 mM $CaCl_2$, for approximately 18 hours at 37°C. DNA content was measured according to Kim et al. (1988). Briefly, to 20 µl of collagenase-digested tendon sample 200 µl Hoechst 33258 dye solution was added, and fluorescence was measured immediately after mixing, using a LS-50 fluorimeter (Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, UK), with excitation at 366 nm and emission at 442 nm. Calf thymus DNA was used as a reference. Results were expressed as µg of DNA per mg dry weight.

Determination of newly synthesized GAG-release rate from extracellular matrix

Newly synthesized GAG that was released from the ECM into the culture medium was precipitated by addition of 50 µl 1 M sodium acetate (pH 5.2) and 750 µl 96% ethanol to 200 µl culture media (which was sampled over the 2 day culture period). Then samples were centrifuged at 13000 rpm for 10 minutes, and the GAG pellet was dissolved in 200 µl 30 mM SDS, 160 mM Na_2CO_3 and 80 mM NaOH. Two ml Lumagel (Lumac LSC BV, Groningen, The Netherlands) was added to the dissolved pellet and ^{35}S was measured by liquid scintillation analysis. Release of the newly synthesized GAGs was expressed as a percentage of the total amount of newly synthesized GAGs: [^{35}S]GAG released / ([^{35}S]GAG released + [^{35}S]GAG in ECM) x 100%.

Determination of GAG-, collagen- and protein synthesis

After measuring DNA content, GAG-, collagen- and total protein synthesis were determined. Non-collagenase digested peptides were precipitated with 10 % of trichloroacetic acid for 20 minutes, and centrifuged at 13,000 rpm for 10 minutes. The supernatant was collected and the pellet was dissolved in 400 µl 30mM SDS, 160 mM Na_2CO_3 and 80 mM NaOH. 2 mL Lumagel (Lumac LSC BV, Groningen, The Netherlands) were added to supernatant and the dissolved pellet and ^{3}H and ^{35}S were measured by liquid scintillation analysis. GAG- and total protein synthesis was calculated from the sum of ^{35}S and ^{3}H incorporation into the tendon explant and the release into the culture media. Collagen synthesis was calculated from L-[2,3-^{3}H]proline incorporation into collagenase-digestible protein. Data was expressed as the number of radioactive disintegrations per minute (dpm) per dry weight.

Statistical analysis

Data from three pieces of each site were averaged and taken as a representative value for each site. Differences between tendon sites were analyzed by one-way ANOVA. For the shock wave experiment, the data was expressed relative to the corresponding

control tendon. Differences between doses were tested by one-way ANOVA. In the shock wave treatment groups, a t-test was used to analyze possible long- and short-term effects. The level of significance was set at $p < 0.05$.

Tendon	Site	Collagen synthesis (dpm/mg dw)	Protein (dpm/mg dw)	GAG synthesis (dpm/mg dw)	GAG release ratio (%)	DNA (µg/mg dw)
SDFT	m	5674 ± 3024	8349 ± 4029	427 ± 193	23.23 ± 7.61	4.7 ± 1.4
SL	p	8229 ± 3549 [a]	12583 ± 3180 [b]	600 ± 191 [c]	20.17 ± 5.23	6.7 ± 2.3
	ins	5672 ± 2296	8425 ± 4244	522 ± 122	23.5 ± 6.59	5.6 ± 0.6
CDET	m	2462 ± 783 [a]	4621 ± 2735 [b]	277 ± 87 [c]	29 ± 5.33	5.1 ± 1.3
	d	4876 ± 944	6442 ± 641 [b]	419 ± 86	20.8 ± 4.15	5.3 ± 1.9
	ins	5027 ± 1412	7017 ± 1427 [b]	515 ± 76	26 ± 9.25	5.9 ± 2.4

Table 1. Mean value (mean ± SD) for newly synthesized collagen, praline and GAGs, and DNA content in tendon explants from the various tendons and sites sampled. Values different superscripts differ significantly from each other ($p<0.05$).
SDFT: superficial digital flexor tendon; SL: suspensory ligament; CDET: common digital extensor tendon; m: middle; p: proximal; ins: insertion; d: distal; GAG: glycosaminoglycans; dpm: disintegrations per minute; dw: dry weight; SD: standard deviation

Results

Anatomical location

No significant differences were found in DNA content and GAG release ratio for the different tendons and sites that were investigated. Significantly higher levels of collagen and GAG synthesis were found in the SL.p compared to the CDET.m. Other anatomical locations investigated were not significantly different with respect to collagen and GAG synthesis. Protein synthesis was highest in the SL.p and was significantly higher than the levels found in the different sites of the extensor tendon (table 1).

Shock wave treatment

No significant differences were found for any of the measured parameters between the different treatment regimens (300, 600 and 1200 pulses). Therefore, data were combined for further analysis.

Three hours after treatment a significant increase was found in DNA content and GAG, protein and collagen synthesis increased over 50% ($p<0.05$) (fig. 1). No significant short-term effect could be detected for GAG release (fig. 2). Six weeks after treatment, there was no effect on any of the parameters measured (fig. 1 and 2).

Discussion

In this study explants from various equine tendons were cultured *ex vivo* to assess the possible effects of the location where the samples originated from and of a treatment that had been applied *in vivo,* respectively three hours and 6 weeks before the samples were harvested. This approach had been used earlier successfully for the determination of proteoglycan metabolism in equine articular cartilage when assessing the effects of osteochondrosis and different exercise regimens (van den Hoogen et al., 1999a; 1999b). In the present study only little effect of treatment or location could be demonstrated using this technique. The reason for this can obviously be twofold: either there was indeed much less effect than expected, or the technique was not sensitive enough to signal existing differences. It cannot, on the basis of the present study, be stated with certainty which of these possibilities, or a combination of the two, is true, but there are reasons to believe that the sensitivity of the technique is limited indeed. The tendons were treated once and then left in place for either 6 weeks (long-term effect), or 3 hours (short-term effect). After euthanasia, all samples were treated and cultured in an absolutely identical manner.

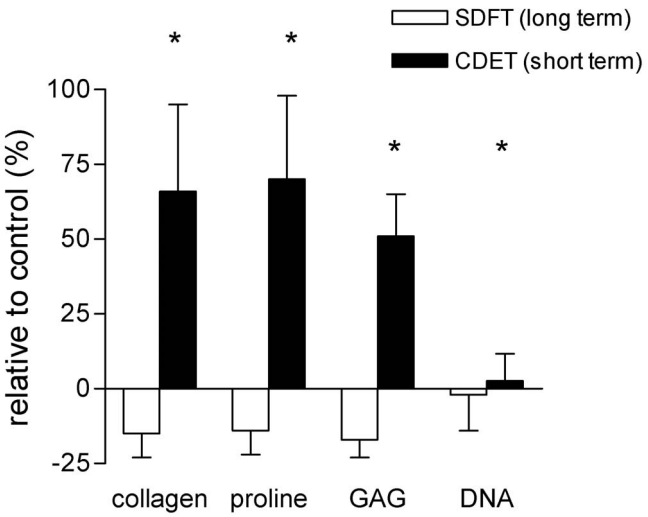

Figure 1. Effect of *in vivo* shock wave (ESWT) treatment as measured through the metabolism of *ex vivo* cultured explants. ESWT was applied 3 hours (short-term) and 6 weeks (long-term) before harvesting of the explant. All measured parameters were expressed as a percentage with respect to the untreated controls. Values are expressed as mean ± SE. *: $p<0.05$

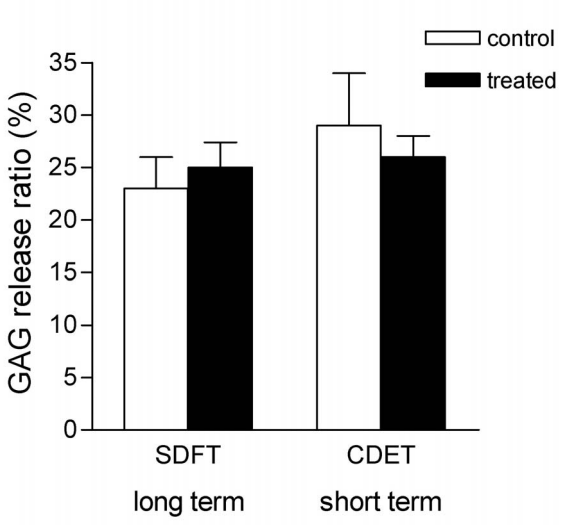

Figure 2. Glycosaminoglycan release by tendon explants cultured *ex vivo* after prior *in vivo* treatment by ESWT 3 hours (short-term) and 6 weeks (long-term) before harvesting of the explant. Values for GAG release are expressed as a percentage of the total GAG synthesis by the explant.

Given the ability of fibroblast-like cells to regenerate and even to dedifferentiate within a short time, it may be not surprising that any differences in metabolic activity induced by shock wave treatment, or due to another site of origin, will be levelled out quickly once the cells are treated in a similar fashion. Arguably, the same would apply to the chondrocytes used in the studies by van den Hoogen *et al.* (1999a; 1999b), but in that case the conditions where they had been subjected to (exercise level and/or presence of osteochondrosis) had existed for many months and were not just single treatments.

If indeed sensitivity of the technique is less than hoped for, the results in this study will most likely be an underestimation of the real effects and false negative results can be expected. On the other hand, false positive results are, in that case, very unlikely to occur.

The biosynthesis in the SL at 1 cm distal to the insertion was found to be higher than at other locations and this difference was significant for collagen and overall protein synthesis in comparison with the entire CDET and for GAG synthesis in comparison with the mid-region of the CDET. The reason for this may be the character of the suspensory ligament, which is a modified muscle and not a real tendon. The difference will become greater towards the centre of the structure, *i.e.* more in distal direction and can be expected to be more pronounced in Shetland ponies than in horse breeds as in Shetland ponies, apart from the existence of other atavisms, it is not uncommon to find a considerable amount of muscle fibres in the suspensory ligament. Apart from the possible influence of muscle fibres, ligaments and tendons are not entirely identical. In rabbits, Riechert *et al.* (2001) found a higher proportion of type III collagen in ligaments as opposed to tendons, which is representative of a more metabolically active state and a concomitant more rapid formation of intra-and intermolecular crosslinks to stabilize the fibrils.

No difference in biosynthesis was found between the SDFT and CDET, although there are distinct differences in biomechanical and biochemical properties between these two tendons (Batson et al., 2003). This is in line with an *in vitro* study of human tendons in which it was shown that there was no difference in the pattern of secretion of total protein or collagen by flexor or extensor tendon cells (Evans and Trail, 2001). In contrast to what was reported by Batson *et al*. (2003), we could not detect a difference in DNA content between the middle sections of the CDET and the SDFT.

The basically similar biosynthetic rate in the different anatomical locations was not unexpected. Tenocytes are generally divided into 3 different categories, based on their morphologic appearance: type 1 are the characteristic elongated cells with spindle-shaped nuclei; type 2 concerns rounder cells with plumper, cigar-shaped nuclei,

which are considered to be more immature and are also called fibroblasts; type 3 tenocytes are more chondrocyte-like and found in fibrocartilaginous areas that experience both compressive and tensional forces (Smith and Webbon, 1996). Little research has been done on the metabolic characteristics of these cell types, but it is generally accepted that type 2 tenocytes are more metabolically active. In agreement with this idea were recent findings in an immunohistochemical study that showed that interstitial collagenase (matrix metalloproteinase 1; MMP-1) was more associated with tenocytes type 2 than with type 1 (Chuen et al., 2004). In the present study no areas were studied subjected to compressive forces, therefore it can be assumed that type 3 tenocytes did not play a significant role. An age-related change in distribution, with a decrease in type 2 cells when ageing, has been reported (Webbon, 1978; Crevier-Denoix et al., 1998), but the ponies of the study population were all mature animals within a relatively narrow range. This leaves anatomical site as the only source of possible differences in tenocyte type ratio. Such a difference in ratio would only significantly influence outcome of the explant as a whole if the differences in cell ratios were very substantial and if the conditioning and handling of the explants would have no levelling effect, as suggested above.

The use of ESWT in the horse has gained large popularity recently, which is mainly based on the reportedly favorable outcome of a number of clinical trials in which shock waves were applied to varying components of the musculoskeletal system (Caminoto et al., 2005; McClure et al., 2004). The underlying events have been studied to some extent in other species than the horse, from which studies it became clear that the biological effect was critically dependent on the way of application, especially on the total energy volume delivered. Tissue damage as a result of a too high energy level has been reported. Shock wave treatment with 500 and 1000 impulses of 0.16 mJ/mm^2 elicited inhibitory effects on tendon repair in Achilles tendonitis (Chen et al., 2004). In rabbit Achilles tendon, an increase in tendon diameter, fibrinoid necrosis and inflammation were found following shock wave application with 1000 impulses of 0.6 mJ/mm^2 (Rompe et al., 1998). Oedema within the paratenon of the quadriceps tendon was seen after treatment with 1500 impulses of 0.5 mJ/mm^2, and an increase in the number of inflammatory cells within the paratenon and a decreased the tensile strength of the tendon itself at 1.2 mJ/mm^2 (Maier et al., 2002). The energy setting used in this study was 0.14 mJ/mm^2 and equal for all applications, but the number of shocks, and hence the total energy volume applied to the tissue, varied from 300 to 1200. In equine clinical practice, an energy setting of 0.14 mJ/mm^2 can be considered normal and the number of shocks that would have been applied under clinical conditions would have been in the order of 500-600 (McClure et al., 2004).

In the present study no differences could be demonstrated that were related to the differences in total energy input. There are basically three explanations for this. First, it may be the case that a threefold difference in total energy input indeed does not induce differences in the response of the tenocytes to shock wave. This explanation is, however, unlikely given the results in other species where deleterious effects could be demonstrated of shock wave applications with numbers of shocks and total energy input comparable to the highest dose in this study (Chen et al., 2004). Second, the insensitivity of the explant culture technique as used in this study and alluded to earlier may have played a role. Third, except for the CDET.m and SDFT.m sites, all sites investigated were close to bone. It is conceivable that at those sites a considerable amount of energy has been absorbed by the bone and not by the tendon. Studies into bone structure and composition at those sites are currently being performed to test this hypothesis.

Apart from the effect of dose, there was no long-term effect of ESWT application on tenocyte metabolism, but in the short-term (3 hours after application) there was a significant increase in DNA content, GAG, and protein/collagen synthesis. This effect was measured despite the apparent insensitivity of the *explant* culture system, indicating a considerable stimulating effect of ESWT application on tenocyte metabolism, at least fro a short while.

The long-term findings corroborate those in sound tendons of rabbits (Maier et al., 2002; Rompe et al., 1998), in which no significant differences in histopathological characteristics could be demonstrated compared to a non-treatment control group at day 28 after shocking with higher energy flux density and/or more pulses than in the present study. Also, in an *in vitro* study a decrease in viability 1 hour after shock wave application was shown, but already after 24 hours no difference in the 48 hours growth potential could be demonstrated anymore between viable shock wave treated cell and control cells (Johannes et al., 1994). It may be not surprising therefore that the initially stimulatory effect of shock waves as evidenced in the short-term part of this study did not last for 4 to 6 weeks.

It is not entirely clear what causes the stimulation of the tenocytes that was demonstrated shortly after shock wave application. A probable cause is the mechanical stress induced by the shock wave and the ensuing cavitation process, thus making use of the responsiveness of fibroblast-like cells that are entangled in an extracellular matrix to deformations of that matrix. The effect might also be due to a minor or temporary acute inflammatory reaction after shock wave treatment. Both protein and GAG metabolism were stimulated, which may be indicative of an overall higher level of activity of the tenocytes, rather than of a specific response. There was no significant

effect on GAG release rate after shock wave treatment. This finding implies that, although GAG synthesis is influenced by shock wave, the turnover rate of GAG is not affected and the proportion of GAG built into the matrix is the same.

It is concluded from this study that the use of *ex vivo* cultured explants from equine tendons to evaluate the effects of a treatment that has been applied earlier *in vivo* is, at least in the way it was performed in this study, not a very sensitive technique. The main reason for this is believed to be the characteristic of fibroblast-like cells to level out individual differences once they are subjected to a similar treatment for some time. This observation to a certain extent limits the interpretation of the results presented in this study concerning the effects of shock wave application to sound equine tendons. Given the supposed low sensitivity of the technique, minor differences may have been missed easily and false negative results are likely. At the other side it lends, however, credibility to the positive results obtained. These concerned principally an overall stimulatory effect on tenocyte metabolism shortly (3 hours) after application of shock waves. These results are in agreement with findings in other species and may be an explanation for the positive clinical effects reported in various studies performed in the horse. The results from this study and those in the clinical trials are far from conclusive yet, but certainly warrant further studies at histological, biochemical and ultrastructural levels to evaluate and to quantify the effects of shock wave application in the horse. Such studies are indispensable to understand the pathways and mechanisms through which shock waves may act and to come to a judicious establishment of science-based criteria for the indications for their use and the mode of application.

Chapter VII

General Discussion

After enjoying the hospitality of the goddess-witch Circe for a year, Ulysses and his crew left the island of Aiea and set sail for the Island of the Sun, their next stop on their long and hazardous journey back from Troy to their homeland Ithaca. On this journey Ulysses had to pass between Scylla and Charibdis, the first a dangerous cliff inhabited by a six-headed monster, the second a whirlpool from which no vessel had ever reappeared. He manages to avoid Charibdis by keeping close to Scylla, but sees six of his men eaten by the monster (approx. 800 BC).

Passing between *Scylla* and *Charibdis* is something as choosing between the bad and the worse. It is a dilemma a veterinary surgeon is often faced with when confronted with new therapies for conditions that are notorious for healing badly. Old therapies are known to be of limited effectiveness, the outcome of new ones is at best unsure.

One solution to solve this dilemma is to conduct well-designed clinical effectiveness studies and to come to so-called *evidence-base medicine*, as currently strongly propagated (Rossdale, 2003). Although the introduction of evidence based medicine in veterinary practice certainly is a great leap forward, clinical proof of therapies that seem to lack a scientific basis is still hard to digest for critical minds and it may, due to various confounding and biasing factors, take many of those studies to come to a final conclusion, as was nicely illustrated by the recent discussion on homeopathy (Vandenbroucke, 2005).

The final, but by far the most difficult and time-consuming solution to this problem is the complete understanding of the biological processes underlying physiology and pathology of specific disorders and to demonstrate the pathways through which given therapies work, or can be expected to never work. This *science-based medicine*, which still needs confirmation through clinical trials, will give the definitive answer, but it is, not unlike Ulysses' journey a difficult, long, hazardous and expensive way to go.

To understand the essential characteristics of the equine tendon, the triangle biomechanical loading – extracellular matrix (ECM) – tenocytes is central. Thanks to the material properties of the ECM, the tendon can withstand the extremely heavy biomechanical challenges it is faced with. The ECM is produced by the tenocytes, which, in their turn, are driven by biomechanical loading, thus closing the circle. A deep insight in the interactions within this triangle, and of the effect (either disruptive or stimulating) of external influences thereon, is essential for the understanding of tendon physiology and tendon pathology, and for providing the rationale for any therapy.

In this thesis the different sides of this triangle are the subjects of study. In the more fundamental first half it is the relationship between ECM composition and loading that is the central theme. The second part tries to assess the effect of two therapy modalities on cultured tenocytes, in an attempt to contribute to the bridging of the gap between basic science and applications.

Extracellular matrix and loading: a reciprocal relationship

Developmental aspects

In articular cartilage of lambs and foals it has been shown that the biochemical composition of their ECM was homogeneous at birth, leading to the concept of a "blank" joint that would be moulded later on through the influence of biomechanical loading (Little and Ghosh, 1997; Brama et al., 2000a). Following a similar line of thought, it was conjectured that the same principle would apply to the equine superficial digital flexor tendon (SDFT). This, however, appeared not to be the case with respect to all biochemical parameters studied (***Chapter II***).

DNA, glycosaminoglycans (GAGs) and pentosidine cross-links were not the same at the two differently loaded sites that were investigated (the mid-metacarpal area subjected to tensile stress only and the sesamoidean area subjected to both tensile and compressive forces). These differences were attributed to movements *in utero*, which can be presumed to have more effect on tendons than on articular cartilage.

Notwithstanding the falsification of the hypothesis of the entirely "blank" tendon, the general concept held. The abrupt changes in loading of the tendons at birth, and probably also the age-related changes in exercise levels in the juvenile period had clear effects on ECM composition. ECM composition at birth was very different from that in the mature horses, and it was shown that fast and substantial changes in all components of the matrix occurred in the period of growth and development after birth. These changes can only be effectuated through the tenocytes, of which certain versatility in young individuals has been shown earlier. Foetal or neonatal tenocytes can be manipulated easily and are to a certain extent pluripotent. Neonatal and foetal bovine tendon cells have been shown to have the potential to follow a tenocytic or chondrocytic pathway (Perez-Castro and Vogel, 1999) and mechanical strains can induce further proliferation (Zeichen et al., 2000). Also tenocytes from equine foetal tendons have been shown to have the potential to follow different pathways, depending on how they are stimulated (Batson, 2002).

The rapid and profound loading-induced changes in matrix composition during the early juvenile period will result in concomitant changes in material properties, and hence in the capacity to sustain load (Batson et al., 2003). Composition of the tendon in biochemical terms and (ultra-)structure are intricately linked in this process. The overall increase in GAGs and hyaluronic acid (HA), as shown in ***Chapter II***, may be related to the changes in collagen fibril diameter that were no subject of study in this thesis, but have been shown in earlier work (Parry and Craig, 1988; Cherdchutham et al., 2001a). In the SDFT, these components may be of special importance as the elastic SDFT is

characterized by a larger population of small diameter fibrils than other tendons such as the deep digital flexor tendon (DDFT) or the common digital extensor tendon (CDET), and GAGs may have a role in the regulation of fibril diameter by preventing lateral growth (Scott, 1990). Total collagen content did not change much, but the rapid decrease of degraded collagen and increase of the mature HP crosslinks are indicative of high turnover and might be related to the replacement of immature crosslinks by mature ones in collagen fibrils that have reached their final size. This will affect the biomechanical properties of the tendons. Equine SDFTs have been shown to gain in strength during development and growth in the early juvenile period, a process that was significantly influenced by the exercise level the animals were subjected to (Cherdchutham et al., 2001b).

Ageing

Adaptive capacity and versatility are terms that are usually not related to ageing and the elderly. In articular cartilage, the process of the functional adaptation of the ECM to loading seems to go on, at a rate that decreases rapidly after birth, until the age of approximately 4 years and no major physiologic change can be found after that (Brama et al., 1999; Vachon et al., 1990). In analogy to what had been done earlier in cartilage, ***Chapter III*** focused at loading-related regional differences in mature individuals, and the possible changes with ageing.

ECM composition differed widely between the areas subjected to tensional load only and to a combination of tensional and compressive load. This applied to both collagenous and non-collagenous parameters, the only biochemical parameter not showing such a difference being the amount of mature HP crosslinks. Although not unexpected, and perhaps even more predictable in tendons than in cartilage because of the more obvious anatomical differences between regions, the observations once more stress the general concept of form following function, as pointed out earlier at the level of different (flexor and extensor) tendons (Batson et al., 2003).

This concept in itself is not new, as it was formulated more than a century ago for bone (Wolff, 1892). However, the implications for the various tissues that constitute the musculoskeletal system are not the same. Bone retains the capacity to remodel throughout life, but articular cartilage does not, as indicated by the static situation after maturity has been reached. Tendon tissue appeared to behave like articular cartilage (***Chapter III***). None of the biochemical parameters measured showed an age-related change, except for pentosidine crosslinks, which are products of a non-enzymatic and solely time-dependent process. This steady state may reflect an adequate basic activity of the tenocytes that under physiological conditions succeed in timely adaptation and

remodeling of the ECM, keeping the levels of the constituents constant. However, it also implies that adult tendon does not have the same potential as immature tendon to remodel the matrix in response to alterations in the mechanical environment, leading to an increased risk of degeneration and injury (Tuite et al., 1997). Indeed, there are strong indications that accumulating micro-damage resulting from the disintegration of large diameter fibrils into fibrils with smaller diameters in the core regions of both the equine SDFT (Patterson-Kane et al., 1997c) and the human Achilles tendon (Birch et al., 2001) lies at the basis of clinical tendinopathy. The present study supported this presumed pathway by demonstrating significantly higher amounts of degraded collagen in the mid-metacarpal region, which is the predilection site of tendon injuries (Genovese, 1993; Webbon, 1977), as opposed to the sesamoidean area where central core lesions hardly occur. Also, higher collagen type III levels (Birch et al., 1998; Webbon, 1977), and the lack of pentosidine accumulation (this study) are indicators of, repetitive loading-induced, micro-damage affecting of the collagen network. Once a certain threshold level has been passed, the limited repair capacity of the mature tendon will be exceeded, resulting in clinical injuries characterized by long healing periods and repair tissue of inferior quality (Watkins, 1999).

Does the application of non-invasive physical therapies affect tenocyte metabolism?

Tendon healing is a painstakingly slow process with frustration as the most frequent result. Tendon repair is accomplished primarily by the resident tendon fibroblasts or tenocytes, which play the key role in the initiation and effectuation of the regenerative responses following injury or degeneration. Because improving the quality of repair by promoting the natural healing processes should always be preferred above artificial interventions such as surgery, improving the metabolic activity of tenocytes or enhancing their regenerative response capacity are natural targets for therapies aiming at improving tendon repair.

Most physical therapy modalities involve the application of some form of physical energy to the tissue, which is expected to result in therapeutic benefit. If this indeed is true, the only mediator through which this energy might influence tissue composition (and hence material properties) is the tenocyte. In our quest for the biological basis of therapies that are advocated to influence tendon healing merely on empirical grounds, we focused on the effect of two treatment devices, micro-current electrical tissue stimulation (METS) and extracorporeal shock wave therapy (ESWT) on tenocyte metabolism.

Micro-current electrical tissue stimulation (METS)

Endogenous electrical currents are known to influence biological processes at the cellular level. For this reason, a large number of devices that generate various types of currents to tissues with the aim to engender a therapeutic effect have been developed with varying success. Micro-current stimulation is a form of bio-electric stimulation as it uses currents in the micro-ampere (μA) range, which are similar to the currents generated during physiologic processes such as the depolarization of cell membranes. This type of therapy is therefore potentially of interest when stimulation of cellular activity is aimed at. The results of ***Chapter IV*** show that micro-current is indeed able to increase cell proliferation, DNA synthesis and protein content. This is in line with previous findings in fibroblasts (Bourguignon and Bourguignon, 1987), chondrocytes (Lee et al., 1982; Rodan et al., 1978) and osteoblast-like cells (Ozawa et al., 1989). However, it became clear that the metabolic effects depend critically on the strength of the current, as shown earlier in fibroblasts from rat skin (Cheng et al., 1982). The margins were small, seemingly minor changes in current level and/or frequency of application could change a beneficial effect at cell level into a negative effect. The apoptosis rate appeared rather sensitive for electric currents, which might due to irreversible membrane permeabilisation, resulting in detachment and finally cell death (Blumenthal et al., 1997). In this context it is important to realise that tenocytes are the sole cells responsible for the repair process; which means that loss of even a relatively minor quantity of these cells may already severely impair the repair capacity of the tissue. Electric therapy should thus be seen as a two-sided sword, and the “therapeutic index” of METS application might be very low. The application of a proper dose of micro-current may act as a light knock to the cell, prompting higher activity, but already slightly higher current levels (about 1mA more) may prove to be a big blow, resulting in knock-out.

The narrow safety margin for METS application as found in ***Chapter IV*** urges utmost care when applying this or similar techniques *in vivo* where conditions are vastly different from those *in vitro*. This big step should not be taken lightly and not without much preparatory work. One of the preparatory studies is done in ***Chapter V*** that focuses on the relationship between transducer placement and actual field strength in the target tissue, *i.e.* the tendon. Results were unequivocal and showed linear relationships between current and field strength in all conditions. This indicated that the bio-impedance of the tendon and leg did not change with variations in applied current, at least not within the relatively limited current range that was used in this study. It was further shown that the effective field strength in the target tissue varies with the plane in which a given current is applied and with the tissues located between electrodes and target tissue. Therefore, the current output of any therapeutic device should be considered a very unreliable indicator of actual field strength in the target tissue, and

hence of potential therapeutic effect. In the equine distal limb, electrode placement in dorsal-palmar direction will be the most efficient in generating sufficient current to influence the tenocytes.

Extracorporeal Shock Wave Therapy (ESWT)

In ***Chapter VI*** it is again the tenocyte and its metabolism that is the central theme. In this chapter it is tried to assess the effect of a single, *in vivo* applied shock wave treatment on the metabolism of tenocytes cultured *ex vivo* in explants. The leading thought in this set-up is that, by culturing as explants, the tenocytes remain in their natural environment, surrounded by extracellular matrix. As in articular cartilage, this will prevent differentiation of tenocytes into fibroblasts. The use of tendon explants in general and of explants of equine tendons in particular has been limited thus far and it had not been tried earlier to use this technique for the evaluation of an *in vivo* applied therapy. In articular cartilage, the method had produced valuable results when assessing the effect of different exercise regimens and the presence of osteochondrosis (van den Hoogen et al., 1999a; 1999b). However, in that case conditions that had existed for many months were tested whereas in the present study single treatments were evaluated, applied shortly (3 hours) or much longer (6 weeks) before harvesting of the explants.

A short-term stimulatory effect on tenocyte metabolism, reflected in an increase of both the protein and glycosaminoglycan synthesis, could be demonstrated and there was a difference in metabolic activity between tissue originating from a site on the suspensory ligament compared to the other sites and tendons samples were taken from. However, overall differences in metabolic activity were less than expected and no influence of various doses of shock waves could be demonstrated. It was concluded that the technique has a relatively limited sensitivity, which was blamed on the well-known characteristic of fibroblast-like cells to level out any morphological or metabolic differences once they are treated in a similar fashion. It might thus be that the identical processing of the samples after harvesting had such an effect, obscuring any differences in metabolic activity that might have been induced by the different shock wave regimens. The same reasoning applies of course to the long-term part of the study: the versatility of the tenocytes make it unlikely that the effect of a single treatment on cell metabolism will still be measurable after 6 weeks.

It is further concluded from this study that the stimulating short-term effect, that was evident despite the apparent insensitivity of the measuring system, together with the reported beneficial effects in a number of clinical studies warrants further investigations into the effects of shock waves at the tissue and molecular level in order to elucidate the pathways that lead to the reported clinical effects.

Limitations to this study

There are a number of factors and circumstances that put some limitations to the work presented in this thesis. With respect to the analysis of the ECM, only biochemical parameters have been measured. As with any building, the mechanical qualities of tissue are determined by both the materials used and the way these are spatially arranged and interconnected, *i.e.* the structure of the tissue. In case of connective tissues that have an important mechanical function such as articular cartilage and tendons, structural aspects may be even more important than biomechanical composition. Analysis of structural aspects was beyond the scope of this thesis, but the detailed study of the (ultra-)structure and fibril arrangement and the consequences thereof for signal transmission to tenocytes deserves probably more attention in tendon research than it has received thus far.

The samples that were used to determine biochemical composition in ***Chapters II*** and ***III*** were only partly of known origin. The samples of the 5- and 12-month-olds came from animals that had participated in a very well-documented research project that focused on the influence of exercise on the development of the equine musculoskeletal system in general and on the development of osteochondrosis (OC) in particular (Van Weeren and Barneveld, 1999). These animals had a known exercise history, and it was of course obvious that the new-born animals had no such history, but the 36-month-olds did not have a known exercise history. The same applies to the series of mature horses used in ***Chapter III***. Although it can be assumed that numbers were high enough to compensate for individual differences and all tendons were macroscopically free from any pathology, use of samples from animals with a known exercise history and pathological record would have provided more focused information.

The application of METS in ***Chapter IV*** was to equine tenocytes cultured in monolayers. Tenocytes are modified fibroblasts that may dedifferentiate and become fibroblasts again, as do chondrocytes, when they are not cultured in the form of explants or using special devices such as agarose beads. The number of passages of the cultured cells was standardized and limited, but it cannot be said with certainty to which extent the reaction pattern of the cultured tenocytes is identical to that of tenocytes *in situ.*

In ***Chapter VI*** a comparable problem was encountered. Here, tenocytes were cultured in explant form and not as monolayers, but the explants that were harvested at the end of the shock wave experiment were all treated in an identical way for some time during and before the measurement of their metabolic activity. It is possible that this

treatment may have influenced cell metabolism, levelling differences that may have existed when they were still *in situ.*

It is realized that restrictions and limitations are inherent to any form of research and that they can not always be avoided. Their presence should, however, be acknowledged and they should be taken into account when interpreting the data.

And in the end….does the story end?

When Ulysses finally reached his homeland Ithaca as the sole survivor of his crew, he found his goods stolen and his faithful wife Penelope besieged by a group of drinking and feasting noblemen. These men, to whom he had entrusted his kingdom when leaving, pretended to marry her and to become the future king. When the Pretenders were gathered in the hall of the castle, Ulysses took his bow, which had been hanging there for 20 years without anybody being able to tighten it, and, aided by his son Telemachus, killed the untrustworthy until the very last (Homer approx. 800 BC).

It has become clear, from this study and many others, that, similar to the situation in cartilage, tendon ECM is able to adapt to mechanical loading and will have to do so in order to function properly. The tenocytes are spiders in the ECM web, signaling and integrating the stimuli received by them and translating them into modifications of the network with ensuing changes in material properties. Stimuli may include biomechanical loading, but also directly applied current, as in METS, or other forms of extraneous energy such as acoustic energy in ESWT.

This basic concept is the fundament of the adaptation of tendons during growth and development, the occurrence of pathology when the system fails, and the response to treatments. However, reality is much more complex than this simplified concept. Elucidation of the pathways along which responses to the stimuli are generated, understanding the molecular mechanisms involved and the identification of crucial elements in these processes that could be targets for therapeutic intervention, are all challenges the researcher in this field is confronted with and in fact form the pieces of a big puzzle. This thesis has contributed to making this puzzle by focusing on same basic aspects of tendon extracellular matrix and the metabolic response of the tenocyte to external stimuli. It may seem that there is no limit to the number of pieces, and the puzzle is certainly not finished yet. However, there will be an end to the puzzle some day as there was an end to Ulysses' seemingly endless voyage. Then, the scientific basis, or lack thereof, of therapies will become clear and the ones that cannot stand the test will be discarded as merciless as Ulysses treated the Pretenders. The road to Ithaca is still long, though.

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Samenvatting

Inleiding

Blessures van de oppervlakkige buigpees bij het paard komen frequent voor en vormen een belangrijke bedreiging voor zowel de atletische carrière van het paard, als voor het welzijn van het dier. Wil men tot een verdieping van het inzicht in zowel de fysiologische processen als in de mogelijke oorzaken van pathologische afwijkingen komen, hetgeen noodzakelijk is voor een adequate therapie en preventie van peesletsels, dan is een gedegen kennis van de normale ontwikkeling van de componenten waaruit de extracellulaire matrix (ECM) van de pees bestaat een eerste vereiste. Een vergelijkbare fundamentele kennis met betrekking tot het reactiepatroon van de peescel of tenocyt is nodig indien men de vele therapeutische mogelijkheden die voor peesherstel aangeprezen worden, wil evalueren.

Doelstellingen van dit proefschrift

Het eerste deel van dit proefschrift betreft een diepgaande analyse van de veranderingen die optreden in de samenstelling van de ECM in het traject van geboorte tot volwassenheid, en in het latere traject van veroudering. Om dit te verwezenlijken is gebruik gemaakt van een groot aantal weefselmonsters van pezen die genomen waren van een grote groep van dieren van verschillende leeftijden (van pasgeborenen tot zeer oude individuen) die géén macroscopisch zichtbare peesletsels hadden. Er is hierbij bewust op twee plaatsen gemonsterd waarvan bekend was dat deze op verschillende wijze belast werden: het uitsluitend op trek belaste mid-metacarpale gedeelte van de oppervlakkige buigpees en de regio ter hoogte van het kootgewricht die op rek én op druk belast wordt.

In het tweede deel van het proefschrift staan de tenocyten in het centrum van de belangstelling. Deze cellen zijn doorgaans weinig actief in gezond peesweefsel van volwassen dieren, maar dienen geactiveerd te worden op het moment dat er peesschade optreedt. In de loop der jaren heeft men al vele niet-invasieve fysische therapieën toegepast om een dergelijke activering of recrutering van cellen te bewerkstelligen, maar er bestaan slechts weinig wetenschappelijke gegevens met betrekking tot hun effectiviteit op cellulair niveau. In dit proefschrift werden schokgolftherapie en een electrische stimulatie techniek die gebruik maakt van stromen in het microampère bereik (de zogenaamde actiepotentiaal simulatie of APS) gebruikt voor het vaststellen van de biologische effecten van dit soort therapieën.

Ontwikkelingsbiologie van de pees

In hoofdstuk II wordt nader ingegaan op de ontwikkelingsaspecten van de pees waarbij de nadruk gelegd wordt op die onderdelen van de extracellulaire matrix die bij uitstek van belang zijn voor de biomechanische sterkte van de pees. Tegen de verwachting (die gebaseerd was op al bekende gegevens betreffende de ontwikkeling van gewrichtskraakbeen) in werd er geconstateerd dat de pees bij de geboorte niet beschouwd kan worden als een homogeen, “blanco” weefsel dat geen verschillen in biochemische samenstelling vertoont. Er werden namelijk al bij pasgeboren veulens significante verschillen in ECM samenstelling gevonden tussen verschillend belaste plaatsen. Aangenomen werd dat deze veroorzaakt werden door intra-uteriene bewegingen die immers meer invloed op pezen zullen hebben dan op kraakbeen dat überhaupt niet belast wordt totdat het dier op zijn of haar eigen benen staat. Wat wel hetzelfde bleek te zijn als bij kraakbeen was dat er in de eerste periode direct na de geboorte grote veranderingen in de samenstelling van bijna alle ECM bestanddelen optraden. Dit zeer actieve ombouwproces heeft zonder meer gevolgen voor de belastbaarheid van de pezen, hetgeen een niet onbelangrijke observatie is gegeven het feit dat in verschillende takken van paardensport de dieren al heel jong zware belastingen dienen te ondergaan.

Veroudering

In hoofdstuk III worden dezelfde technieken als uit hoofdstuk II gebruikt om te zien wat er ná de volwassenheid, dus gedurende het proces van veroudering gebeurt. Het blijkt dat er in deze periode geen essentiële veranderingen in de ECM samenstelling meer optreden. Op dat moment is de samenstelling van de ECM al aangepast aan de belasting die er gevraagd wordt en vermindert de metabole activiteit van de tenocyten tot op een zeker basaal niveau. In het mid-metacarpale gebied is er een hogere collageendichtheid dan in de kogelregio hetgeen duidt op een grotere belastbaarheid op rek. In de mid-metacarpale regio is er echter ook sprake van een grotere hoeveelheid beschadigd collageen en blijkt het zo te zijn dat de (normale) tijdsgerelateerde accumulatie van producten van de niet-enzymatische glycosylering (NEG), zoals pentosidine cross-links, niet optreedt. Beide bevindingen duiden op het bestaan van een zekere mate van degeneratie en (subklinische) schade. Dit komt overeen met de veel grotere incidentie van klinische letsels in deze regio in vergelijking met de kogelregio waar minder beschadigd collageen te vinden is en er wél accumulatie van NEG-producten optreedt.

Actiepotentiaal Simulatie (APS)

Bij vele fysiologische processen worden electrische stromen gegenereerd. Qua sterkte bevinden deze zich in het microampère bereik. Om deze reden ligt een eventueel positief therapeutisch effect meer voor de hand in geval van apparatuur die met deze stroomsterkten werkt, dan in geval van apparatuur die met veel zwaardere stromen werkt, zoals met name in het verleden veel gedaan werd. Deze overweging leidde tot de keuze van een APS-apparaat voor het bepalen van het effect van electriciteit op tenocyten. In hoofdstuk IV wordt aangetoond dat tenocyten inderdaad gevoelig zijn voor electrische stimulatie. Het bleek echter dat het stroombereik waarbinnen er een positief effect gevonden kon worden relatief beperkt was. Kwam men boven een bepaalde stroomsterkte, dan veranderde de stimulatie van de celproliferatie en de eiwitsynthese in een remming en ook nam het aantal cellen dat aan apoptose ten onder ging duidelijk toe. Met name dit laatste effect zou het helend vermogen van het peesweefsel sterk nadelig kunnen beïnvloeden. Er werd dus geconcludeerd dat APS zeker een potentieel bruikbare techniek is, maar dat er zeer behoedzaam mee moet worden omgesprongen wil de positieve werking niet omslaan in een negatieve. De techniek kan derhalve nog niet aanbevolen worden voor klinische toepassing.

Het blijkbaar nogal beperkte bereik waarbinnen electrische stroom toegepast kan worden, maakt de vraag in hoeverre uitwendige omstandigheden de uiteindelijke veldsterkte in het weefsel bepalen des te prangender. In hoofdstuk V wordt op één van deze uitwendige omstandigheden, de plaatsing van de electroden, nader ingegaan. In dit hoofdstuk wordt aangetoond dat de wijze waarop de electroden worden aangebracht inderdaad een duidelijk en significant effect heeft op de veldsterkte in de pees. Plaatsing in dorsopalmare richting resulteert in een geringer verlies aan veldsterkte dan plaatsing in mediolaterale of proximodistale richting. Dit soort informatie is van groot belang op het moment dat klinische toepassing van dit soort apparatuur overwogen wordt.

Schokgolfbehandeling

Behandeling met schokgolven van letsels van het spierskeletstelsel is de afgelopen tijd erg populair geworden en er zijn een aantal klinische studies gepubliceerd waarin een positief effect gerapporteerd wordt. In hoofdstuk VI wordt een experiment beschreven waarin het effect van schokgolven op het metabolisme van tenocyten centraal staat. De studie moet voornamelijk gezien worden als een eerste poging die met name bedoeld was om het concept uit te testen waarbij schokgolven bij het levende dier toegepast worden en het effect op het metabolisme gemeten wordt op peescellen die opgekweekt worden vanuit stukjes weefsel die na euthanasie van het

dier verkregen zijn. Uit deze studie bleek dat er wél een korte termijn effect aangetoond kon worden (een verhoging van het celmetabolisme van cellen die 3 uur na behandeling geoogst werden), maar geen lange termijn effect (waarbij er een tijdsverloop van 6 weken was tussen de behandeling en het oogsten van de cellen). Ook kon er geen verschil aangetoond worden tussen drie verschillende hoeveelheden schokgolven (van dezelfde zwaarte). De eindconclusie was dat er óf sprake was van een relatief geringe gevoeligheid van gezond peesweefsel voor schokgolven, óf dat de gebruikte techniek (opkweken van cellen vanuit stukjes weefsel die geoogst werden kort of lang na de behandeling) onvoldoende gevoelig was om eventueel geïnduceerde verschillen aan te tonen.

De puzzelstukjes vallen op hun plaats

Op basis van het gehele werk kan er geconcludeerd worden dat er al bij veulens duidelijke verschillen bestaan in de samenstelling van de extracellulaire matrix, waarschijnlijk veroorzaakt door de beweeglijkheid van de vrucht voor de geboorte. Na de geboorte vindt er vervolgens een proces van functionele aanpassing plaats dat vergelijkbaar is met dat bij gewrichtskraakbeen en dat onder invloed van mechanische belasting lijkt plaats te vinden. Gezien de zeer trage herstructurering van met name collageen bij het volwassen dier, is dit proces van functionele aanpassing gedurende de jeugd van cruciaal belang voor de ontwikkeling van een zo gering mogelijke blessuregevoeligheid.

Voor wat het effect van de niet-invasieve fysische technieken betreft die in dit proefschrift onderzocht werden valt op te merken dat zowel APS als schokgolftherapie een zeker effect op het metabolisme van de tenocyten bleken te hebben. Blijkbaar vertalen de cellen de door hen waargenomen veranderingen in hun directe omgeving die ontstaan door mechanische belasting of anderszins, zoals door stroom, in een herstructurering van de matrix. Alhoewel er op dit moment nog een onvoldoende inzicht bestaat in de voor- en nadelen van dit soort apparatuur, lijken de vooruitzichten goed en is verder onderzoek in deze richting zeker geïndiceerd.

Summary

Introduction

Injuries to the superficial digital flexor tendon (SDFT) play a prominent role in equine orthopaedic disorders and form an important threat to both the athletic potential and welfare of sport horses. A thorough knowledge of normal development of the extracellular matrix (ECM) components of tendons is a prerequisite for the understanding of physiological processes and of possible causes of pathological aberrations, and hence crucial for the prevention and treatment of tendon injuries. Although large numbers of treatments have been investigated for the often severely disabling tendon injuries, much knowledge is still lacking, with respect to both physiological events such as development and the mechanistic aspects of some advocated treatments.

Goals of this thesis

The first part of this thesis aims at the in-depth analysis of the changes occurring in the ECM from birth to maturity, and subsequently during ageing. To this end, a detailed biochemical analysis was performed of SDFT samples harvested from animals ranging from neonates to elderly individuals that all were free of macroscopic lesions in the tendon. It was a deliberate choice to analyze tendon samples from two regions that were known to be very differently loaded. The first region (mid-metacarpal) almost exclusively sustains tensile strain and the second (sesamoidal) region sustains both tensional and compressive loading.

In the second part of the thesis attention focuses at the cellular part of the tendon because the tenocytes, which are rather quiescent with a low profile in healthy mature tendon, have to be activated once injury occurs. Many non-invasive physical therapy modalities have been used to achieve such an activation and/or recruitment of cells, but few scientific studies have been performed to evaluate their effect at tissue level. Micro-current electrical therapy and shock wave therapy were chosen in this thesis for the evaluation of the biological effects of this kind of therapy.

Tendon development

Chapter II concerns developmental aspects and focuses on those elements of the extracellular matrix that are known to contribute directly to the biomechanical strength of the tissue. Different to what was anticipated on the basis of pre-existing knowledge about the development of articular cartilage, it was shown that the tendon of the newborn was not a homogeneous “blank” tissue without any site-related differences in biochemical composition. In contrast, already in neonates significant differences in ECM composition were found between tensile and compressive regions. Intra-uterine

movements, which may lead to a certain loading of tendons whereas their influence on articular cartilage may probably be neglected as long as the animal is not yet on its feet, might cause this. Directly after birth many changes in the collagenous and non-collagenous components of the ECM of these two regions were observed. It was shown that the functional adaptation of tendon to weight bearing and loading took place for the larger part during the first months of life. This ongoing process of maturation of the various ECM components in the juvenile animal can be assumed to affect the loading capacity of the tissue and thus may imply potentially serious consequences of early athletic performance.

Ageing

A study on ECM composition in sound, mature animals (Chapter III) made clear that no dramatic alterations took place with ageing, once maturity had been reached. This indicates that the metabolic activity of the tenocytes decreases during maturation, to maintain only a certain basic level afterwards. By that time the significant regional differences in the biochemical composition of the ECM that match loading have developed and do not alter essentially during the rest of life. In mature tendons the tension-loaded mid-metacarpal region is characterized by a higher cellularity and collagen density than the sesamoidean region. The lack of any age-related accumulation of pentosidine cross-links and the higher level of degraded collagen in this region suggest a certain amount of degeneration and micro-trauma, which might be a long-term effect of accumulated peak loads. In contrast to the tensile region, the sesamoidean region, which is loaded by both tensional and compressive forces, had higher glycosaminoglycan content, making the tissue better able to resist compression while giving in on tensile resistance. The lower levels of degraded collagen and the linear increase in pentosidine during ageing in this region are in line with the much lower incidence of injuries at this site.

Micro-current electrical tissue stimulation (METS)

Electric currents that are generated during physiologic processes belong to the micro-ampere range. Devices using micro-currents are therefore of more potential interest for the stimulation of healing processes than others using higher currents. For this reason, a METS-device was chosen to assess the effect of electrical stimulation on tenocytes. The results in chapter IV show that tenocytes are indeed sensitive for micro-current therapy. However, only a narrow range of current is beneficial to promote the proliferation of tenocytes and stimulate their metabolism. If the threshold is passed, not only cell proliferation and metabolic activity will decrease, but there is also an increase in tenocyte apoptosis, which might severely impair the reparative capacity and delay

the repair process in vivo. Although METS still can be considered potentially useful for the promotion of tendon healing, the effect seems to be a two-sided sword and results are too ambiguous yet to warrant recommendation for clinical use at this moment.

The apparently narrow range for the application of electric currents urges a thorough knowledge of the effects of factors that might influence the eventual current levels in the target tissue. Chapter V reports a preparatory study into the effect of transducer placement on actual field strength in the target tissue. It is shown that transducer placement indeed has a significant effect on field strength at tissue level, and hence may affect any possible therapeutic effect. Application of current in dorsal-palmar direction appears to result in less loss of field strength than application in medio-lateral or proximo-distal directions. This kind of information is essential once clinical application of this type of devices is considered.

Extracorporeal Shock Wave Therapy (ESWT)

Extracorporeal shock wave therapy (ESWT) has become one of the most popular therapeutic devices for musculoskeletal injuries in recent times and success has been reported in various clinical studies. In Chapter VI a preliminary study is described that investigated the effect of ESWT on the metabolism of tenocytes from normal tendons. Tenocytes harvested from explants that were cultured after sacrificing the animal had a higher metabolism 3 hours after treatment, with collagen, protein and glycosaminoglycan (GAG) synthesis increased by more than 50%. However, 6 weeks after application no effect could be demonstrated anymore. Further, there was no dose effect when comparing different numbers of pulses with the same energy flux density. It was concluded that this preliminary data suggested that normal tendon is not very sensitive to ESWT, or that the *ex* vivo measuring system was not sensitive enough to detect accurately the effects of a single treatment.

Putting the pieces of the puzzle together

It was concluded from the entire work that already in neonates regional differences exist in tendon ECM composition, probably induced by movements *in* utero. After birth, a process of functional adaptation takes place that is similar to articular cartilage and seems to be load-driven. As collagen turnover is relatively slow at adult age, the process of functional adaptation of the collagen network of tendon at young age may be of great importance for the development of resistance to injury.

With respect to the effect of non-invasive therapeutic devices on tenocytes, micro-current and shock wave were both shown to affect tenocyte metabolism to a certain extent. The tenocytes apparently perceive the loading or other extraneous

stimulation that is transmitted to them through the environment, and translate this into a modification of the extracellular matrix. Although a full insight into the effects of these devices is still lacking, there might be a great potential for non-invasive physical therapy, warranting further fundamental scientific evaluation.

摘 要

序

腳部的遠端淺屈肌肌腱受傷（腿筋拉傷扭傷發炎斷裂等）是現役馬常見的運動傷害，對往後的運動潛力以及生活品質都形成一股嚴重的威脅。因此在運動醫學的研究上期能藉由對肌腱細胞外基質的組成及其在生長發育過程的變化全盤瞭解，進而對於病理變化和疾病的預防及治療上有更進一步的發展。目前雖有許多治療方法，但對於肌腱組織的整體了解及治療上仍有許多不足之處有待研究。

本論文的目標

本論文分為二部分，在第一部分是探討馬自出生到老年其肌腱細胞外基質組成物的生長曲線。組織則選自位於掌骨的淺屈肌肌腱兩處受力不同的部位，一為拉力承受處，解剖部位於掌骨兩端中間點；另一部位則為同時承受壓力及拉力之處，行經近端種子骨。第二部分則著重於物理治療對於肌腱細胞的代謝的作用。成年後大部分的肌腱細胞呈現靜態休息期，代謝率相當低，平時只維持低需求量，一旦肌腱受到傷害時，肌腱細胞需要立刻再度運作。目前雖有許多物理治療的儀器及臨床使用的報告，但卻少有縝密的科學驗證。鑑於基礎理論的不足，此論文第二部分深入探討為微量電流及體外震波對肌腱細胞生理的影響。

肌腱生長

第二章著重於瞭解肌腱細胞外基質的生長發育。腳關節軟骨在一出生時不同壓力承受點的細胞外基質組成皆相同，相對於此，遠端淺屈肌肌腱上受力不同的兩個部位其細胞外基質成分在出生時即已呈現相異之處。造成此差異的原因可能是當胎兒在子宮裡伸展之力傳至拉力承受處而促使細胞因應此伸展拉力而製造所需之外基質，而壓受力處的肌腱及腳關節軟骨則發生在出生承受重力之後。出生後的前幾個月在此兩個不同受力部位其膠原蛋白及其他組成外基質的物質逐漸因應體重及運動量增加等生長需求而有所調適。肌腱的細胞外基質在成長發育過程中持續產生變化，一般認為此對日後運動表現具有影響。

成年至老年

馬成年後至老年的肌腱細胞外基質並沒有太大的變化，此表示肌腱細胞的代謝趨於緩和，只維持基本需求。兩處不同受力部位的肌腱細胞外基質在發育過程中所發展出不同的特質在馬成年後也已定型。在拉力受力處含較多的細胞數及膠原蛋白，且 pentosidine crosslink (一種非酵素性蛋白質糖化反應的最終產物)並無隨年齡增長而增

加，此外此處亦含較多變性膠原蛋白，種種現象皆顯示出肌腱退化及肌腱小纖維反覆拉扯或過度運動而失去連續性。相對的，在種子骨部位的淺屈肌肌腱則具有較多的氨基葡聚糖 (glycosaminoglycan, GAG)，使得此部位的肌腱得以承受種子骨所帶來的壓迫及肌腱本身的拉力。此外，此部位的肌腱細胞外基質的變性蛋白質含量相當低，且 pentosidine 含量和年齡增長成正比關係，皆符合組織低受傷率的特徵。

微電流治療 (METS)

在生理反應過程中所產生的電流量為微安培,因此有許多微電流治療儀器是根據此電流大小的範圍所設計的，其作用可活化細胞及加速修補受傷細胞。第四章即探討微電流治療對肌腱細胞的作用。研究結果顯示微電流只有在很小的安全範圍內具有加速細胞新陳代謝的正面效果。如果給予過高的電流則不僅效果打折且會造成細胞形態變化最後死亡 (apoptosis)。此負面反應可能會延長組織修復時間及降低肌腱癒後的品質。雖然微電流治療具有治療肌腱的潛力，但其效果也像一把雙刃劍，因此在有更進一步基礎及臨床研究之前並不建議冒然使用。

由於前一實驗結果得知使用微電流時的安全範圍很窄,因此在接下來的實驗中(第五章)為測試肌腱在不同的電流供應量下所得到之實際電場大小。研究結果顯示貼片式電極的放置部位對於在組織內所形成的電場大小具有顯著的影響力。將兩貼片放置在前腳的前側及背側較其他放置部位（置於兩側，以及背側的上下端）的效果好。此研究方法提供了對這類儀器於臨床使用上的具體建議。

體外震波治療

體外震波治療於近年來在骨骼肌肉傷害的應用上逐漸熱門，也有不錯的成效。因此在第六章所探討的是體外震波對正常肌腱細胞生理代謝的初步研究。在給予震波治療後三小時所採樣的組織其肌腱細胞所產生的膠原蛋白、蛋白質、及氨基葡聚糖 (GAG)，皆增加百分之五十以上，但於六週後已檢測不到震波的作用效果。在給予相同電通電密度(energy flux density) 但不同震波數上對肌腱細胞代謝並沒有顯著差異性（原因尚在研究中）。初步結果並不如預期，推測可能為正常肌腱對於體外震波並不是非常敏感，或檢測體外組織代謝的方法上仍有待改善。

總結

肌腱上受力不同的兩個部位其細胞外基質成分在出生時即已具相異性，此可能是由於胎兒在子宮裡伸展之故。出生後肌腱細胞外基質發育過程因應荷重的調適性機制和腳關節軟骨的發育相似。由於成年後膠原蛋白代謝減緩，因此肌腱在生長期的發育對日後承受傷害的能力具重大的影響力。

微電流及體震波等非侵入性治療皆對肌腱細胞的代謝具有一定的作用。肌腱細胞接受到重力及運動負荷或外在刺激訊息後，因應其需求產生細胞外基質。雖然現階段對物理治療在體內的詳細作用機制尚未全盤瞭解，但相當值得更進一步研究。

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Is it the end? I think it just starts now…

Curriculum Vitae

YiLo Lin was born on February 17th, 1975 in Taipei, Taiwan. After finishing high school in Taipei, she started her studies in Veterinary Medicine in 1993 at the National Chung-Hsing University (國立中興大學) in Taichung, Taiwan, which she concluded in 1998. In that year she started work for her master degree at the same university, carrying out an epidemiological survey on the occurrence of equine Japanese encephalitis in Taiwan for 2 years under the supervision of Dr. C. L. Mao. From November 2001, she has worked on her PhD thesis at the Department of Equine Sciences of Utrecht University. This PhD study focused on physiological and developmental aspects of the extracellular matrix components of the equine superficial digital flexor tendon and on the effect of certain modalities for physical treatment on the metabolism of tendon cells. This thesis gives an account of the work and will be defended in public on December 14th, 2005.