Can we improve tendon healing in the horse?

A multi-angle study of a multi-facet problem

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Can we improve tendon healing in the horse?

A multi-angle study of a multi-facet problem

Hoe kan peesherstel bij het paard bevorderd worden?

Een complex probleem van verschillende zijden belicht

(met een samenvatting in het Nederlands)

Proefschrift

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à ma grand-mère, *Germaine*

Contents

Chapter 1

General Introduction:

"*Tendons are the body's marionette strings, connecting bones to muscles that raise an eyebrow or propel us into a full run*"[1]

Tendons constitute an incredibly complex tissue. For this reason they have been widely avoided by researchers until the late fifties. Although a paper from Girdlestone in 1882 mentions that (kangaroo) tendons were considered as an alternative to silk or gut for ligatures and sutures [2], the real interest in tendon research came only with an increasing number of tendon injuries that arose in the era of modern industrialization (Fig.1). The increase of repetitive movement at work (Fig. 2), as well as the introduction of defined limits of working time that enabled leisure activity after work were both highly detrimental to tendons [3]. The incidence of tendon overuse injuries including complete, spontaneous ruptures has consequently increased over the last decades in industrialized countries [4]. Current tendencies towards obesity, hypertension, diabetes, steroid or estrogen exposure as well as the popularity of the Nintendo wii have also been cited as causes of the increased number of clinical cases [5-8].

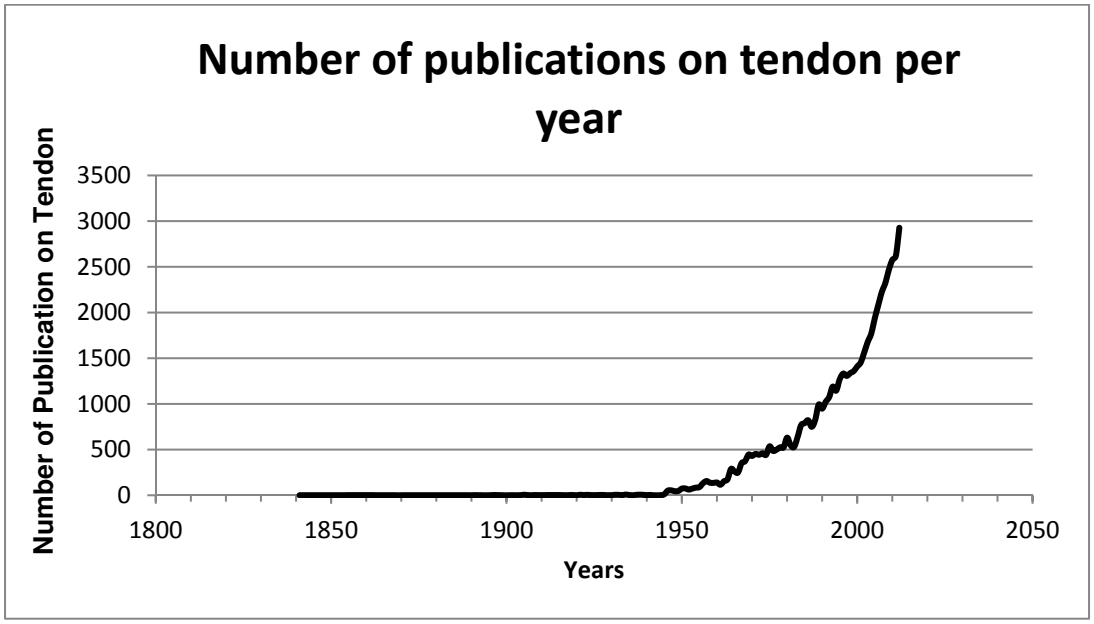


Figure 1: Number of publications on tendon per year based on Pubmed search. The figure of 500 publications per year was only reached in 1977.

Figure 2: Repetitive work injuries increased with the Taylor/Ford concept of manufacturing. Dividing the assembling process of small parts of lamps in the early 1920s is an example of this change in work organization.

The two most frequently studied tendons in humans are the Achilles tendon and the rotator cuff tendon in the shoulder. (The patellar tendon is also frequently studied, but this is strictly speaking not a tendon but a ligament as it is linking two bones and not a bone to a muscle). The Achilles tendon is the largest and strongest tendon in the human body. Nevertheless, disorders of the Achilles tendon are among the more common injuries seen by physicians specializing in sports medicine [9]. Despite the high prevalence of these injuries, there is still a lack of knowledge on their pathogenesis.

Tendon injuries in the equine world are also a major concern. Horses have been bred and selected for centuries for their athletic prowess and the costs involved in raising a foal to become an equine athlete are substantial. In horse racing the betting industry is an important economic factor with annual turnovers that may run into billions of dollars, depending on the country. Tendon injuries are among the most common and often career-ending disorders in horse racing [9].

The industrialized era has also profoundly changed the lifestyle of horses. The traditional role of the horse for millennia after domestication of the species was to deliver speedy transport and

hauling power for the military, civil and agricultural activities [10]. The rapid mechanization of society that started during World War I and was basically finished after the Second World War has bereft the horse of all these classic roles. Horses have been replaced by tanks and armored cars in the modern-day cavalry and by tractors in the fields and a wide variety of motorized vehicles on the road. Horses are now principally used as sports and leisure activities and like humans, are often asked to produce a sudden effort after many hours of little activity. Although hard epidemiological proof is lacking, it is likely that these lifestyle changes have affected equine health and may be implicated in the rather high prevalence of tendon injuries, which now constitute 10% of all diagnosed cases of lameness in racehorses [11]. Reliable figures for other equestrian disciplines are lacking, but there are indications that tendon injuries play an important role in wastage in other of these disciplines as well [12]. The notoriously bad healing capacity of tendons, the prolonged recovery time and the considerable risk of recurrent injury are highly detrimental to the horse industry [13].

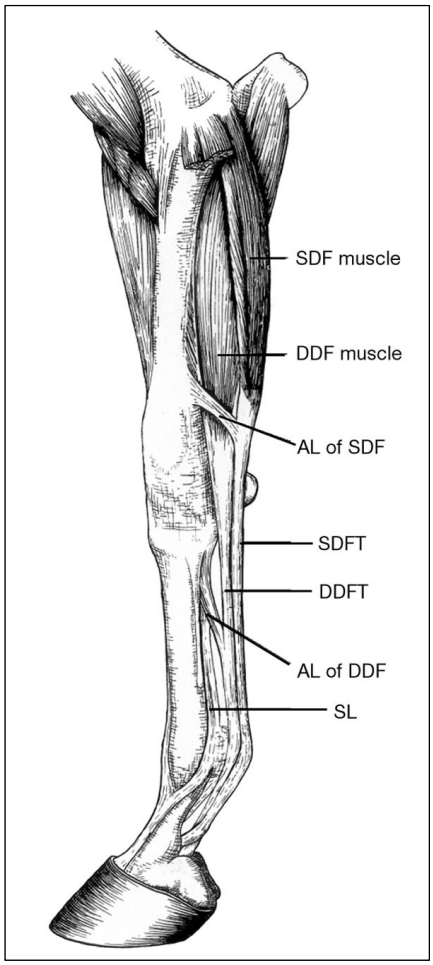
When looking at the anatomy, composition and function as an elastic, load-bearing structure, there are striking similarities between the human Achilles tendon and the equine superficial digital flexor tendon (SDFT)[14]. Descriptions of human tendon pathology frequently cite equine literature [15-17]. The similarity of the SDFT tendon injury in horses with Achilles tendinopathy in humans has led to mutual benefit. Investigations into equine tendons have had a significant impact on human tendon research, as analogies could be made. In the horse, it is possible to collect interesting tendon samples from freshly slaughtered animals; something that is of course not conceivable with human patients. New therapies have also been tested first on horses. On the other hand, equine tendon research has profited from human funding and facilities.

It is generally agreed that most tendon injuries originate from repeated microscopic damage for undefined periods of time prior to rupture, implying that there is a window of opportunity for intervention [14]. Although our understanding of tendon disorders has increased over recent years, our knowledge is still limited in terms of:

- the effects of immobilization after acute injuries;
- the role of intrinsic/extrinsic repair;
- how and why certain tendons form poor quality scar tissue;
- why lipid or calcium depositions are found in degenerative tendons;
- many aspects of fundamental tendon cell and matrix biology

Anatomy of the equine superficial digital flexor tendon:

The superficial digital flexor (SDF) muscle originates from the medial epicondyle of the humerus and runs distally along the caudal side of the radius (Fig. 3). The transition from the SDF muscle to the SDF 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 [18].



SDFT: Superficial Digital Flexor Tendon
DDFT: Deep Digital Flexor Tendon
AL: Accessory Ligament
SL: Suspensory Ligament

Figure 3: Schematic drawing of the medial aspect of the equine forelimb (from Wilson et al. Nature, 2001[19])

Anatomy of the Achilles tendon:

The Achilles tendon in humans originates from the merging of the soleus muscle with the two bellies of the gastrocnemius (Fig.4). As the tendon descends, the fibres twist 90 degrees until it attaches to its insertion at the back of the calcaneus or heel bone.

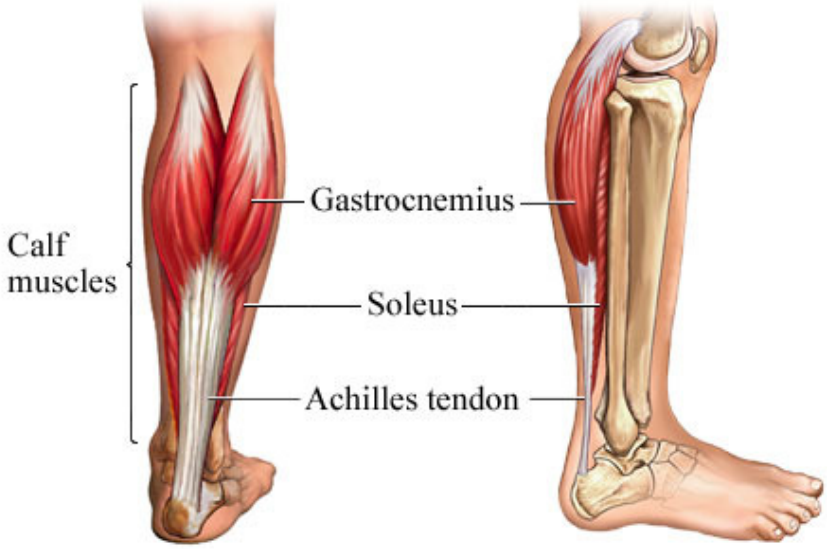


Figure 4: Schematic drawing of the caudal and lateral aspects of the human leg. (From footandankleinstitute.com)

Tendon constituents:

Tendons have a typical white glistening appearance due to collagen and water being their main components (65-80% of the dry mass of the tendon is collagen, water accounts for up to 70% of wet weight) [20]. The predominant type of collagen is type 1 (~60% of dry mass). Collagens II, III, IV, XII and XIV are also present but in small quantities [20; 21]. Type III collagen is found in considerable amounts in immature tendons and injured tendons [22; 23]. The mechanical properties of tendon collagen depend largely on intra- and intermolecular cross-linking, which is essential for the stability of collagen fibrils [24; 25].

Aside from collagen, another important constituent of the tendon extracellular matrix (ECM) is formed by proteoglycans and their associated glycosaminoglycan (GAG) side chains such as

dermatan sulfate (DS), chondroitin sulfate (CS) and keratan sulfate (KS)[26]. Proteoglycans form bridges and bonds between the collagen fibrils in longitudinal as well as transverse directions [27]. Therefore, although, they constitute only ~1-1.5% of tendon dry weight, they probably play a key role in tendon function [28].
Proteoglycans are also believed to influence many important physiological processes, such as ion transport, the diffusion of nutrients and water retention [29]. Further, they play a role in fibrillogenesis and matrix architecture [30-34]. Changes in the glycosaminoglycan and proteoglycan composition have been described as occurring during maturation and ageing, after immobilization and exercise in response to mechanical cues and also after tendon injury [35-38].

The prevalent cell types in tendon tissue are tenocytes and tenoblasts. They account for 90-95% of the cell population in tendons. The remaining 5 to 10% are made up of chondrocytes, endothelial cells, tendon-derived stem/progenitor cells and synovial cells.
At birth, tenoblasts form the majority of cells. They form many long chains of cells parallel to the collagen fibres. Inside the chains, tenoblasts are linked via gap junctions or adherent junctions, enabling chemical as well as mechanical communication between the cells. Tenoblasts may have various shapes, varying from spindles to round or rectangular forms. Their size varies also from 20 to 70 µm. As they display a high secretory activity, the endoplasmic reticulum and the Golgi apparatus of these cells are well developed. Furthermore, the ratio between the nucleus and the cytoplasm is low. With aging, tenoblasts become tenocytes which have a spindle shape and which may attain a length of 300 µm. In these tenocytes, the nucleus takes most of the intracellular space, leaving relatively little for the cytoplasm such that the organelles are smaller, indicating a decreased metabolic activity. They have elongated protrusions to maintain the communication between the cells. Overall, cellularity is little and the extracellular matrix (ECM) is the predominant component of mature tendons.

Recently, tendon stem/progenitor cells (TSCs) have been identified in mice and humans [39]. TSCs have multipotent differentiation capacity. De Mos et al. showed that tendon-derived fibroblasts (TDFs) from adolescent human hamstring tendons were also able to differentiate into adipocytes, chondrocytes, and osteocytes suggesting that tendon fibroblasts or tenocytes may have trans-differentiation potential [40]. In several animal models, stem cells have been used to repair connective tissue defects. However, the guidance of stem cell differentiation is still not fully understood and, as the study of Harris et al. shows, mesenchymal stem cells may create

ectopic bone formation in rabbit tendons [41]. Further investigations are still needed to understand and control the mechanisms that influence differentiation.

Tenocytes have an elaborate mechano-sensitive apparatus consisting of various components. First, complex focal adhesions anchor their cytoplasmic membrane to the ECM, secondly, gap junctions between the cells link them together [42; 43] and finally, primary cilia complete their mechano-sensory apparatus [44-46].

When loaded, tenocytes adjust their metabolic activity with respect to the production of extracellular matrix components. Male runners were found to have ~30% larger Achilles tendon cross-sectional areas (CSA) than non-runners. Similarly, male athletes who perform frequent weight bearing exercise (running, jumping) have relatively thick tendons compared to athletes in non-weight bearing sports (e.g. kayakers) (~20% larger CSA). It could of course be argued that these differences are due to natural selection. However a recent study found 30% greater CSA of patellar ligaments in the leading leg of male athletes competing for at least 5 years in sports like fencing or badminton[47]. It has also been demonstrated that collagen synthesis in vitro is upregulated when cyclic tensile strain is applied over a 24h loading period in isolated tendon fascicles [48; 49].

It should be noted that different types of loading (tension, compression) elicit different metabolic responses [50].

Tendon structure:

Tendons present a hierarchical structure with their collagen bundles predominantly ordered in a longitudinal direction (Fig.5). The basic structural unit is tropocollagen, a triple helix polypeptide chain. Five tropocollagen molecules form each collagen filament. These are then organized into the larger structural units of the tendon: subfibrils, fibrils, fibres, sub-fascicles, fascicles, tertiary fibre bundles and the tendon itself [51; 52].

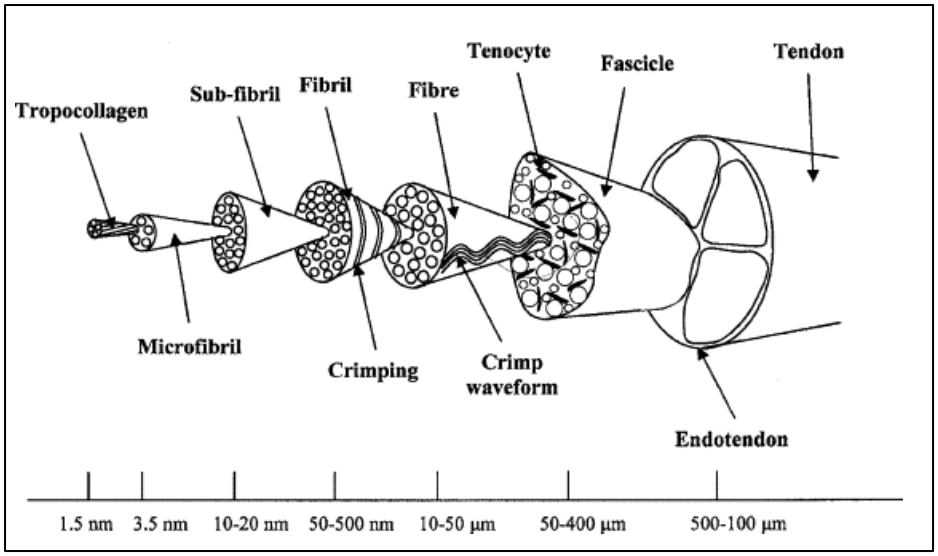


Figure 5: schematic diagram representing the hierarchical structure of tendon (from Screen et al.[53])

On the outer layer of the tendon, a sheath of loose fibrillar tissue called the paratenon functions as an elastic sleeve, permitting free movement of the tendon between the surrounding tissues [20]. The collagen fibre system of the paratenon is well defined. The main components of the paratenon are type I and type III collagen fibrils, as well as elastic fibrils [54]. The paratenon is lined on its inner surface by synovial cells [55].

Beneath the paratenon, the entire tendon is surrounded by a fine connective tissue sheath called the epitenon. The epitenon is contiguous at its outer surface with the paratenon and at its inner surface with the endotenon [20].

The epitenon is a relatively smooth fibrillar network of collagen with strands of 8-10 nm in thickness. This network contains longitudinal, oblique as well as transversal fibrils. It shows little variation in density or fibril orientation. Occasionally, the fibrils of the epitenon are fused with the superficially located tendon fibrils [56].

The endotenon is a thin reticular network of connective tissue inside the tendon that has a well-developed crisscross pattern of collagen fibrils [51; 57; 58]. The endotenon fibrils invest tendon fibres and bind fibres together. Along with this important function of binding, the endotenon network allows the fibre groups to glide along each other and carries blood vessels, nerves, and lymphatics to the deeper portion of the tendon [57; 59].

Tendon biomechanics:

Only a minority (~2%) of all Achilles tendon complaints and injuries are a result of systemic, predisposing disease; most tendon problems in a population can be traced to sports and exercise-related overuse [60]. Tendon biomechanics are therefore of primary importance.

Flexor tendons, by definition, flex the digit when the limb is in the swing phase. In the stance phase they are under very high tensile stress as, depending on the gait, they may be challenged by loads of more than two times body weight [61]. In humans, studies have shown that the Achilles tendon experienced forces as high as 9.0 kN, corresponding to 12.5 times body weight, during sprinting [62]. Furthermore, the efficiency of locomotion is vastly enhanced by the capacities of the flexor tendons to store and release energy; a function which requires elasticity [63; 64].

The relationship between load (force) and deformation (strain) of tendons can be depicted in a so-called stress-strain curve (Fig. 6). The curve has four important regions. The first region, the "toe" region, represents the straightening out of the crimps of the fibrils and fibres (Fig. 6). In this region, minor forces are required to get considerable extension. For the equine SDFT, this region ends at 3% strain. Stretching the tendon in this region will cause no damage, as the tendon will completely return to its original length after unloading [65; 66]. Before testing to failure, a tendon is normally preconditioned by stretching it a couple of times in this toe region, to get a standardized starting point for all samples.

The second region (Fig. 6) represents a phase where the fibres of the tendon become arranged in a more parallel fashion and is almost linearly elastic. In this elastic phase, the degree of deformation is dictated by the structure of the collagen. Strains of up to 4-5% are still completely reversible. However, loading beyond this region, will lead to slippage of the interfibrillar cross-links and subsequent dissociation of the fibrils [67] (Fig. 6, region 3). Strains up to 8% [68; 69] cause irreversible plastic deformation, finally resulting in microscopic failure followed by a complete rupture (Fig. 6, region 4) at strains of about 12.5% and at a load of approximately 12,000± 1300 N [70; 71].

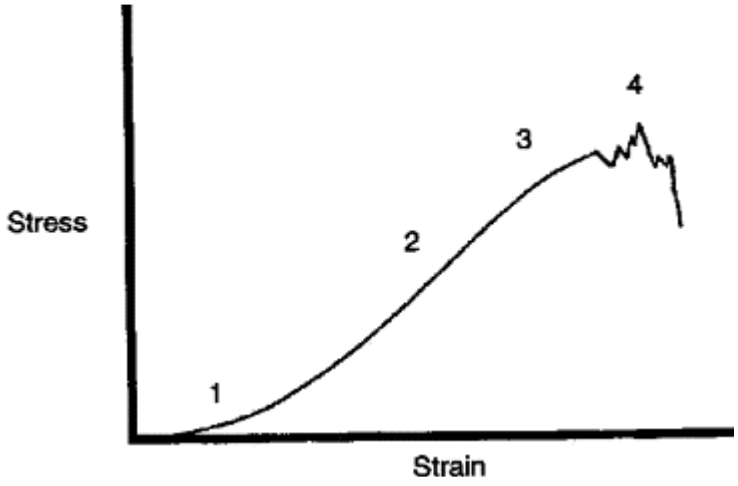


Figure 6: Simplified stress–strain curve for the equine SDFT (from Goodship et al., 1994)[63].
Key: 1, toe region; 2, linear deformation; 3, yield; 4, rupture.

Tendinopathy:

The most commonly accepted cause for tendinopathy is overuse in possible combination with intrinsic and extrinsic factors leading to what may be seen as progressive failing of the innate healing response.

The clinical symptoms of tendinopathy in horses and humans differ slightly. In both species the damaged tendon is swollen and tender upon palpation. However, whereas pain is a hallmark of the initial phase in both species, persistent pain is common in humans but not in horses. In the equine species the loading capacity of affected tendons is severely reduced and the risk of re-injury is high, but horses generally become relatively quickly free of lameness. Under light microscopy, the pathological aspects of tendinopathy are similar in both species and are characterized by disrupted collagen, increased proteoglycan content, more prominent and numerous tenocytes and neovascularization [72-75] (Fig.7). Lipoid degeneration as well as calcification have also been observed in some cases of tendinopathy.

The etiology, pathogenesis and natural course of tendinopathy are largely unknown [76]. Tendon injuries can be caused by intrinsic or extrinsic factors, either alone or in combination [77]. In case of acute trauma, extrinsic factors predominate while chronic injuries are generally

multifactorial. Extrinsic factors are for example trauma, repetitive movement or excessive loading. The list of intrinsic factors that are known to influence tendon disorders is long. Among others, they include: mal-alignment, diabetes, age, hypertension and genetics.

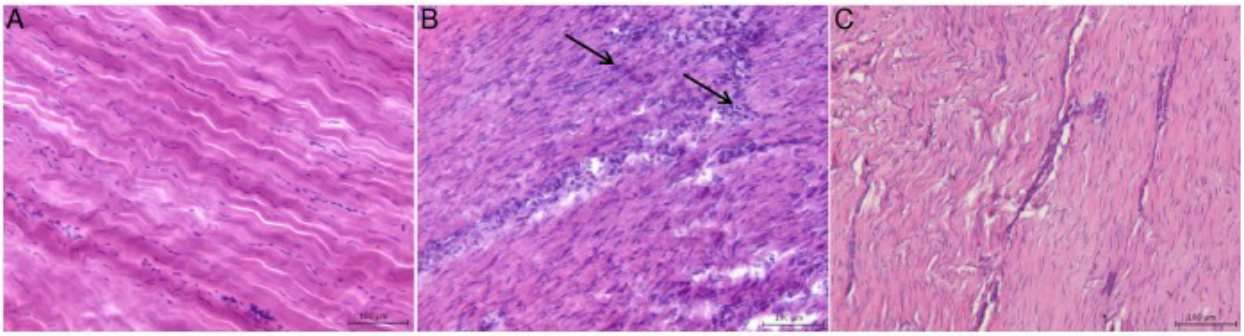


Figure 7: Longitudinal histology sections stained with Haematoxylin and Eosin showing: (A) normal superficial digital flexor tendon (SDFT) from a 6-year-old horse showing regular arrangement of parallel collagen fibrils. (B) Sub-acutely injured SDFT 3 weeks post injury from a 4-year-old horse showing marked cellular infiltration (black arrows). (C) Chronically injured SDFT >3 months post injury from a 12-year-old horse showing increased cellularity and poor organization of collagen fibrils compared to (A). Scale bar in all pictures = 100 µm. From Dakin et al. 2012 PLoS One [75].

In humans, inflammatory cell infiltration has not been shown in biopsies of tendons with chronic disorders or in ruptured tendons with degenerative changes [60; 72; 78]. However, as no study has been performed on the early stages of tendinopathy, the presence of inflammation cannot be conclusively excluded. In the horse, where tendon samples can be harvested in the acute phase of tendinopathy, Dakin et al. observed the presence of prostaglandins E_2 (PGE_2)[75]. Several studies support the involvement of PGE_2 in the development of tendinopathy via inflammatory processes [79; 80]. More investigations are still needed to understand the possible role of inflammation in tendinopathy and whether it is species specific or not.

The mechanical theory of tendon overuse injury proposes that, when a tendon is repeatedly stretched by submaximal loads, it is unable to endure further tension, whereupon injury occurs. The tenocytes are overcharged by the repetitive micro-traumatic process and are unable to repair the tendon accordingly. The collagen fibres start to slide past one another, breaking their cross-links and, in the worst case scenario, the collagen fibrils themselves rupture [76].
The structure of the tendon and also the natural alignment of the cells are disrupted by this repetitive strain. Two theories for the cause of this degenerative process are conceivable: either the tenocytes are being over-stimulated during repetitive mechanical loading or they are, after initial trauma, being under-stimulated as damaged fibres are no longer able to transmit loads.

Researchers first thought that the repetitive high strains on the cells would result in increased cell death and potentially inappropriate synthetic and degradative responses which became responsible of tendon degeneration [81]. Tenocytes are vulnerable cells as they can only withstand loads of 0.9 ± 0.2 μN, which is minimal compared to what the entire tendon endures (up to 9kN for Achilles tendon) [82]. However, Screen et al. showed that in the physiological situation the cells inside the tendon are experiencing a minimal strain compared to the overall strain applied to the tendon [83]. The lower local strains can be attributed to the realignment of collagen fibres, or they can be a result of slippage between individual collagen fibres [84]. In this way, the cells are rarely experiencing repetitive high strains. Recent in-vitro studies in which tendon cells were over-stimulated showed an increase in inflammatory cytokines and degenerative enzymes [79; 85-88]. However, these cells had been removed from their natural environment, and the applied strain might not have been representative of that actually received by the cells *in situ*. Furthermore, many of these investigations feature potentially confounding external factors or have utilized non-physiologic strain patterns to elicit these cellular responses. Thus, the outcome of those experiments must be evaluated with some reservation.

The second theory was developed by the group of Arnoczky et al. Their group was able to show that mechano-biological under-stimulation of tendon cells can also produce a pattern of catabolic gene expression that results in extracellular matrix degradation [89-94]. The idea is that initially repetitive mechanical loading creates micro-rupture of the collagen fibrils. Then, as these damaged fibrils are no longer able to transmit extracellular matrix loads to the tendon cells, an under-stimulation of the cells occurs which, in turn, initiates a catabolic response. It has been shown that the number of myofibroblasts, a type of cells responsible for fibrosis, increases in tendons kept under no tension for several days [95]. Further, the rounding up of the cells, which is another characteristics of tendinopathy, has also been observed in dog flexor tendons kept in culture without tension [96]. These observations add support to the theory that under-stimulation of tenocytes can lead to degenerative mechanisms in tendons.

To summarize, tendinopathy is a multi-factorial disease, of which the etiology is still not completely understood. Repetitive strain injury is the most accepted initial factor, but two diametrically opposed theories of over- and under-stimulation respectively of the cells are still competing to explain the following steps towards tendon degeneration.

Treatment options:

There are several treatment options for tendinopathy in humans which have been scored with respect to the degree of evidence with respect to their relative efficacy [97]. None of these has been given an A-score which would indicate consistent, good-quality, patient-oriented evidence (Table 1). Current clinical use of conservative and operative treatment modalities varies considerably from country to country and even from clinic to clinic and often relies on empirical evidence without supporting scientific data. Most of the therapeutic studies have been retrospective, and only a few have included objective, well-controlled criteria to evaluate the outcome [76].

New treatment options have often been initially tested in horses. Platelet rich plasma as well as cell therapies might improve the actual treatment options. However, more studies still need to be performed to assess their efficacy and safeness [98].

Table 1: KEY RECOMMENDATIONS FOR CLINICAL PRACTICE

Clinical recommendation	Evidence rating	References
Relative rest and reduced activity will prevent further damage and promote healing and pain relief. There are no clear recommendations for the duration of rest and avoidance of activity.	C	*
Cryotherapy provides acute relief of pain in case of acute tendinopathy and its use is widely accepted. Repeated applications of melting ice water through a wet towel for 10-minute periods are most effective.	B	[99-101]
Strengthening through eccentric loading is an effective treatment of tendinopathy and may reverse degenerative changes.	B	[102-104]
NSAIDs° are recommended for short-term pain relief but have no effect on long-term outcomes. Topical NSAIDs° are effective and may have fewer systemic side effects. It is unclear whether NSAIDs° are better than other analgesics.	B	[105; 106]
Locally injected corticosteroids may be more effective than oral NSAIDs° in acute-phase for pain relief but might alter long-term outcomes.	B	[107-109]
No conclusive recommendations can be made for the use of orthotics and braces in patellar tendinopathy or elbow tendinopathy. Clinical experience and patient preference should guide therapy.	B	[110; 111]
Therapeutic ultrasonography, corticosteroid iontophoresis, and phonophoresis are of uncertain benefit for tendinopathy.	B	[112; 113]
Extracorporeal shock wave therapy appears to be a safe, noninvasive, effective but expensive means of pain relief for a number of chronic tendinopathies.	B	[114; 115]
Surgery is an effective option in carefully selected patients who have failed three to six months of conservative therapy.	B	[116; 117]

*—Generally accepted practice.

°NSAIDs = non-steroidal anti-inflammatory drugs.

A = consistent, good-quality, patient-oriented evidence; B = inconsistent or limited-quality, patient-oriented evidence; C = consensus, disease-oriented evidence, usual practice, expert opinion, or case series. For information about the SORT evidence rating system, see http://www.aafp.org/afpsort.xml.

Scope of this thesis:

Tendinopathy of the equine superficial digital flexor tendon still is one of the major causes of wastage in athletic horses [118]. In humans, tendon ‘‘over-use’’ injuries have been claimed to account for 30–50% of all sports-related injuries [119], and almost half of all occupational illnesses in the United States [120]. Thus, there is a need to investigate the pathological mechanisms leading to tendinopathy, as well as to improve our understanding of the innate healing process in injured tendons.

In this thesis an attempt is made to improve the current knowledge regarding the healing mechanisms in (energy-storing) tendons. This was approached from different angles.

In **Chapter 2** an experimental model of tendinopathy in the horse has been characterised. The establishment of a good model for tendinopathy is of great importance for the evaluation of different treatment options. This model had already been used for different studies, but a complete characterization was still lacking. At the time of writing, there is still no optimal model for tendinopathy. The experimental model described in this thesis is showing many characteristics of naturally occurring tendinopathy and also has the advantage of being standardized. However, on the other hand the artificial creation of lesions in this model does not mimic the etiology of naturally occurring tendinopathy.

In **Chapter 3**, an investigation into the effects of immobilization following acute trauma in tendons using this same model is described. After lesions were created in both front legs of 6 horses, one leg was cast to prevent mobilization of the lower joint. Monitoring the size of the injury was performed using computerized ultrasonographic tissue characterization over time as well as by macroscopic assessment of the dissected tendon 42 days post-surgery.

The same model was also used in **Chapter 4**, where the efficacy of platelet-rich plasma treatment was compared to placebo treatment with respect to the healing process of artificially injured tendons. Twenty-four weeks after surgery, the tendons were harvested for biochemical, biomechanical, and histological evaluations.

In **Chapter 5**, genipin, a natural exogenous cross-linker which is frequently used in Chinese medicine, was tested for its cytotoxicity and for the exhibition of any mechanical effects on tendons *ex vivo*. Previous studies on genipin in tendons had shown promising mechanical results but only at high concentrations. In our study, the mechanical effects were evaluated at doses that were not cytotoxic for the cells.

In **Chapter 6** we investigated the healing potential of populations of cells originating from the peritenon compared to populations of cells from the tendon core. This chapter examines intrinsic versus extrinsic healing in tendons.

In **Chapter 7**, the reasons of the appearance of myofibroblasts in tendons were investigated. Myofibroblasts are fibroblasts with a developed contractile apparatus that are often observed in wound healing and which are also responsible for fibrosis. The presence of myofibroblasts in tendons has not been investigated in detail so far. This study investigates the link between the formation of tendon scar tissue resulting from poor healing and the presence of myofibroblasts.

Finally, **Chapter 8** presents a general discussion of the main findings of the studies contained in this thesis and attempts to put them into perspective.

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Chapter 2

Further characterization of an experimental model of tendinopathy in the horse.

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Further characterisation of an experimental model of tendinopathy in the horse

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Summary

Reasons for performing study: Injuries in energy-storing tendons are common in both horses and man. The high prevalence of reinjury and the relatively poor prognosis for returning to preinjury performance levels warrant further research, for which well characterised models would be very helpful.
Objectives: Given the clinical similarities in tendinopathy of energy-storing tendons, we hypothesised that a recently developed experimental model of equine tendon injury would display many of the characteristics of clinical tendinopathy and could therefore be of use for both species, thus providing comparative insight to the human condition and offering direct potential impact to equine medicine.
Study design: *In vivo* experimental study.
Methods: Surgical lesions were created in the superficial digital flexor tendon (SDFT) of 6 horses. Clinical examination, as well as biochemistry, histology and immunohistochemistry were performed on the harvested samples at 6 weeks post surgery.
Results: Disrupted collagen fibres, increased glycosaminoglycan content, increased presence of tenocytes with plump nuclei, the scarcity of inflammatory cells, increased matrix metalloproteinase (MMP) activity and neovascularisation were observed and found to be consistent with clinical tendinopathy.
Conclusion and relevance: This model displays the key features of the most common human and equine degenerative tendon disorders and is therefore an appropriate, if still imperfect, model of tendinopathy.

Keywords: horse; experimental model; surgical lesion; energy-storing tendon; histopathology

Introduction

The horse is prone to some orthopaedic disorders that are similar to those in man [1]. Tendinopathy is one of the main causes for the retirement of racehorses [2] and accounts for up to 50% of all sports injuries in man [3,4]. Under light microscopy the pathological aspects of tendinopathy are similar in both species, being characterised by disrupted collagen, increased proteoglycan content, more prominent and numerous tenocytes and neovascularisation [5–10]. In human patients, Achilles tendinopathy is characterised by pain with minimal classical signs of inflammation whereas equine tendon disease frequently has a sudden onset with a short and variable phase of classic inflammation. Equine superficial digital flexor tendon (SDFT) disease rarely, if ever, leads to long-term lameness while other tendon injuries, especially deep digital flexor tendon injuries, frequently do [11]. Continuing efforts need to be made to improve the effectiveness of treatment and to expedite recovery. Toward this end, validated experimental models of tendinopathy are essential to gain a better understanding of the tendon repair process and to optimise clinical strategies for treatment.

Numerous *in vivo* and *in vitro* tendinopathy models have been developed. Many of these *in vitro* models remain very distant from the clinical situation and the translation of findings from such models to naturally occurring pathologies remains largely speculative. Forced treadmill running and artificial muscle stimulation have been used to provoke tendon disorders in small animals [12–16]. Despite the solid aetiological justification of those models, the reproducibility of the injury is variable between individuals and depends strongly on the targeted tendon. Furthermore, the tendon lesions created in these models often heal spontaneously when overuse training is discontinued, a phenomenon that only rarely occurs in clinical cases [14,16,17]. Another widely employed model, which has the advantage of not showing spontaneous repair, is the collagenase-induced lesion model whereby bacteria-derived collagenase is injected into the tendon to cause widespread fibre disruption. Histological, clinical and functional evidence indicates a certain degree of similarity of this model to naturally occurring tendinopathy [18,19]. However, the suitability of this model has been questioned due to the poor consistency of the lesions and the fact that intratendinous injection of collagenase initiates a strong inflammatory response, also involving the surrounding peritendinous tissue that is rare in the more chronic stages of natural tendon injury [18].

Other models using cytokines, inflammatory mediators, corticosteroids or fluoroquinolone have been developed but these have never been thoroughly characterised with respect to their similarity to naturally occurring tendinopathy [20–24]. There is thus both a need for, and a persistent lack of, an appropriate model for tendinopathy. Many models continue to be used in the absence of validation, thereby leading to potentially misinterpreted treatment effects and an excessive burden on research animals. Our aim in this study was to characterise a recently developed and seemingly promising equine tendinopathy model based on the surgical creation of lesions that is gaining widespread use [25–29] and to discuss its strengths and limitations compared with more established models. We hypothesised that this model would closely mimic the well-known features of naturally occurring lesions in both horses and man and have collected clinical, computerised ultrasonographic, macroscopic, biochemical, histological and immunohistochemical data at 6 weeks after surgery to verify this.

Materials and methods

Experimental animals

Six Standardbred horses (aged 2–5 years; 3 geldings and 3 mares; 489 ± 28 kg) were used. The horses were free of lameness prior to the experiment. The SDFTs showed no signs of tendinopathy upon initial clinical and ultrasonographic investigation. The study was approved by the Ethical Committee of Utrecht University in compliance with the Dutch act on animal experimentation.

Surgical procedure and post operative treatment

Surgery was performed under general anaesthesia using a technique adapted from Schramme *et al*. [25]. A small skin incision was made in the palmar midline of the limb, just proximal to the digital flexor tendon sheath, in both forelimbs. Through a small (1 cm) longitudinal incision into the core of the SDFT, an arthroscopic burr (C9110)[a] (Ø4.0 mm) was inserted proximally over a length of 6 cm. Incisions in the paratenon and skin were

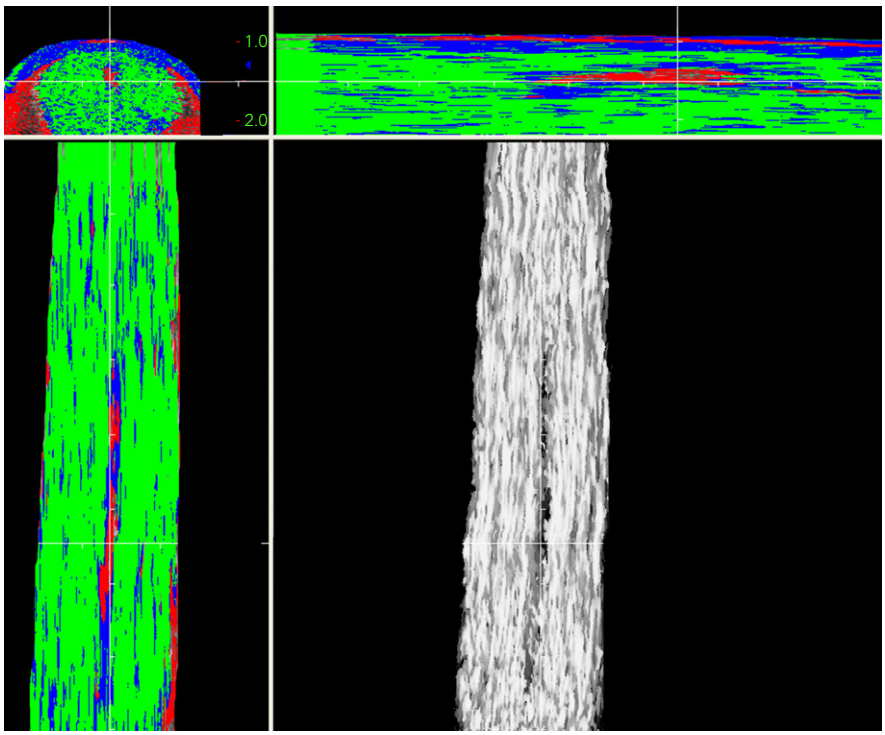

Fig 1: Transverse (top-left), sagittal (top-right), coronal (bottom left) and coronal 3-D ultrasonographic image of surgically induced standardised core lesion in equine SDFT 10 days post surgery. The lesion is clearly visible as a loss of structural integrity in the centre of the tendon indicated here by red intratendinous colour coding.

closed in a routine fashion. Horses received oral meloxicam (Metacam)[b] 0.6 mg/kg bwt preoperatively and for 3 days post operatively. A 2-layer bandage was applied to the limbs for 10 days. After that period the skin sutures and bandages were removed under sedation (detomidine 10 μg/kg bwt). Horses were box-rested for 3 weeks and then walked daily for 10 min until the end of the study.

Clinical assessment

Body temperature, heart and respiratory rates and appetite were evaluated daily throughout the study. Surgical wounds were inspected daily for signs of infection (Days 0–42). The horses were examined for lameness by an equine veterinarian at 6 weeks post surgery.

Computerised ultrasonographic tissue characterisation

Horses were sedated with detomidine 10 μg/kg bwt i.v. and the palmar aspect of the metacarpus clipped, shaved and washed before each ultrasonography session. Using established protocols [6,27], computerised ultrasonographic tissue characterisation was performed on both forelimbs on Days 0, 10, 15, 21, 28, 35 and 42 post surgery. The system consists of both hardware and software (ultrasonographic tissue characterisation imaging)[c] for quantitative evaluation of tendon structural integrity. Ultrasonographic tissue characterisation is driven by a precision instrument that automatically moves a high frequency ultrasonographic probe (10L5)[d] along the long axis of the tendon, collecting transverse images at regular intervals of 0.2 mm over a length of 9.6 cm. These images are stored in real-time for later processing by compounding contiguous transverse images into a 3D ultrasonographic data-block that can be used 1) for tomographic visualisation and 2) for the ultrasound tissue characterisation and quantification of the architecture and integrity of the collagenous matrix. Further, the procedure allows for accurate calculation of lesion size.

Regions of interest within the 3D ultrasonographic data-block were visualised in the transverse, sagittal and coronal planes, as well in a 3D rendered coronal view (Fig 1). The ultrasonographic tissue characterisation algorithms were applied to quantify the echo patterns in contiguous images and integrity of the collagenous matrix was assessed. Four different echo-types were discriminated and related to histopathological stages (Table 1). All datasets were blindly analysed by the same operators (F.D. and H.v.S.). Once the paratenon suture material was identified in greyscale, transverse images as a superficial hyperechogenic structure casting acoustic shadows, the suture was used as a distal reference point (0 cm). Subsequently, in the 3D block of ultrasonographic tissue characterisation-processed images, the most proximal aspect of the lesion was identified and its length measured for each imaging session. The tendon border was manually delimited, defining the cross-section at 3, 4 and 5 cm proximally to the suture. Interpolation was performed over this 2 cm long tendon segment. A computerised count of the 4 different echo types was provided for each cross-section image and averaged for this tendon segment. The percentages of the 4 echo types were then calculated and the same procedure repeated for each imaging session. Echo *types III* and *IV* together represent the amount of injured tissue (absence of tendon bundles) over the whole tendon cross-section vs. normal tendinous tissue (Echo *types I* and *II*). This measurement was used as a morphometric estimate of the width of the lesion over this 2 cm long tendon segment. Each measurement was repeated 3 times and the average of the 3 values thus obtained was calculated and used for further statistical analysis.

TABLE 1: Ultrasonographic tissue characterisation grade and equivalent histological composition (adapted from Bosch *et al*. Vet J. 2011 [27])

Ultrasonographic tissue characterisation Grade (colour code)	Echo source according to histology
Echo-*type I* (Green)	Generated by reflections at intact and aligned tendon bundles
Echo-*type II* (Blue)	Generated by reflections at discontinuous or waving tendon bundles
Echo-*type III* (Red)	Generated by interfering echoes from mainly fibrillar components
Echo-*type IV* (Black)	Generated by mainly cellular components and fluid in amorphous tissue

Sampling procedure and handling

Six weeks after the start of the experiment, horses were subjected to euthanasia by barbiturate overdose (200 mg/kg bwt i.v. pentobarbital, [Euthesate][e]) after sedation with detomidine 0.01 mg/kg bwt i.v. (Domosedan)[f]. Immediately after euthanasia, one SDFT was harvested per horse. The tendons were divided transversally every 1 cm starting from the entry point of the burr to 12 cm proximally, covering more than the lesion area. Digital photographs were taken of each cross-section. The sections were divided further in the core and in the area adjacent to the core, based on macroscopic evaluation of tendon tissue colour and structure. Half of the samples were snap frozen in liquid nitrogen and stored at -80°C for later biochemical analysis. The remaining samples were fixed in 10% formalin for 48 h, dehydrated and then embedded in paraffin for histopathology.

Histology

Representative sections from each tendon area were selected for the various staining procedures. The samples were cut in 6 μm sections. After deparaffinisation and rehydratation, the sections were stained with haematoxylin & eosin (H&E), Alcian Blue and Masson Trichrome. The area of the specimen showing the most advanced pathological changes was assessed by semi-quantitative evaluation based on a standardised protocol used by Movin *et al*. [30]. Fibre structure, fibre arrangement, roundness of the nuclei, regional variations in cellularity, increased vascularity, decreased collagen stainability, hyalinisation and glycosaminoglycan (GAG) content were assessed using a 4 point scoring system, where 0 is normal, 1 slightly abnormal, 2 moderately abnormal and 3 markedly abnormal. Three observers individually evaluated each specimen. The presence of inflammatory cells was assessed by an experienced pathologist. Masson Trichrome staining enabled assessment of matrix arrangement and composition. Surface areas of blue GAG stain were compared between samples from the core area and the adjacent healthy tissue. For this purpose, 4 sections per condition and per horse were analysed.

Collagen architecture was analysed using confocal laser scanning microscopy [31]. Samples from the core of the lesion, the adjacent tissue as well as sections from horses suffering from naturally occurring tendinopathy were used. After deparaffinisation and rehydration of the tissue sections, the specimens were counterstained with H&E. Confocal pictures were generated with the 543 nm line of helium-neon laser. Fluorescence of the collagen fibres was observed using a 60 × magnification lens. The emission light beam was recorded by a photomultiplier after passing a pinhole aperture and emission filter. The depth of the optical section (confocal z resolution) was kept constant by holding the size of the aperture pinhole constant in the emission pathway. The scanning time-setting for image collection was 8 s with the average set to reduce photon noise. The bandwidth filter was set to 1 for the sharpest images.

Immunohistochemistry

To characterise the presence of myofibroblasts (cells involve in wound healing and potentially fibrosis), a mouse monoclonal antibody recognising alpha-smooth muscle actin[g] was used. The slides were treated for 60 min at room temperature (1:1200) followed by an ABC/PO-complex solution for another 30 min at room temperature. The slides were then counterstained with haematoxylin to highlight the nuclei.

Biochemical analysis

Collagen and cross-link analysis: Collagen and cross-links were analysed using a method adapted from Colgrave *et al*. [32]. After lyophilising for 24 h, the tendon samples were hydrolysed (110°C, 18–20 h) in 6 mol HCl for mass spectrometric determination of hydroxyproline (Hyp) and the collagen cross-links hydroxylysylpyridinoline (HP). The hydrolysed tendon samples were vacuum-dried and dissolved in an internal standard solution (2.4 mmol/l homo-arginine). After centrifugation at 13,000 ***g*** for 10 min, the supernatants were subjected to high-performance liquid chromatography (HPLC) and mass spectrometric using a 4000 Q-TRAP mass-spectrometer (MDS Sciex)[h] at a source temperature of 300°C and a spray voltage of 45 kV. Amino acids were separated on a Synergi MAX-RP 80A[i] (250_3 mm, 4 mm) column at a flow rate of 400 ml/min. Elution was performed with a isocratic flow of MilliQ1water/acetonitrile (80/20 v/v) for 2 min, followed by a linear gradient to MilliQ1water/acetonitrile (20/80 v/v) for 16 min, after that the column was washed with 100% acetonitrile for 4 min and conditioned with MilliQ1 water/acetonitrile (80/20 v/v) for 2 min (all buffers contained 1.2 mmol/l of tridecafluoroheptanoic acid and 2.5 mmol/l ammonium acetate [pH 5.6]). Amino acids were identified by mass spectrometric in multiple reaction modes using the mass transitions 429.0/82.0 (HP), 189.2/143.7 (homo-arginine) and 131.8/67.8 (Hyp). A known amount of Homo-arginine was used as an internal standard to measure recovery after processing the samples. Collagen content was calculated as follows: mg collagen 0.25*(pmolHyp/300)*0.3 (300 being the number of Hyp residues in one collagen triple helix, 0.3 being deduced from the molecular weight of collagen, 300,000 Da).

Glycosaminoglycan analysis: After lyophilising for 24 h and recording dry weight (dwt), the tendon samples were digested with papain. The sulphated GAG concentration was analysed using the modified 1,9-dimethylmethylene blue (DMMB) dye binding assay described by Farndale *et al*. [33]. Shark chondroitin sulphate was used as a standard. Results were expressed as μg/mg dwt.

Matrix mettaloproteinase (MMP) activity analysis: General MMP activity was measured by means of a fluorimetric assay based on cleavage of the fluorogenic peptide substrate FS-6[i]. This substrate shows enhanced sensitivity for measurement of collagenase (MMP-1,8,13) activity in biological fluids [34]. 80 μl of buffer (0.1 mol/l Tris, 0.1 mol/l NaCl, 10 mmol/l $CaCl_2$, 0.05% [w/v] Triton X-100, 0.1% [w/v] PEG 6000, pH 7.5) and 100 μl of 10 μmol/l FS-6 solution was added to 20 μl of homogenised tendon samples after which the fluorescent signal was monitored over 10 min. The linear slope (RFU/s) was calculated as a measure of general MMP activity.

Data analysis

Samples were grouped into 2 categories: samples from the core of the artificially created lesion and samples from the area adjacent to the visible lesion. Data were tested by use of a paired *t* test. A value of $P<0.05$ was considered significant for all statistical analyses.

Results

Clinical evaluation

Throughout the experimental period, the general health status of the horses, as assessed by a daily routine check, remained good. Horses were not recumbent more frequently or for longer durations than before the intervention. Lameness was checked by hand walking along a straight line on a hard surface only and horses were not lame at 6 weeks post surgery. All skin wounds had healed completely by that time. However, there was a palpable irregular thickening of the skin at the site of injury and the tendons appeared to be tender.

Computerised ultrasonographic tissue characterisation

Ultrasonographically, the surgically created lesions in the 6 horses appeared at D10 as areas with a loss of organisation and structural integrity in the core of the tendon. This was visualised as a marked decrease in structure-related echo-*types I* and *II* that is indicative of the loss of intact tendon bundles. These structure-related echo-types were replaced by an increased amount of echo-*type IV*, which indicates accumulation of fluid and cells in the gap that results from the induced lesion (Fig 1). These findings are consistent with those described earlier in the tendons of injured horses [6,27].

At Day 42, the lesions had enlarged from the initial 6 cm to 8.2 ± 0.2 cm along the proximo-distal axis. The size of the lesion became larger until Day 35 and was 10.9 ± 1.2% of the total cross-sectional area. The small standard deviations for both length and cross-sectional area indicated that the lesions were still highly standardised after 6 weeks and were independent of the individual animal.

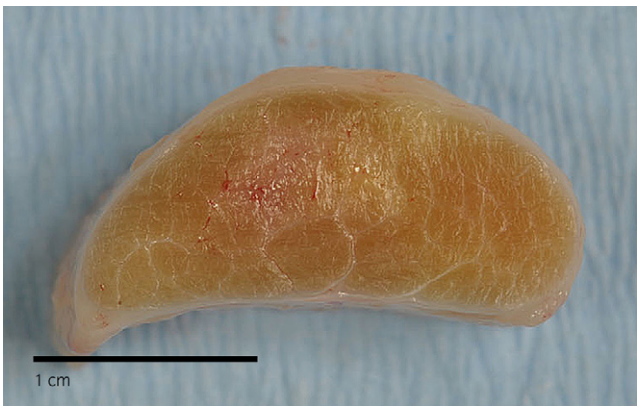

Fig 2: Macroscopic image of a tendon cross section with a surgically induced lesion (light discolouration in the core of the tendon) 6 weeks post surgery. The tendon is still swollen and neovascularisation can be seen in the core of the lesion. The section was taken 2 cm proximally from the entry point of the burr.

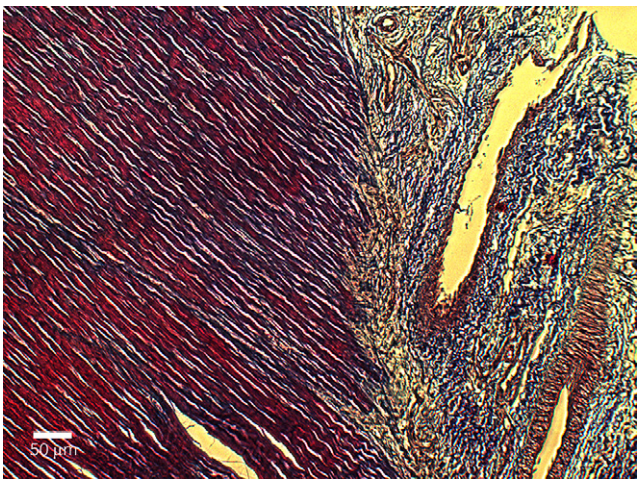

Fig 3: Masson trichrome staining (20×) showing the clear delineation of the artificially created lesion. Healing characteristics are observed on the right side whereas the aligned pattern of collagen fibres is conserved outside the created lesion (left side).

Macroscopic evaluation

The dissected tendons also demonstrated evidence of tendinopathy. Upon macroscopic inspection the tendons were noticeably swollen and their normal crescent shaped cross-section had assumed a half disc shape. The paratenon and endotenon were thickened compared with healthy tendons. In only 2 horses, signs of localised haemorrhages were observed. On cross-section, a generalised light-pink discolouration was observed in the core of the lesion (Fig 2). This discolouration could be seen up to 9 cm proximal to the place where the burr had entered the tendon (avg: 8.3 ± 0.3 cm; n = 6). There was increased vascularisation in the centre of the lesion. The tissue next to the discoloured area had a normal macroscopic appearance (Fig 2).

Microscopic evaluation

Light microscopic examination of the core of the lesions disclosed many hallmarks of naturally occurring tendinopathy (Figs 3, 4). The highly organised structure characteristic of healthy tendons had been profoundly altered with heavily disrupted collagen fibres in the core of the lesion, thereby losing the natural alignment along the loading axis. Small randomly aligned fibres were clearly observed using confocal laser scanning microscopy (Fig 5). The collagen organisation was more affected in the artificial lesion than in the sample with naturally occurring tendinopathy. The mean Movin sum-score [30] of sections from the core of the lesion was greater ($P<0.001$) than the mean sum-score of sections adjacent to the lesions (15.1 ± 2.7 vs. 2.4 ± 1.6) (Table 2). In more detail, the score of the core of the lesions was a median of 2 vs. 0 for the adjacent tissues for all the following criteria: fibre structure, fibre arrangement, rounding of the nuclei, regional variations in cellularity, decreased collagen stainability and hyalinisation. For GAG content, the score of the core of the lesions was a median of 2.5 vs. 1 for the adjacent tissues and the score of the core of the lesions was a median of 1 vs. 0 for the adjacent tissues for increased vascularity. Few lymphocytes and macrophages were noticed in the core of the lesion around vessels. Myofibroblasts, as detected by alpha smooth muscle actin antibody, were mostly located around the newly formed vessels (Fig 4a).

Biochemical evaluation

Mass spectrometry detected a small but significant decrease in collagen content ($P = 0.04$) and HP crosslinks ($P = 0.009$) in samples from the core of the lesions compared with samples from the adjacent area. The values for total collagen content were 0.03 (± 0.01) mg of col/mg dwt and 0.05 (± 0.02) mg of col/mg dwt for the samples from the core and the samples from the adjacent area respectively and the values for the HP crosslinks were 10.8 (± 5.1) nmol/ mg dwt for the samples from the core and 18.2 (± 6.7) nmol/mg dwt for the samples from the adjacent area respectively. Alcian Blue staining also showed an increased presence of GAG in sections of the core of the lesion compared with sections of the area adjacent to the lesion (Fig 4b). This was confirmed quantitatively by the DMMB dye binding assay

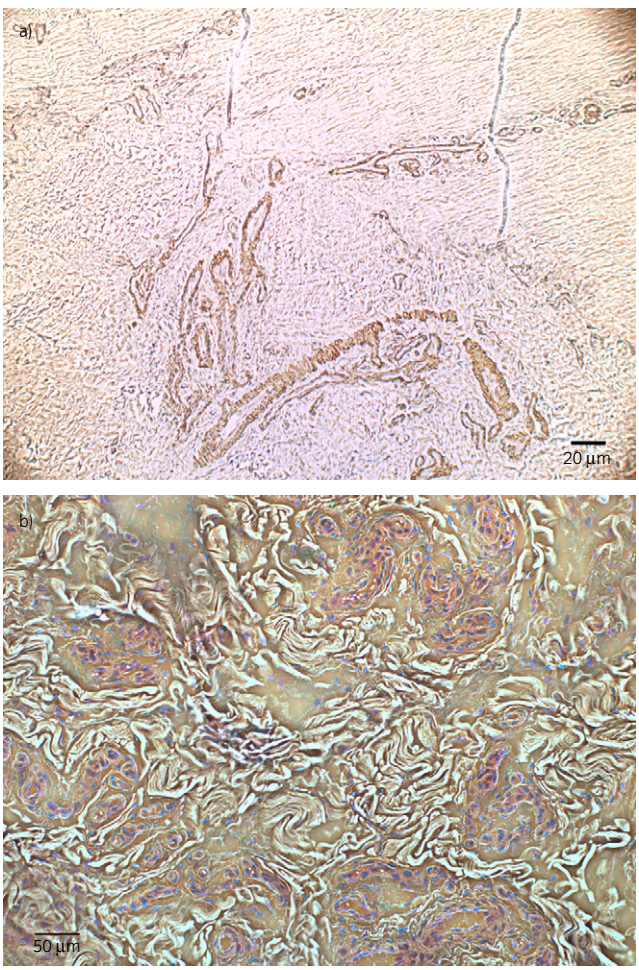

Fig 4: Healing activity is present in the core of the lesion. a) Immunostaining with alpha smooth muscle antibody clearly outlines the new vascularisation (brown) (10×). b) Alcian Blue staining demonstrates the newly deposited glycosaminoglycan (blue). Heterogenous cell distribution is clearly visible (20×).

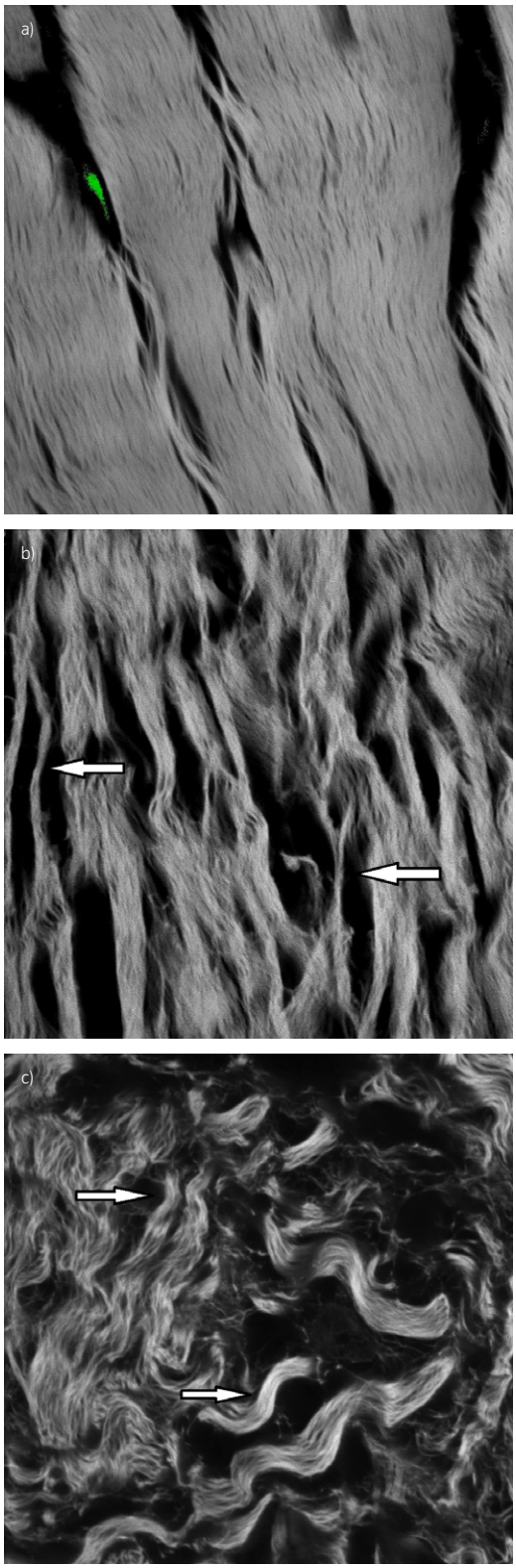

Fig 5: Haematoxylin & eosin slides viewed under a confocal microscope (60×). a) Sample from the adjacent area of the lesion, b) sample from a horse suffering from naturally occurring tendinopathy and c) sample from the core of the lesion. The lesion disrupted the aligned collagen fibre pattern demonstrated in (a) and newly formed collagen fibrils with small diameters (indicated by arrows) are present in the core of the artificially induced lesion similarly to those seen in naturally occurring tendinopathy (b).

TABLE 2: Summary of pathological score of sections from the core of the lesion and from section adjacent to the lesions (Movin score)

Total pathological score	Core of the lesion	Adjacent tissue
Mean	15.1	2.4
Median	14	2.2
s.d.	2.7	1.7
Range	12–19.7	0.7–5.4

Normal or slight tendon changes (0–8), Moderate changes (9–16), severe changes (17–24).

(P = 0.008). The average GAG content for the samples from the core was 172 (± 74.7) μg/mg dwt and the average GAG content for the samples from the adjacent area was 112.9 (± 30.4) μg/mg dwt (Fig 6b). There was a significant increase in total MMP activity in the core of the lesion (P = 0.005). The FS-6 cleavage rate was of 0.7 RFU/s for the core and 0.18 RFU/s for the adjacent area (Fig 6a).

Discussion

To validate models for clinical disorders, clear assessment criteria must be established that relate the model to the clinical condition. For

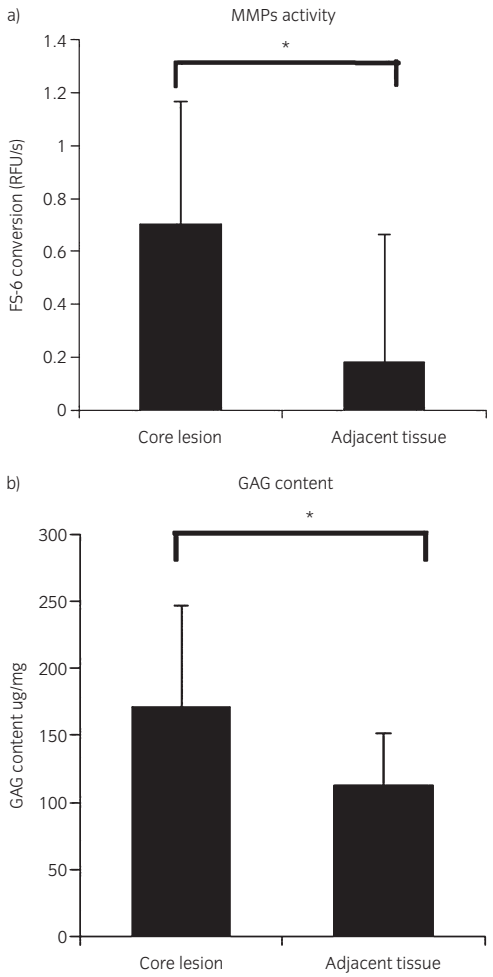

Fig 6: Quantitative measurement of remodelling activity in the core of the lesion compared with the adjacent tissue. Both matrix metalloproteinase (MMP) activity (a) and glycosaminoglycan (GAG) content (b) were significantly increased in the core of the lesion compared with samples from the adjacent area.*P<0.05. The general MMP activity was measured based on the linear slope of the peptide SP-6 fluorescent signal conversion.

tendinopathy, these criteria should include organisational, compositional, imaging and clinical parameters, together with parameters related to function [35]. In the present study we sought to apply this high standard of assessment to evaluate a recently developed experimental equine SDFT lesion model originally proposed by Schramme *et al*. [25,28].

In the surgically created lesions the organisational parameters based on histopathology revealed hypercellularity, hypervascularity, loss of matrix organisation and rounding of cell nuclei. These correspond to characterised clinical cases of tendinopathy [5,36,37]. These findings, while qualitative, represent an essential point of model validation with regard to capturing key aspects of the disease [9]. Any model lacking these traits would be of questionable clinical relevance.

Compositional parameters were also investigated, based on the objective evaluation of the content of the extracellular matrix. Since samples were harvested from animals 6 weeks post surgery, we opted for analysis of proteins rather than gene expression. Interestingly, total collagen content and HP crosslinks were still slightly lower in the core of the lesion compared with the adjacent area. This illustrates the slow healing capacity of tendons after such injury. An increase in total GAG content is observed in chronic tendinopathy and this was also observed in the core of this artificial lesion [5,38]. Increased MMP activity in the core of the lesion can be seen as being indicative of increased tissue turnover and a dysregulation of cellular activity. Again, this is also observed in clinical cases of tendinopathy [38].

With regard to clinical parameters, the tendons were swollen and still tender 6 weeks following surgery. Computerised ultrasonographic tissue characterisation revealed a strong hypoechogenicity and an altered longitudinal fibre pattern in the centre of the tendons (Fig 1). This is also one of the hallmarks of equine naturally occurring tendinopathy [39]. Given the fixed time point of the present study, longitudinal healing characteristics in the model could not be investigated. However, in a previous study from our group [26] in which the same surgery was performed and tendon sections were harvested 24 weeks following surgery, the biomechanical properties were still altered at this much later time point. Collectively these studies indicate that the model captures the long-term deficiency in healing that is emblematic of tendinopathy. Additionally, the severity of the lesion can be assessed as moderate based on the dimension of the lesion compared with the total tendon cross-sectional area (>10% of the total cross-sectional area) [40].

In assessing function-related parameters such as altered gait or activity-related tendon pain, all horses appeared to be sound at walk. However, any lameness may have been obscured since both front legs were operated on and it should be noted that, unlike human patients, horses with tendinopathy are not typically lame for long periods. Mechanical testing of tendon samples *ex vivo* was not performed, but mechanical results from our previous study using samples harvested 24 weeks post surgery still demonstrated lower failure force compared with healthy tendons [26].

In this study, tendon lesions were induced by a surgical intervention leading to the development of core lesions that are very similar to clinically observed tendinopathy. However, it should be noted that, despite the observed similarities to naturally occurring tendinopathy, the model does not reflect the aetiology of the disease. The pathogenesis of natural tendon lesions is thought to be linked with recurrent mechanical overloading [41] (e.g. damage accumulation) and this is therefore the preferred means of creating tendinopathy in laboratory animals. However, it has been shown that up to 30% of severe Achilles tendinopathy cases requiring surgery have causes not related to physical activity [7]. Various other intrinsic and/or extrinsic factors, as for example: age, body composition and genetic factors can be involved in the pathogenesis of tendinopathy [42–44]. In any case, a single animal model can never be representative of this diversity of causative factors.

It is commonly accepted that tendinopathy is a result of a defective healing response to accumulated micro-injuries that occur in the tendon tissue, which for largely unknown reasons is unable to effectively repair itself [45]. In this regard, the current model has a clear advantage. The standardised injury in the core of the tendon offers a concrete and reproducible baseline for characterising, and eventually understanding, downstream healing mechanisms in tendons. The magnitude of the lesion can be standardised, facilitating post surgical comparison between legs and among different horses. Bilateral application of standardised surgery offers a convenient means of assessing the effects of 2 different treatments. On the other hand, it is clear that acute induction of degenerative changes, as practiced in this model, cannot reflect the natural, chronic development of the disease [46]. In our model, the onset of tendinopathy was acute but the healing process remained visible until at least 24 weeks post surgery [29].

Apart from all advantages and the similarities with clinical tendinopathy, the model also presents some limitations when used comparatively for human research. Horses, as large animals, are expensive to use and public acceptance may be limited. Also, current availability of molecular tools is limited in the horse. Importantly, horses often have an unknown exercise history when assigned to an experiment. However, a large advantage of horses over other animals in tendinopathy research is that tendon injury represents a major clinical issue for horses themselves [2]. Any therapeutic modality that shows promise in the horse as a large animal model of human disease will have inherent value for veterinary clinical application in horses.

In conclusion, the macroscopic, microscopic and biochemical assessments all indicated that tendons 6 weeks post surgery very closely resembled naturally occurring tendinopathy. Therefore, although the model cannot be used to study insidious degenerative changes that precede the clinical manifestation of tendinopathy, this equine tendinopathy model provides a satisfactory standardised lesion that may serve well to study the healing mechanisms of tendon tissue with applicability in both equine and human tendon research.

Authors' declaration of interests

No competing interests have been declared.

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Authorship

J. A. Cadby, F. David, G. Bosch, H. T. M. van Schie and P. R. van Weeren have contributed to the study design and the study execution. J. A. Cadby, F. David and H. T. M. van Schie have contributed to the data collection. All authors have contributed to data analysis, interpretation and preparation of the manuscript.

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[e]CevaSante Animale, Libourne, France.
[f]Pfizer Animal Health, New York, USA.
[g]Biogenex Laboratories Inc, Fremont, California, USA.
[h]Applied Biosystems Foster City, California, USA.
[i]Phenomenex Inc., Torrance, California, USA.
[j]Calbiochem, San Diego, California, USA.

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Chapter 3

Short-term cast immobilisation is effective in reducing lesion propagation in a surgical model of equine superficial digital flexor tendon injury.

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Short-term cast immobilisation is effective in reducing lesion propagation in a surgical model of equine superficial digital flexor tendon injury

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Summary

Reasons for performing the study: Larger superficial digital flexor tendon (SDFT) injuries have a poorer prognosis than smaller lesions. During the inflammatory phase enlargement of the initial lesion is frequently noted, with biomechanical loading being recently proposed to play an important role.
Objectives: To evaluate the effect of lower limb cast immobilisation on tendon lesion propagation in an equine model of surgically induced SDFT injury.
Methods: Core lesions were surgically induced in both front SDFTs of 6 young mature horses. At the end of surgery, one leg was randomly placed in a lower limb cast and the other leg (control) was bandaged for 10 days. Computerised ultrasonographic tissue characterisation performed at Days 10, 15, 21, 28, 35 and 42 allowed measurement of lesion length (cm) and width (expressed as a percentage of whole tendon cross-section). On Day 42 horses were subjected to euthanasia and both SDFTs were sectioned every centimetre to assess the lesion length macroscopically. Statistics were performed to compare cast vs. control legs with significance set at $P<0.05$.
Results: When all time points were combined, lesion length was 19% shorter ($P<0.0001$) and lesion width 57% smaller ($P = 0.0002$) in the cast legs (6.13 ± 0.12 cm; 6.90 ± 0.64%) than in the control legs (7.30 ± 0.21 cm; 10.85 ± 1.22%). On Day 42 the lesion length on macroscopic evaluation was 19% shorter ($P = 0.04$) in the cast (7.00 ± 0.36 cm) than in the control legs (8.33 ± 0.33 cm).
Conclusions: Cast immobilisation for 10 days effectively reduced lesion propagation (length and width) compared to bandaging in an *in vivo* model of artificially-induced tendon lesions.
Potential relevance: A short period of cast immobilisation during the early phase of tendon healing may be an easy and cost-effective way to reduce the initial enlargement of lesion size and hence to improve prognosis.

Keywords: horse; tendonitis; tendinopathy; immobilisation; cast; ultrasonography

Introduction

Superficial digital flexor tendon (SDFT) injuries are frequently encountered in equine practice [1–3]. Larger tendon lesions seem to have a poorer prognosis for functional repair than smaller lesions [4,5]. Tendon lesions have a tendency to propagate significantly after initial injury [6]. Although lesion size directly influences prognosis, damage control in the sense of prevention of propagation of the original lesion seems pivotal.

The healing process of tendon injury basically follows the general pattern of wound healing, with 3 overlapping, phases: acute inflammatory, proliferation and maturation [7]. During the inflammatory phase, lasting approximately 10–21 days, disrupted tendon fibres are digested by proteolytic enzymes and then removed by phagocytosis, leading to an apparent enlargement of the initial lesion [6,8]. However, based on findings in experimental studies, it has recently been proposed that biomechanical loading also plays an important role in the initial propagation of tendon lesions [9,10].

Motion restriction of the lower limb during the acute phase has been advised to minimise propagation of the initial lesion [6]. As biomechanical load on the SDFT is maximal during hyperextension of the metacarpophalangeal joint [11], the extension of this joint should be limited. However, even if horses are strictly box-rested, the SDFT remains loaded for as long as the limb is weight-bearing, which is most of the day. Therefore, the application of supportive bandages has been advised [12,13], but a conventional bandage provides little resistance to metacarpophalangeal joint extension [14]. A lower limb cast, as advised by some authors [10,13], prevents hyperextension of the metacarpophalangeal joint. Recently, in an *ex vivo* equine model of acute SDFT injury, casting appeared efficient to reduce lesion propagation [9]. However, the effect of cast immobilisation on the progression of tendon lesions during the early phase of healing has not yet been studied in live horses.

It was hypothesised that immobilisation with a lower limb cast should significantly reduce tendon lesion propagation compared to immobilisation with a Robert-Jones bandage in an equine model of surgically induced SDFT lesions.

Materials and methods

Animals

Six young mature Standardbred horses (mean ± s.d. age 52 ± 13 months; 3 geldings and 3 mares; bodyweight 489 ± 28 kg) were used. The horses were free of lameness and ultrasonographic evaluation did not reveal any signs of present or previous tendon injuries. The experimental protocol was approved by the ethics committee of our institution.

Surgery

The horses were premedicated with detomidine 10–20 μg/kg bwt i.v. and butorphanol 20 μg/kg bwt i.v. and anaesthesia was induced using ketamine 2.2 mg/kg bwt i.v. and midazolam 0.06 mg/kg bwt i.v.. They were positioned in lateral recumbency and anaesthesia was maintained with isoflurane 1.2% (end tidal) and a continuous rate infusion of detomidine 10 μg/kg bwt/h. Using a technique adapted from Little and Schramme [15], core lesions were created in the SDFT of both front legs. This procedure was chosen in order to mimic lesions observed clinically as closely as possible [16]. Briefly, a 10 mm skin incision was made in the palmar midline of the limb, just proximal to the proximal limit of the digital flexor tendon sheath. The incision was continued into the paratenon and core of the SDFT with a No. 15 scalpel blade, taking care not to penetrate the dorsal surface of the SDFT. A blunt arthroscopic obturator (diameter 3.5 mm) was then introduced and guided proximally over a distance of 6 cm through the

central part of the SDFT, in order to create an intratendinous tunnel. Following withdrawal of the obturator, a 4.0 mm diameter arthroscopic oval burr (Linvatec)[a] was introduced along the previously created tunnel over a length of 6 cm. With the blade facing palmar, the burr was activated and slowly retracted at a speed of 0.003 m/s. The burr was manually rotated during removal in order to damage palmar, lateral and medial tendon fascicles while taking care not to penetrate the dorsal surface of the tendon. No suction was applied during burring. Incisions in the paratenon and skin were closed routinely. Horses received meloxicam (Metacam)[b] 0.6 mg/kg bwt i.v. preoperatively and for 3 post operative days orally. To ensure repeatability of the procedure, all lesions were created by the same operator (F.D.).

Immobilisation and post operative treatment

With the horse still in lateral recumbency, one randomly chosen leg was immobilised with a half-limb cast while the other was placed in a 2-layer Robert-Jones bandage (control).

For the cast, the cannon and digit were maintained aligned and the leg was wrapped in a light one-layer bandage. Three rolls of 10 cm fibreglass material (Cellacast)[c] were then applied from the proximal region of the cannon down to and including the foot. The bottom of the cast was moulded with 2 rolls of plaster of Paris (Plastrona)[d]. Finally a piece of rubber was secured to the bottom of the cast using an additional roll of fibreglass material. A 2-layer Robert-Jones bandage was placed on the other leg and the horses were recovered from anaesthesia. To ensure uniformity, all casts and the first Robert-Jones bandages were applied by the same operator (F.D.). During the first 10 post operative days the Robert-Jones bandages were replaced every third day.

On post operative Day 10 the cast, Robert-Jones bandage and skin sutures were removed under sedation (detomidine 10 μg/kg bwt i.v.) and a stable bandage was applied on each forelimb for 5 days.

The horses were kept stall-rested until post operative Day 28, then were hand-walked for 10 min daily until the end of the study (Day 42).

Clinical assessment

Body temperature, heart rate, respiratory rate and appetite were evaluated daily throughout the study. Comfort level was carefully monitored during the immobilisation period (Days 0–10). Casts and Robert-Jones bandages were inspected daily to assess the need for reinforcements or replacements. Surgical wounds and SDFTs were inspected regularly for signs of infection (Days 10–42).

Computerised ultrasonographic tissue characterisation (UTC)

Using a standardised protocol, the UTC procedure was performed on both forelimbs on Days 0, 10, 15, 21, 28, 35 and 42 post surgery. Horses were sedated and the palmar aspect of the cannon was clipped, shaved and washed before each ultrasound session.

To ensure repeatability of the procedure, the ultrasound scanner settings were standardised (preset gain, focus, time–gain compensation) and all datasets were collected by the same operator (H.v.S.). Once ultrasound gel was applied, a 10 MHz linear-array transducer (Smartprobe 10 L5)[e] coupled to a custom-built tracking device[f] was applied over the palmar aspect of the cannon. To ensure that scanning was accomplished in a gradual, sliding movement, the transducer was fixed in a motorised tracking device with an in-built stand-off. It moved at a constant speed over a distance of 9.6 cm, in approximately 45 s. Perpendicularly to the tendon's long axis to maintain a correct angle of insonation, 480 raw digital transverse images were collected at a regular distance of 0.2 mm [10]. The images were stored and subsequently processed into a 3D data block for tomographic visualisation of the SDFT in transverse, axial and coronal views. The 3D stability of the echo pattern, based on intensity and distribution of grey levels of corresponding pixels in contiguous images, was analysed and quantified by means of custom-designed algorithms for image analysis as developed on isolated equine tendons [17,18]. In these studies 4 echo types were assigned. *Echo type I* is highly stable and generated by intact and aligned tendon bundles. *Echo type II* is medium stable and generated by less continuous and/or waving tendon bundles. *Echo type III* is highly variable and generated by interfering echoes from mainly fibrillar components. *Echo type IV* is constantly of low intensity and variable distribution and appeared to be generated by mainly cellular tissue and fluid. The same UTC algorithms were used in the present study.

All datasets were blindly analysed by the same operators (F.D. and H.v.S.). Once the paratenon suture material was identified in greyscale, transverse images as a superficial hyperechogenic structure casting acoustic shadows, the suture was used as a distal reference point (0 cm). Subsequently, in the 3D block of UTC-processed images the most proximal aspect of the lesion was identified and the UTC length of the lesion was measured for each imaging session.

The tendon border was then manually delimited, defining the cross-section at 3, 4 and 5 cm proximally to the suture. Interpolation was performed over this 2 cm long tendon segment. A computerised count of the 4 different echo types was provided for each cross-section image and averaged for this tendon segment. The percentages of the 4 echo types were then calculated and the same procedure was repeated for each imaging session. *Echo types III* and *IV* together represent the amount of injured tissue (absence of tendon bundles) over the whole tendon cross-section vs. normal tendinous tissue (*Echo types I* and *II*). This measurement was used as a morphometric estimate of the UTC width of the lesion over this 2 cm long tendon segment.

Each measurement was repeated 3 times and the average of the 3 values obtained was calculated and used for further statistical analysis and graphical representation.

Macroscopic evaluation

On Day 42 (6 weeks post surgery) horses were sedated with detomidine 10 μg/kg bwt i.v. and then anaesthetised with a combination of ketamine 2.2 mg/kg bwt i.v. and midazolam 0.06 mg/kg bwt i.v.. The horses were then humanely subjected to euthanasia using an overdose of pentobarbital (200 mg/kg bwt i.v.).

Directly after euthanasia, all forelimb SDFTs were harvested and 1 cm thick transverse slices of the tendons were obtained at the paratenon suture (0 cm) and then every centimetre up 9 cm proximally, with a last section at 12 cm proximal to the suture. Macroscopic digital photographs of each transverse section were taken and tendon tissues were collected for the purpose of another study. The sets of photographs were then blindly analysed by the same operator (F.D.). The most proximal extent of the lesion was identified and the macro length of the lesion was recorded for each limb and horse.

Data analysis

Data were analysed using GraphPad Prism 4.3 statistical software and normality of data was tested using the Shapiro-Wilk method. When data were normally distributed, lesion length (macro and UTC), amount of *Echo types III* and *IV* and lesion width were compared between groups (Cast vs. Control) using a paired *t* test. Otherwise, a nonparametric test (Wilcoxon's signed ranks test) was performed. To compare the effects of time and immobilisation on lesion length, amount of *Echo types III* and *IV* and lesion width, a 2-way analysis of variance (ANOVA) was performed. When significance was found, Bonferroni *post hoc* comparisons were made. All data were displayed as mean ± s.e. The threshold of statistical significance was set to $P<0.05$ for all comparisons.

Results

Clinical observations

No complications were observed intraoperatively. All the horses were fully weightbearing on both forelimbs after surgery and remained comfortable during the whole immobilisation period. One horse developed a cast sore on the dorsoproximal aspect of the metacarpus. Seven days after cast removal, the horse developed a focal secondary cellulitis requiring systemic antibiotic therapy for 5 days and local wound treatment. As the comfort level remained good and no significant lameness was observed, no additional treatment was required.

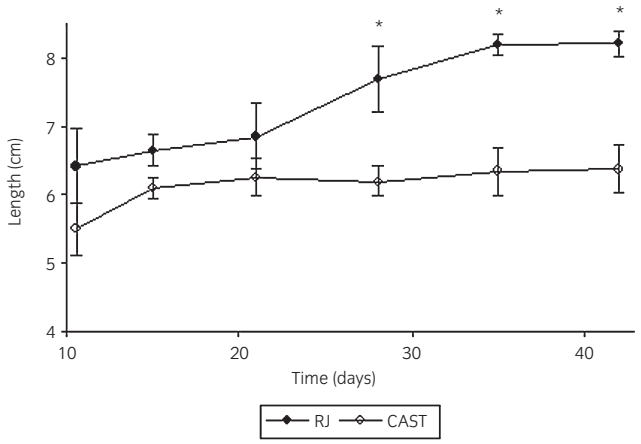

Fig 1: UTC length (mean ± s.e.) of tendon lesions over time. The asterisk (*) indicates a significant difference between the cast and Robert-Jones (RJ) bandaged legs.

UTC length

In the cast legs, the lesion length increased subjectively for the first days following cast removal (Days 10–15) and then stabilised (Fig 1). In the control legs, the lesion length increased from Day 10 to Day 35 and then stabilised. When all time points were combined, the lesion length was 19% shorter (P<0.0001) in the cast legs (6.13 ± 0.12 cm; n = 30) than in the controls (7.30 ± 0.21 cm; n = 24). There was a significant effect of treatment (P<0.0001) and time (P = 0.0016) on lesion length. The effect of interaction between treatment and time was not significant (P = 0.21). Treatment and time respectively accounted for 34% and 22% of the total variation observed. Further *post hoc* analysis showed that the lesion was significantly shorter in the cast legs (Day 28: 6.20 ± 0.21 cm; Day 35: 6.35 ± 0.35 cm; Day 42: 6.68 ± 0.35 cm) than in controls (Day 28: 7.7 ± 0.47 cm; Day 35: 8.17 ± 0.17 cm; Day 42: 8.22 ± 0.19 cm) on Day 28 (P<0.05), Day 35 (P<0.05) and Day 42 (P<0.001). For all other time points (Day 10, Day 15, Day 21), differences were not statistically significant.

UTC width

In the cast legs, the lesion width represented by the combined *Echo types III* and *IV* increased subjectively up to Day 28 and then decreased slowly (Fig 2). A similar tendency was observed for the controls, which peaked at Day 35 instead. When all time points were combined, the amounts of *Echo type III* (P = 0.0071) and *IV* (P<0.0001) in the tendon cross-section were

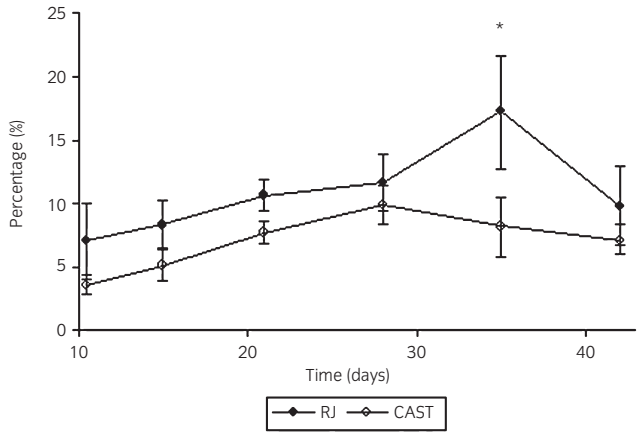

Fig 2: UTC width (mean ± s.e.) of tendon lesions over time. The width of the lesion is expressed as a percentage of the total cross-sectional area. The asterisk (*) indicates a difference between the cast and Robert-Jones (RJ) bandaged legs.

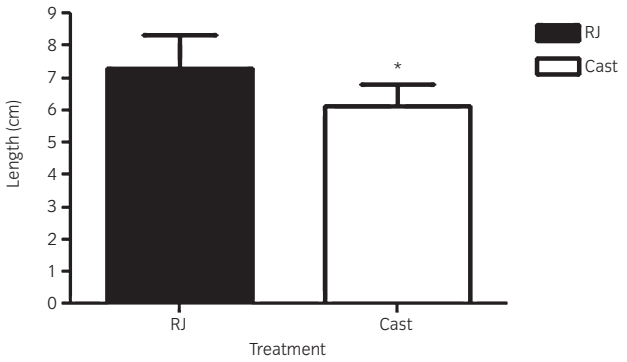

Fig 3: Macroscopic length (mean ± s.e.) of the tendon lesions at Day 42. The asterisk (*) indicates a significant difference between the cast and Robert-Jones (RJ) bandaged legs.

significantly lower in the cast legs (*III*: 2.15 ± 0.36%; *IV*: 1.91 ± 0.32%; n = 36) than in controls (III: 4.07 ± 0.68%; IV: 3.52 ± 0.59%; n = 36). When *Echo types III* and *IV* were considered together, the lesion width with all time points combined was significantly lower (P = 0.0002) in the cast legs (6.90 ± 0.64%; n = 36) than in controls (10.85 ± 1.22%; n = 36). This significant difference corresponded with a 57% larger lesion width in the controls than in the cast legs. There was a significant effect of treatment (P = 0.0022; P = 0.019) and time (P = 0.023; P = 0.040) on the amounts of *Echo types III* and *IV*, respectively, within the tendon cross-section. The interaction between treatment and time was not significant (P = 0.23; P = 0.90). Treatment and time accounted for 12% and 16%, respectively, of the total variation observed for *Echo type III* and for 7% and 15%, respectively, of the total variation observed for *Echo type IV*. *Post hoc* analysis showed that the amount of *Echo type IV* was significantly lower in the cast legs (3.73 ± 1.11%) than in controls (8.82 ± 1.98%) on Day 35 (P<0.05). For all other time points with *Echo type IV*, and at all time points with *Echo type III*, differences were not significant.

Macro length

The lesion length on macroscopic evaluation (Fig 3) was 19% shorter (P = 0.04) in the cast legs (7.00 ± 0.36 cm; n = 6) than in controls (8.33 ± 0.33 cm; n = 6). Although not objectively measured, the lesions in the cast legs seemed smaller on cross-section than in the control legs (Fig 4).

Discussion

In support of our initial hypothesis, this study clearly demonstrates, that cast immobilisation for 10 days significantly reduced lesion length and width propagation compared to bandage application in an equine model of surgically induced SDFT core lesions.

The pattern of lesion propagation was significantly modified by 10 days of cast immobilisation. Although not significant at Day 10, lesions seemed smaller (length and width) in the cast legs. Loaded and unloaded situations alternate many times a day during locomotion (fetlock flexion and extension) or when the horse shifts its weight from one leg to the other. This alternation translates at the tendon level by an alteration in fibre packing density. This creates a kind of 'pump' phenomenon that has been described in a study on the functional anatomy and biomechanics of collagen fibrils [19]. The 'pump' forces interstitial fluid between the collagen bundles, enhancing the dispersion of soluble substances such as proteolytic enzymes within the tendinous matrix. It can be speculated that cast immobilisation would slow down this phenomenon and hence may limit expansion of the lesion. Further, under the influence of weightbearing, progressive tearing of some fibre bundles can be expected after surgery [10]. As a cast prevents fetlock hyperextension [14], the stress-shielding effect of the cast may also have played a role in reducing length extension.

It is interesting to note that a nonstatistically significant rebound was noticed following cast removal (Day 15) for the lesion length in the cast legs.

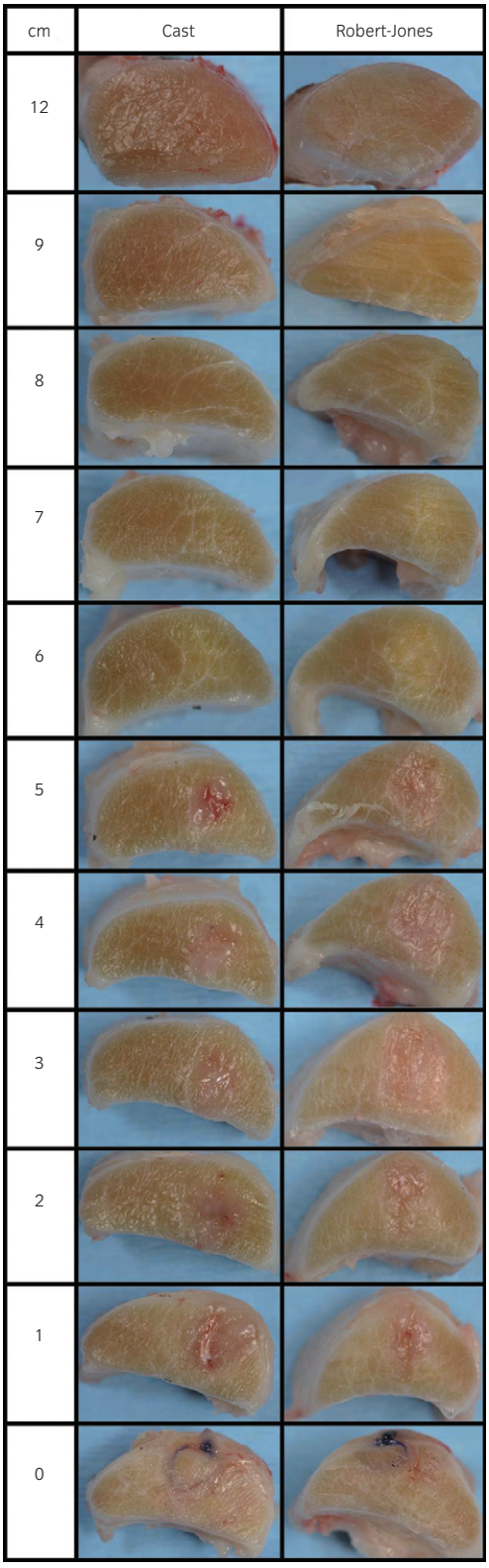

Fig 4: Macroscopic appearance of surgically induced core lesions from a representative horse on Day 42. Transverse sections were made every centimetre from the suture (0 cm) to 9 cm proximally, with a final section made at 12 cm. The lesion is macroscopically shorter (length) and narrower (width) in the cast leg (left column) than in the Robert-Jones bandaged leg (right column).

This is likely to be due to the sudden increase in load and the ensuing increase in fetlock extension after cast removal. This observation is indirect proof of the protective effect of the cast during the early phase of injury. A longer period of casting might potentially have limited this rebound effect. However, despite this rebound, lesions treated with the cast were still significantly smaller than those in the Robert-Jones bandage on Day 42, indicating that the benefit from this short period of initial casting kept the cast lesions constantly ahead of the control lesions. Little scientific evidence exists on the optimal period for immobilisation; it has been advised in human patients that immobilisation is to be used only during the acute phase for about 5–7 days [20]. Further work is needed in horses to determine the optimal duration and best type of immobilisation for tendon injuries.

Cast immobilisation had a stronger protective effect on width (57%) than on length propagation (19%). During the acute phase after a tendon injury the affected area should be protected to prevent inflammation, which, besides cooling, rest and medical intervention, may be achieved by immobilisation in humans [21,22]. In veterinary medicine the same feeling exists, although this is based on anecdotal evidence rather than on scientific work [6]. Tendon inflammation and swelling can be limited by adequate counter-pressure from a proper bandage [12,13]. As cast material is inextensible, the thin bandage applied after the surgery under the cast remained tight during the whole immobilisation period. On the contrary, a Robert-Jones bandage has the tendency to loosen over time. It is possible that this loosening reduced counter-pressure and allowed enlargement of the injured tendon and enhanced lesion propagation in the transverse plane. On Day 35, i.e. 7 days after having started the hand-walking programme, a significant difference in lesion width was observed between Robert-Jones and cast treated legs. During movement, tendons are exposed not only to longitudinal but also to transversal and rotational forces [23]. Cast immobilisation may have not only negated transversal forces and provided a constant counter-pressure that consequently prevented lesion width extension but also prepared the repair tissue to resist the additional mechanical stimulation from the walk.

During the initial post injury phase enlargement of the lesion has been documented in naturally occurring tendon lesions [6,7,24], although the specific time frame was not reported. In our study, lesion size (length and width) increased progressively up to Day 35 post injury in the controls. Maximum length, width/cross-sectional area or core lesion volume have been reported between 4 and 6 weeks post injury in studies using similar experimental designs [10,15]. Naturally occurring tendon lesions may behave in a similar way and therefore should be re-evaluated clinically and ultrasonographically on a weekly basis until Week 4–6 post injury to ensure that the maximal extent of the damage is assessed. Ultrasound images obtained at this time can therefore be considered to represent the lesion at its maximum size and can be used as a reference for further comparison in a rehabilitation programme.

Cast immobilisation has been shown transiently to deteriorate the biochemical and biomechanical properties of tendons in many species, with stress deprivation being regarded as the essential causative factor [25]. With respect to tendons, those effects are well known to be time and dose dependent, completely reversible with remobilisation, and significant variations have been reported between species, with a faster and stronger effect noted in light species (rabbit and rat) [25]. Recently, no detectable effect was reported on human Achilles tendons following a short period of cast immobilisation (2 weeks) [26]. Effects of cast immobilisation have been evaluated on equine bone [27,28], but neither short-term nor long-term effects of immobilisation have been studied in equine tendons and muscles. Although in the present study this cannot be substantiated by biochemical or biomechanical evidence, we presume that significant deteriorations in tendon properties secondary to cast immobilisation were unlikely to occur within 10 days, owing to the notoriously very slow tendon metabolism [29].

Lack of mechanical stimulation has been shown to affect tendon repair negatively [30]. Early mobilisation and a gradual increase in physical load improve healing by enhancing the synthesis of collagen in the proliferation phase and the proper orientation of collagen fibres in the remodelling phase [12,30]. Despite these studies, there is still a lack of consensus regarding the best post operative rehabilitation protocol after Achilles tendon repair in humans [31]. Early functional therapy seems preferable

over strict cast immobilisation [31,32]. However, although there are abundant data on Achilles tendon rupture in man and animal models of tendon transection, those lesions poorly correspond to the core lesions observed in equine cases. Therefore, any extrapolation should be made with caution. Nevertheless, questioning of whether cast immobilisation could have negatively affected the healing process in our study is legitimate. Although it is difficult to determine the exact amount of stress deprivation in our immobilisation model, the type of cast used (cast over a one-layer bandage) has certainly not produced a complete stress-shielding effect and some degree of mechanical stimulation is likely to have remained. The UTC results do not support a negative effect of the cast on healing, but further biochemical, histological, and biomechanical analyses and longer-term studies would be required to fully elucidate this point.

Cast sores are common complications of long-term casting in horses [33]. Although the cast was only kept in place for 10 days, one of the horses developed a superficial cast sore. Cast rub sores at the level of the proximal sesamoid bones are also common and may limit access to intralesional therapy. Alternative immobilisation techniques such as bivalve casts or half-limb boots that can be opened and replaced every day may reduce the risk of cast sore development.

Our study is not without limitations. Core lesions in SDFTs were created surgically and do not correspond exactly to naturally occurring tendon lesions. However, this experimental model allowed for a better control of time, location and size of the tendon injury. As a low limb cast including the foot was applied unilaterally, it is possible that load on the contralateral limb was increased artificially. This extra load on the control leg may have caused additional lesion expansion. However, it is important to note that all horses remained comfortable and weight bearing on both limbs during the whole immobilisation period. A block could have been placed under the foot of the control leg to make the leg length even. This was considered unproductive, as horses were kept in box stalls copiously bedded with shavings. So, the elevation of a few cm on the cast leg was likely to be compensated for by levelling of the bedding. An in-shoe pressure measurement device could have helped to assess the load applied on both forelimbs objectively [34]. Alternatively, 2 groups of horses (one group with cast, one group with bandage) could have been used to minimise the effect of potential uneven weightbearing due to cast application.

The results of this study add to the understanding of the initial enlargement of lesions that is commonly seen after acute tendon trauma. It confirms the idea that application of a lower limb cast for a short period after injury can effectively reduce tendon lesion propagation in an *in vivo* model of artificially induced tendon lesions in horses. A combination of a true immobilisation effect and a constant counter-pressure on the injured tendon are likely to be responsible for the beneficial signs detected on the casted legs. Based on our findings, similar positive effects of immobilisation on naturally occurring tendon lesions can be considered likely but still need to be proved. Importantly, potential detrimental effects of immobilisation need to be investigated before clinical use can be advised.

It is concluded that a short period of cast immobilisation during the early phase of tendon healing may be an easy and cost-effective way to reduce the initial enlargement of lesion size and hence to improve prognosis.

Authors' declaration of interests

Hans van Schie has co-developed the UTC technology, is co-founder and owns a minority share of UTCimaging.

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Manufacturers' addresses

[a]Linvatec, Largo, Florida, USA.
[b]Boehringer Ingelheim, Ingelheim, Germany.
[c]Lohman & Rauscher, Neuwied, Germany.
[d]Paul Hartmann AG, Heidenheim, Germany.
[e]Terason 2000, Teratech, Burlington, Massachusetts, USA.
[f]UTCimaging, Stein, The Netherlands.

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Chapter 4

Effects of platelet-rich plasma on the quality of repair of mechanically induced core lesions in equine superficial digital flexor tendons: A placebo-controlled experimental study.

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Effects of Platelet-Rich Plasma on the Quality of Repair of Mechanically Induced Core Lesions in Equine Superficial Digital Flexor Tendons: A Placebo-Controlled Experimental Study

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ABSTRACT: Tendon injuries are notorious for their slow and functionally inferior healing. Intratendinous application of platelet-rich plasma (PRP) has been reported to stimulate the repair process of tendon injuries, but there is little conclusive evidence for its effectiveness. A placebo-controlled experimental trial was performed to test the hypothesis that a single intratendinous PRP treatment enhances the quality of tendon repair, as evidenced by improved biochemical, biomechanical, and histological tissue properties. In six horses, tendon lesions were created surgically in the Superficial Digital Flexor Tendons (SDFT) of both front limbs, one of which was treated with PRP and the other with saline. After 24 weeks, the tendons were harvested for biochemical, biomechanical, and histological evaluations. Collagen, glycosaminoglycan, and DNA content (cellularity) was higher in PRP-treated tendons ($p = 0.039$, 0.038, and 0.034, respectively). The repair tissue in the PRP group showed a higher strength at failure ($p = 0.021$) and Elastic Modulus ($p = 0.019$). Histologically, PRP-treated tendons featured better organization of the collagen network ($p = 0.031$) and signs of increased metabolic activity ($p = 0.031$). It was concluded that PRP increases metabolic activity and seems to advance maturation of repair tissue over nontreated experimentally induced tendon lesions, which suggests that PRP might be beneficial in the treatment of clinical tendon injuries. © 2009 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 28:211–217, 2010

Keywords: platelet-rich plasma (PRP); tendon; collagen; histology; repair

Tendon injuries are common in both human and equine orthopedic practice.[1,2] They heal slowly and the repair tissue is often functionally inferior to normal tendon tissue, leading to a high re-injury rate.[3,4] The intrinsic repair process of injured tendons relies on the ability of tenocytes to proliferate and produce extracellular matrix (ECM), which is composed mainly of collagen and proteoglycans.[5] The poor healing tendency of tendons has been attributed to the high ECM–cell ratio.[6]

Recently, platelet-rich plasma (PRP), an autologous concentrate of blood platelets has been introduced as a possible new therapy for the treatment of tendon injuries. PRP has been used successfully in maxillofacial surgery to accelerate soft tissue and bone healing,[7] and is currently under investigation for a possible role in regeneration of tendons.[8,9] PRP treatment of tendon disorders appeared to be effective in human and equine patients,[10–12] and studies using laboratory animals or in vitro methods have elucidated some of the working mechanisms.[8,9,13,14]

Platelets are known to play a crucial role in the cascade of tissue healing by delivering growth factors to the site of injury.[15] Upon activation, platelets release growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF)-1, from their α-granules.[16] PDGF, TGF-β, VEGF, and IGF-1 play key roles in tendon repair through effects on proliferation, cell migration, and synthesis of collagen.[15,17–19] A variety of commercial systems for the production of PRP are available; they differ in platelet separation technique and in final PRP characteristics.[20] It is generally accepted that a three- to five-fold increase of platelet numbers, yielding a concentration of at least 1×10^6 platelets/μL, should be achieved.[21]

Tendon repair studies in laboratory animals have been limited in duration, even though it is known that the final stage of tendon repair in humans and horses may only be reached after a year or even longer.[3,22] Furthermore, the degree of mechanical loading, which is vastly different between laboratory species and larger animals, may be important for the ultimate quality of repair. Therefore, longer lasting studies in humans or horses would allow a better judgement of the effectiveness of PRP treatment. Studies in clinical patients would require large numbers because of the large variation in lesions, and do not provide information at the tissue level. These problems can be overcome by using a standardized model of tendon injury.

Several models to study tendon injuries in vivo have been developed. Most are based on application of collagenolytic enzymes[23,24] or on direct surgical damage,[8,25] but they poorly represent real life injuries. Recently, a new, more realistic, surgical model for tendon injuries in the equine Superficial Digital Flexor Tendon (SDFT) has been described.[26]

In the present study, the effect of PRP treatment on standardized, surgically induced lesions in the SDFT of

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horses was evaluated, after a recovery period of 23 weeks from treatment. It was hypothesized that a single PRP treatment 7 days after the original trauma would result in better biochemical, mechanical, and histological properties of the repair tissue than in placebo-treated controls.

MATERIALS AND METHODS

Experimental Animals

Six 3–5-year-old Standardbred horses (five geldings and one mare; bodyweight 448 ± 31 kg) were used. The horses were free of lameness, and ultrasonographic evaluation did not reveal any signs of present or previous tendon injuries. The study was conducted in compliance with the Dutch Act on Animal Experiments, and approved by the Utrecht University Committee on the Care and Use of Experimental Animals.

Surgical Procedure and Postoperative Treatment

Surgery was performed under general anesthesia using a technique adapted from Little and Schramme.[26] Briefly, a small skin incision was made in the palmar midline of the limb, just proximal to the digital tendon sheath. A small (1 cm) longitudinal incision was made into the core of the SDFT, through which an arthroscopic burr (Ø 3.5 mm, Linvatec, Largo, FL) was bluntly, and in inactivated state, inserted proximally over a length of 7–8 cm. Then, the burr was activated and slowly retracted in approximately 20 s. The incisions in the paratenon and skin were sutured in a simple interrupted pattern with resorbable polyglactin 910 (Vicryl Rapide; Ethicon, Somerville, NJ) and unresorbable nylon (Ethilon; Ethicon), respectively. Horses received NSAIDs (Meloxicam, 0.6 mg/kg BWT, Boehringer Ingelheim, Ingelheim, Germany) for 3 days postoperatively. A Robert Jones bandage was applied to the limbs for 14 days. Horses were box rested for 3 weeks, and received an intratendinous PRP or placebo treatment 1-week postsurgery. From week 4 onwards, an increasing exercise protocol was started (Table 1).

PRP Treatment Protocol

Seven days postsurgery, one randomly assigned front limb was treated with PRP, whereas the contralateral limb was placebo-treated. PRP was produced using a commercially available system (GPS II, Biomet, IN). The PRP was divided into 3 mL for treatment and 3 mL for analyses; 0.5 mL of the latter fraction and a sample of whole blood were used for platelet and leukocyte counts (Medonic CA 530; Boule Medical, Stockholm, Sweden). The remaining PRP and separately obtained serum were frozen at −80°C until further analysis.

Table 1. Exercise Protocol of Horses after Inducing Lesions in the SDF Tendons Bilaterally and PRP or Placebo Treatment at 1 Week

Week	Walking (min/day)	Trot (min/day)
1–3	—	—
4–6	10	—
7–10	20	—
11–14	30	—
15–18	40	—
19–21	35	5
22–24	30	10

After sedation with detomidine (0.01 mg/kg bwt. i.v.; Domosedan®, Orion Pharma, Espoo, Finland), the limbs were clipped and prepared aseptically. A local nerve block of both the lateral and medial metacarpal nerve and palmar digital nerve was applied above the site of treatment. Under ultrasonographic guidance (MyLab 25 X Vision with 12–15 MHz linear array transducer; Esaote-PieMedical, Maastricht, the Netherlands), 3 mL of PRP or saline was injected into the core of the induced lesions.

Clinical Assessment

Twenty-four weeks after the start of the experiment, horses were scored for lameness on a 0–5 scale[27] by an equine orthopedist, unaware of treatment site. SDFTs were carefully palpated for signs of pain or irregularities, and semi-quantitatively scored on a 0–4 scale.

Sampling Procedure and Sample Handling

At 24 weeks, the animals were humanely euthanized using pentobarbital (200 mg/kg bwt i.v., Euthesate®; Ceva Sante Animale, Naaldwijk, the Netherlands). Immediately after euthanasia, all treated and control SDFTs were harvested. After localization of the surgical entrance in the tendon, a transverse slice of 1 cm was taken 2-cm proximal to the entrance portal. Proximal to this slice, another segment of 3 cm was harvested. The core of the lesion in the 1-cm slice, that could easily be identified (Fig. 1), was sampled with a 4-mm biopsy punch in longitudinal direction. This sample was divided into three pieces that were frozen and stored at −80°C for biochemical analysis. The 3-cm piece was divided longitudinally into two halves through the center of the lesion. One half, destined for biomechanical testing, was wrapped in PBS-soaked gauze, frozen, and stored in airtight sealed tubes at −20°C. The other half, for histological evaluation, was fixed in 4% formalin for 48 h and then placed in 96% ethanol for 5 days.

Growth Factor Quantification

Commercially available assays were used to quantify active concentrations of PDGF (subtype BB), TGF-β (R&D systems, Minneapolis, MN) and IGF-1 (Diagnostic Systems Laboratories, Webster, TX) in duplicate. Cross-reaction of the antibodies in these human kits with equine samples had been demonstrated previously,[28,29] or has been assumed on homology between equine and human protein structure.[9]

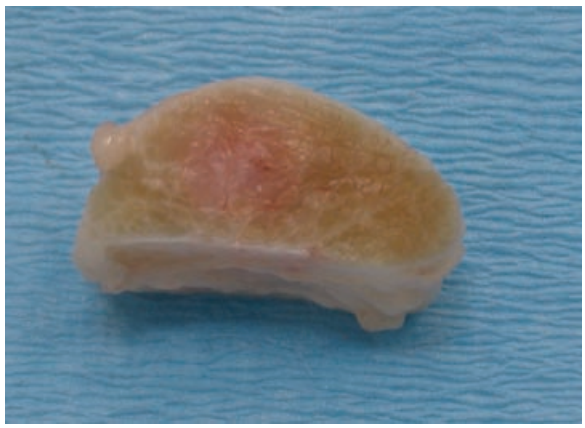

Figure 1. Transverse image of a SDFT core lesion 24 weeks after surgical induction.

Glycosaminoglycans and DNA Analysis

After lyophilizing for 24 h and recording dry weight, the tendon samples were papain digested. The sulphated GAG concentration was analyzed using the modified 1,9-dimethylmethylene blue (DMMB) dye binding assay described by Farndale et al.[30] Shark chondroitin sulphate (0–100 μg/ml) was used as a standard. Results were expressed as μg/mg dry weight. DNA content was measured as described by Kim et al.[31] Salmon sperm DNA (0–500 μg/ml) was used as a reference. Results were expressed as μg/mg dry weight.

Collagen and Cross-Links Analysis

Collagen and cross-links were analyzed using a method adapted from Colgrave et al.[32] After lyophilizing for 24 h, the tendon sample was hydrolyzed (110°C, 18–20 h) in 6 M HCl for mass spectrometric (MS) determination of hydroxyproline (Hyp) and the collagen cross-links pentosidine, hydroxylysylpyridinoline (HP), and lysylpyridinoline (LP). The hydrolyzed tendon samples were vacuum-dried and dissolved in an internal standard solution (2.4 mM homo-arginine). After centrifugation at 13,000*g* for 10 min, the supernatants were subjected to HPLC and MS, using a 4,000 Q-TRAP mass-spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) at a source temperature of 300°C, and a spray voltage of 45 kV. Amino acids were separated on a Synergi MAX-RP 80A (250 × 3 mm, 4 μm) column (Phenomenex Inc., Torrance, CA) at a flow rate of 400 μl/min, using a gradient from MilliQ® water (Millipore, Billerica, MA) to acetonitrile, both containing 1.2 mM of tridecafluoroheptanoic acid and 2.5 mM ammonium acetate. Amino acids were identified by MS in multiple reaction mode using the mass transitions 429.0/82.0 (HP), 413.0/84.0 (LP), 379.3/250.0 (pentosidine), 189.2/143.7 (homo-arginine), and 131.8/67.8 (Hyp). Pentosidine was analyzed simultaneously by fluoroscopy (FP-1520; Jasco, Great Dunmow, UK) with excitation at 297 nm and emission at 400 nm. Data were related to the recovery of internal standard. Collagen content was calculated as follows: μg collagen = (*pmol* Hyp/300)*0.3 (300 is the number of Hyp residues in one collagen triple helix, 0.3 is deduced from the molecular weight of collagen, 300,000 Da).

Biomechanical Testing

Tendon specimens were thawed at room temperature for approximately 1 h before mechanical testing. Two longitudinal slices with a cross-sectional area of 4 mm^2 were cut from the visible lesion, using a cutting device as described by Dudhia et al.[33] Slices were tested to failure in a loading device (LRX; Lloyd, Fareham, UK) at a speed of 0.5mm s^{-1} after preconditioning at 1 Hz and 3% strain for seven cycles. For each slice, a stress–strain curve, and the force at failure (F_{max}) was determined. The stress at failure (σ_{max}) was calculated [$\sigma_{max} = F_{max}$/cross-sectional area ($N/mm^2 = MPa$)] and the modulus of elasticity (EM), was deduced from the slope of the linear part of the curve.[12] Results of the two slices were averaged.

Histology

Longitudinal 5-μm–thick sections were cut and stained using hematoxylin and eosin. The sections were independently scored by two blinded researchers following the semi-quantitative, four-point scale described by Astrom and Rausing,[34] where 0 indicates a normal appearance, 1 slightly abnormal, 2 moderately abnormal, and 3 markedly abnormal. The underlying features were: fiber structure (0 = linear, no interruption; 3 = short with early truncation), fiber alignment (0 = regularly ordered, 3 = no pattern identified), morphology of tenocyte nuclei (0 = flat; 3 = round), variations in cell density (0 = uniform; 3 = high regional variation), and vascularization (0 = absent; 3 = high). Sub-scores were given for structural integrity (fiber structure and fiber alignment), and for metabolic activity (morphology of nuclei, cell density, and vascularization). The scores of the two observers were averaged.

Statistical Analysis

Data were analyzed statistically using SPSS® 15.0 (SPSS Inc, Chicago, IL). Data for biochemical and biomechanical testing, which were continuous and had a normal distribution, were tested using a paired sample Student's *t*-test. The discrete clinical parameters and histomorphological scores were not normally distributed and thus compared using a Wilcoxon Signed Ranks test. Interobserver repeatability of histological scores was assessed using the Intraclass Correlation Coefficient (ICC). The significance level was set at $p \leq 0.05$.

RESULTS

Clinical Assessment

All horses developed bilateral tendon injuries with clinical and ultrasonographical features identical to clinical cases of SDFT injury (Fig. 2). At 24 weeks, five horses were sound and one horse showed slight lameness of the placebo-treated limb. The score for pain and deformation of the PRP-treated tendons (1.33) did not differ significantly ($p = 0.44$) from the placebo-treated tendons (1.83).

Growth Factor Content

Platelets and leukocytes in PRP were enriched with factors 3.78 and 6.00, respectively. Levels of PDGF-BB (2.94 times) and TGF-β (4.47 times) were increased, compared to blood serum levels. The concentration of IGF-1 was slightly lower in PRP than in blood serum (Table 2).

DNA, GAG, Collagen, and Cross-Link Content

PRP-treated tendons had a significantly higher content of collagen and GAG ($p = 0.039$ and 0.038, respectively). Concomitantly, an increase in cellularity, indicated by DNA, was observed ($p = 0.034$). HP, LP, and pentosidine cross-links did not differ (Table 3).

Biomechanical Testing

The tensile properties of the slices from PRP and placebo-treated tendons differed significantly (Fig. 3). The average σ_{max} in the PRP group (9.36 ± 2.52 MPa) was higher than in the placebo group (6.81 ± 2.82 MPa)

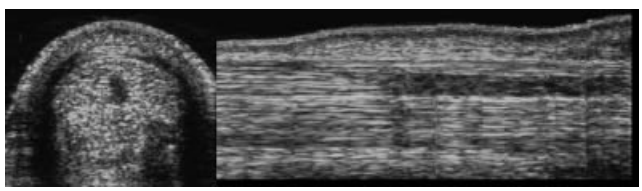

Figure 2. Transverse and longitudinal ultrasonographic image of surgically induced core lesion in equine SDFT 1-week postsurgery.

Table 2. Hematological Parameters and Growth Factor Concentrations in PRP and Serum

Parameter	Serum ($n = 6$)	PRP ($n = 6$)	Ratio PRP: serum
Platelets Giga/l	172.2 ± 21.9	639.7 ± 103.2	3.78
Leukocytes Giga/l	7.0 ± 1.1	42.1 ± 16.7	6.00
TGF-β ng/ml	107.7 ± 26.8	481.9 ± 153.5	4.47
PDGF- ng/ml	1.70 ± 0.27	5.00 ± 0.83	2.94
IGF-1- μg/ml	37.1 ± 17.1	26.3 ± 12.8	0.71

($p = 0.021$). The Elastic Modulus was higher in the PRP group (0.98 ± 0.44 GPa) compared to the placebo controls (0.53 ± 0.30 GPa) ($p = 0.019$).

Histology

No significant differences in total Astrom score were observed between the two groups (Fig. 4). The sub-score for structural integrity was slightly lower in the PRP-treated group ($p = 0.031$), indicating a better structural organization in this group (Figs. 4–6). The sub-score for indications of metabolic activity was higher in the PRP-treated tendons ($p = 0.031$) (Figs. 4–6). ICC for interobserver repeatability was 0.65.

DISCUSSION

This study shows that a single injection of PRP can influence the repair of surgically induced tendon lesions. Twenty-three weeks after PRP injection, the repair tissue from the treated tendons had significantly different biomechanical and biochemical properties compared to placebo-treated controls. The exact mechanisms of action of PRP remain unclear in an end-point evaluation, but the elevated levels of PDGF and TGF-β in PRP can be assumed to have played an important role, as both have been shown to positively influence tendon healing.[22,35] Platelet-derived growth factor plays a role in the acute phase after tendon damage, and stimulates the production of other growth factors; TGF-β is active during the inflammatory phase as an inhibitor of MMP-activity,[36] and it has several effects in cell migration and proliferation.[15] IGF-1 was not

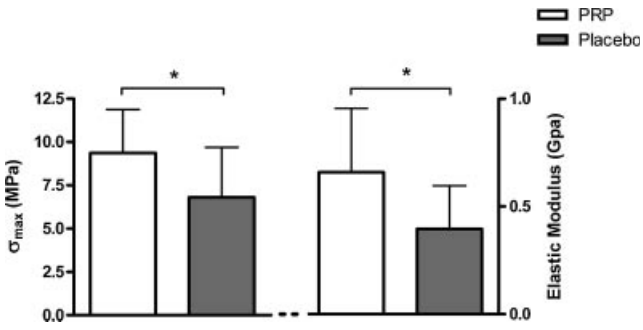

Figure 3. Biomechanical parameters (average ± SD) of PRP and placebo-treated SDFT lesions 24 weeks after sustaining lesions. σ_{max}, stress at rupture (MPa); Elastic Modulus, measure of tensile stiffness of materials (GPa). $^*p < 0.05$.

increased in the platelet fraction, which is in agreement with earlier reports,[9,37] but an indirect effect on the IGF-1 level through a PDGF-induced increase in IGF-1 synthesis might have occured.[15,38] IGF-1 increases cell proliferation and migration, and collagen production, and it was shown to enhance the repair of collagenase-induced tendon injuries.[17]

In both treatment groups, DNA and GAG levels were above those reported for noninjured SDFTs,[39] illustrating cell proliferation and migration and increased ECM production during the naturally occurring repair process. In the PRP-treated tendons DNA, GAG levels and collagen levels were higher than in the placebo-treated group. Although all three variables increased on a dry weight basis, collagen and GAG content expressed per DNA did not increase. This suggests that the principal effect of PRP on the healing process of tendon injury is a (lasting) effect on cell proliferation and migration, and hence on overall metabolic activity. These findings are supported by histological findings, with PRP-treated tendons showing a higher regional cell density and more vascularization, indicating a higher metabolic activity.

Probably the most important parameter in evaluating tendon repair is long-term functionality. Functionality is closely related to the functional capacities of the repair tissue. Although only one (placebo-treated) horse showed a slight lameness after 24 weeks, it cannot be stated that functional repair was achieved, as tendon loads at trot are much less than during heavy athletic activities.[40]

Table 3. Effect of PRP Treatment versus Placebo Control on Biochemical Parameters of Repair Tissue of Surgically Induced Core Lesions in Equine SDFTs 23 Weeks after PRP Injection

	Group		
Parameter	PRP ($n = 6$)	Placebo ($n = 6$)	p-Value
DNA μg/mg dwt	5.89 ± 1.17	4.30 ± 0.27	0.034
GAG μg/mg dwt	31.8 ± 6.9	24.9 ± 2.8	0.038
Collagen mg/mg dwt	0.84 ± 0.10	0.60 ± 0.21	0.039
HP mol/mol col	0.38 ± 0.12	0.54 ± 0.24	0.24
LP mol/mol col	0.027 ± 0.008	0.031 ± 0.011	0.55
Pentosidine mol/mol col	0.0035 ± 0.0021	0.0052 ± 0.0029	0.39

GAG, glycosaminoglycans; HP, hydroxylysylpyridinoline; LP, lysylpyridinoline.

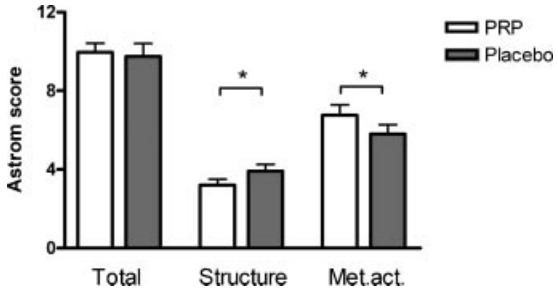

Figure 4. Average (± SD) histomorphological score of PRP and placebo-treated SDFT lesions 24 weeks after sustaining lesions. Total, combined score for all items; Structure, score for structural integrity; Met. act, score for items related to metabolic activity. $^{*}p < 0.05$.

Further, scoring of lameness in horses with a bilateral problem is not unequivocal, making lameness a poor indicator of repair in this experiment. In both groups, palpable abnormalities were still present, which is an indication of the ongoing repair process. Nevertheless, the biomechanical properties of tissue taken from the core of PRP-treated tendons were closer to those of healthy tendons compared to the placebo-treated tissue.[41] Results indicate that PRP-treated tendons show an advanced repair after 23 weeks over placebo-treated tendons. This may result in the need to adapt current rehabilitation protocols after PRP treatment, since it is known that loading plays an important role during the repair process of tendon injuries.[42,43] The difference in biomechanical properties is corroborated by the histological findings indicating a better structural integrity in the PRP-treated tendons. Cross-link levels did not differ between both treatment groups, although numerical values for all three cross-links were lower in the PRP-treated group. In a metabolically active environment, there will be more turnover of ECM components that may lead to a relatively young collagen network with less cross-linking. This might be beneficial to the final quality of the repair tissue, as it has been suggested before that early cross-linking may prevent proper realignment of collagen fibers.[44]

In this study, tendon lesions were induced by mechanical damage to the core of the SDFT without removing much tissue, leading to the development of core lesions very similar to clinical tendon injuries. The created compartment allowed intralesional treatment, which is not feasible in enzymatically induced lesions or the surgical lesions created thus far. Despite the similarity to clinical lesions in horses, the pathophysiology still differs from clinical cases where repetitive

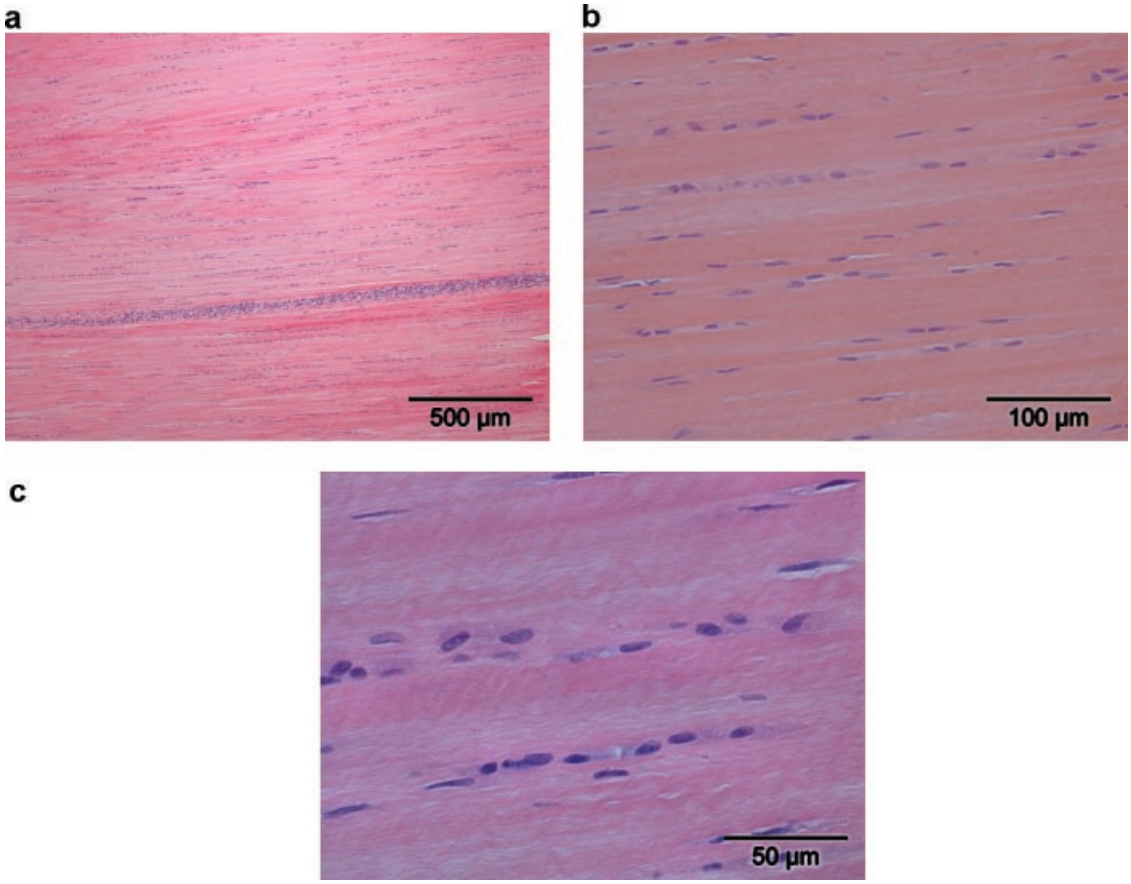

Figure 5. Photomicrographs showing H&E-stained sections of a PRP-treated surgically induced core lesion in an equine SDFT. (a) There is regular organization of extracellular matrix with closely packed fibers and parallel arrangement of tendon bundles. (b) Cellularity is increased; cells are arranged in rows between tendon bundles, and the nuclei are slightly rounded. (c) Fibrils show unidirectional arrangement with a regular wave pattern.

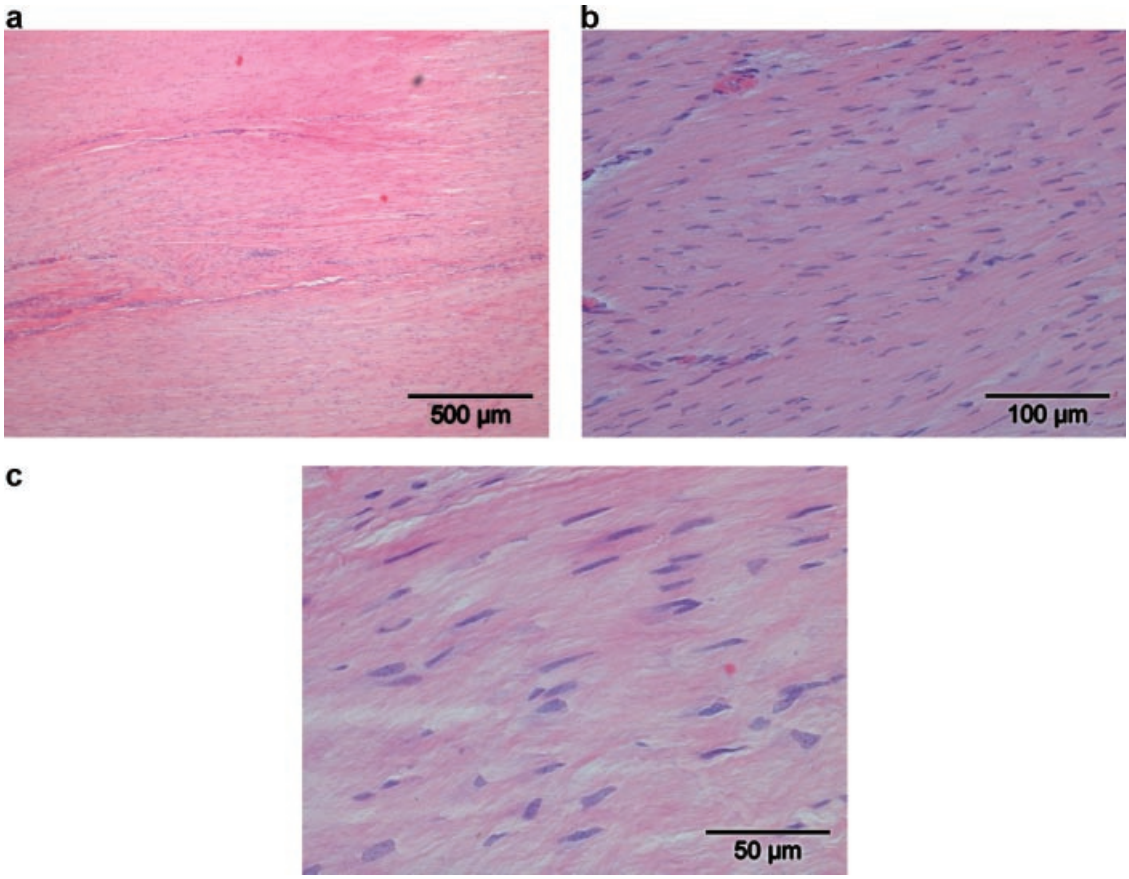

Figure 6. Photomicrographs showing H&E-stained sections of a placebo-treated surgically induced core lesion in an equine SDFT. (a) There is lack of unidirectional arrangement of tendon bundles. (b) The repair tissue shows increased cellularity, fibroblast distribution is fairly homogeneous throughout the tissue, and the nuclei are more elongated compared to Figure 5b. (c) Irregular fibrils are loosely arranged, without a regular wave pattern.

overload is thought to be the primary etiological factor.[6,21] Even though tendon injuries in man also have an acute onset, in many cases they are often only recognized and subjected to treatment in a more chronic stage. It remains therefore to be investigated whether findings using this equine model are valid for human tendon injuries as well. Nevertheless, the findings in this study make it more than probable that PRP treatment will result in a relevant biological effect in the human species as well.

The hypothesis that a single intratendinous PRP treatment, administered early in the proliferative phase of tendon healing, has a significant effect on biochemical, biomechanical, and histological properties of the repair tissue in surgically induced core lesions in SDFTs of horses could be confirmed. Repair tissue of PRP-treated tendons was stronger and had a higher collagen, GAG, and DNA content than control-treated tendons. Based on these findings, it seems likely that a PRP treatment is beneficial for the treatment of acute clinical tendon injuries. The effect on more chronic lesions in both equine and human patients will have to be assessed in double-blinded, placebo-controlled clinical trials. Further research is also necessary to determine the most appropriate timing for PRP treatment in relation to the phase of repair, and procedures to determine the actual stage of the healing process, and should be developed to guide the revalidation scheme.

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Chapter 5

Dose and time dependent effects of exogenous genipin cross-linking on tendon cytotoxicity and tissue mechanics

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Running title: Dose and time dependent genipin cross-linking

Abstract

The crosslinking agent genipin is increasingly invoked for the mechanical augmentation of collagen tissues and implants, and has been demonstrated to arrest mechanically-driven tissue degeneration. In this study we established an *in vitro* dose-response baseline for the effects of genipin treatment on tendon cells and their matrix, with a view to *in vivo* application to the repair of partial tendon tears. Regression models based on a broad range of experimental data were used to delineate the range of concentrations that are likely to achieve functionally effective cross-linking, and predict the corresponding degree of cell loss and diminished metabolic activity that can be expected. We conclude that rapid mechanical augmentation of tissue properties can only be achieved by accepting some degree of cytotoxicity, yet that post-treatment cell survival may be adequate to eventually repopulate and stabilize the tissue. On this basis, we conclude that development of delivery strategies and subsequent *in vivo* study is warranted.

1. Introduction

Cross-linking has long been employed to buttress the mechanical properties of collagen-based implants for the repair or replacement of musculoskeletal and cardiovascular tissues [1, 2]. The physiological environments of these systems can expose implants to extreme physical demands that include high mechanical stresses, high mechanical strains, and/or highly repetitive loading. Such loading regimes can overwhelm even native tissues, a fact that is evidenced by high clinical rates of connective tissue disease and injury [3].

Although tissue and biomaterial cross-linking strategies have almost exclusively focused on *ex vivo* chemical treatments of an implant prior to its application, *in vivo* exogenous cross-linking has more recently been pursued (as we recently reviewed in detail [4]). In this paradigm, the collagen matrix of injured tissue is bolstered by judicious application of low-toxicity cross-linkers. The idea here is to augment a tissue at the margins of a damaged region, arrest mechanically driven tissue degeneration, and possibly provide a foothold for eventual recovery of tissue homeostasis. The use of ultra-violet radiation (in combination with riboflavin as a photosensitizer) to augment the biomechanical properties of connective tissues within the eye has by now become a common clinical treatment of keratoconus, a disorder where local matrix

weakness leads to tissue bulging under ocular pressure. Proof of concept studies using low toxicity cross-linkers in orthopedic applications are also emerging [5, 6].

Among known low-toxicity collagen cross-linking agents, one of the best characterized is genipin (GEN), a naturally occurring organic compound derived from the fruit of the gardenia plant (Jasminoides, Rubiceae). The feasibility and benefit of employing GEN as an alternative to higher toxicity cross-linkers like glutaraldehyde has been demonstrated in a range of applications including heart valves [7], pericardial patches [8], conduits for nerve growth guidance [9], scaffolds for tissue-engineered cartilage [10], decellularized tracheal transplantation [11], and as a more general application to augment the strength and degradation properties of collagen-based gels [9, 12-14].

Our own efforts have focused on *in situ* application of GEN to arrest the progression of tendon lesions that often follows acute injury [6], in an attempt to address this urgent and unmet clinical need (see [15] for background on prevalence and clinical outcome). Tendon injuries are also widespread in equine athletes, with much pathophysiological similarity to tendon injury in man [16]. Using equine tendon we demonstrated that immersion in a high concentration GEN solution (20 mM) could significantly recover post-injury tendon function, bringing it to levels similar to uninjured controls. This functional recovery was reflected in reduced tissue strains at a given mechanical stress, increased tissue elasticity, and the arrest of mechanical damage accumulation during high-cycle dynamic loading. Although functional efficacy of these GEN treatments was clearly demonstrated, the physiological implications (e.g. cell toxicity) of the treatment were not investigated. More specifically, it was unclear what effect GEN treatment has on resident cell populations, and whether GEN concentrations at levels reported by others as non-cytotoxic [10, 17-21]) could be sufficient to elicit recovery of mechanical integrity. This information is critical to guide further development of GEN based clinical approaches to *in situ* tissue augmentation of dense collagen-based connective tissues, including tendon.

The first aim of the present study was to investigate *in vitro* dose-dependent tendon cell toxicity, exploring effects of both treatment time and concentration. Previous studies have similarly investigated a range of other cell types [17-22], with variable results indicating that tissue specific investigation of relevant cell phenotypes is warranted. The present series of studies focus on tenocytes as a class of fibroblastic cells derived from dense collagen connective tissue. The second aim was to investigate the functional effects of *in situ* GEN treatment on tendon explants

to establish dependency of these effects on treatment concentration and duration. Ultimately our goal was to determine whether a balance between non-cytotoxicity and functional (biomechanical) efficacy of GEN dosing could be achieved, widening the potential range of viable clinical applications for this increasingly used collagen cross-linking agent. Method

1.1. Study design

All studies were carried out on isolated cells or tissue explants of the superficial digital flexor tendon (SDFT) from the front limbs of freshly slaughtered horses collected from a local abattoir. All experimental factors (treatment time and concentration) were generally performed with tissue extracted from the same animal and then replicated using tissue from additional animals. Samples were subjected in a random manner to either sham-treatment (incubated in genipin-free cell expansion medium) or in medium supplemented with genipin at concentrations (C_{GEN}) ranging from 0.01mM to 20mM. Incubation durations of 24 h, 72 h and 144 h were investigated.

The experiments were conducted starting with a broad approach and progressively focusing on a more limited range of dosages and their effects. First, we performed cell-culture experiments to assess cytotoxicity in terms of relative cell-viability and metabolic activity. Using tissue explants we assessed penetration of the cross-linking agent, quantified the homogeneity of cross-link distribution by inherent fluorescence, and assessed the physical effects of the treatments as changes in denaturation temperature. All these experiments were performed over a wide range of concentrations and treatment times, aiming to identifying dosing regimes capable of altering the physical properties of collagen with minimal cytotoxicity. In a second phase, we assessed gene expression and cell motility within a reduced range of dosing regimes. Finally, we assessed tissue-level mechanics for a targeted range of GEN dosing, to identify minimal dosing thresholds able to achieve functionally relevant changes in biomechanical properties.

1.2.Isolation of cells and tissue explants

Tissue explants were dissected from the core of the SDFT to a standardized size of approximately 2x2x2 mm^3 under sterile conditions using previously described dissection methods [6, 23] and then incubated in either GEN supplemented or control medium. For isolated cell-culture experiments, tendon cells were extracted by digestion of explanted tissue using Protease Type XIV (Sigma-Aldrich, St. Louis, MO) for 2h at 37°C and Collagenase B solution (Roche, Burgess

Hill, UK) for 16h at 37°C. After the digestion process, the mixture was filtered and centrifuged. The cell pellet was re-suspended, seeded at a density of 10^4 cells/cm^2, and then cultured at 37 C and 5% CO_2 in expansion medium (Dulbecco's modified Eagle's medium, 10% fetal calf serum (FCS), 50 µg/ml gentamicin and 1.5 µg/ml fungizone (all from Life technologies, Paisley, UK)). Cells used in experiments were either freshly digested or passaged once at subconfluency.

1.3. Genipin cross-linking

A 20mM stock solution of GEN (Challenge Bioproducts Co., Taiwan) was freshly prepared in expansion medium for each experiment and then sterile filtered (Millipore, Carrightwohill, Cork, IRL). GEN was solubilized by adding 0.1% DMSO and heating at 40°C. The stock was then diluted to the required concentrations. For explant cell-viability, differential scanning calorimetry (DSC) and tissue mechanics explants were incubated in Falcon tubes containing GEN supplemented medium at 37°C and 5% CO_2. Explants used for biochemical analysis, assessment of penetration and cross-linking distribution were snap frozen after treatment and stored at -80°C until later use.

1.4. Penetration and color changes

After GEN treatment, excess treatment solution was removed by blotting the samples on clean cellulose tissue. Superficial formation of blue pigmentation that qualitatively indicates GEN cross-linking [24] was documented using a digital camera under consistent illumination. The same samples were then embedded in paraffin according to standard methods and cut into 6µm sections (RM2265, Leica, Wetzlar, Germany). Inherent sample fluorescence (excitation wave length (λ_{ex}): 510-560nm; emission wavelength (λ_{em}): 590nm) corresponding to cross-link formation [25] was measured using a fluorescence equipped upright microscope (Nikon Eclipse E600).

1.5. Cell-culture viability and metabolic activity

Cell viability and metabolic activity was compared to a sham/positive control group (cells incubated in expansion medium only) and to a negative control group (cells were killed with 70% methanol for 1 hour prior to the assay). For both tests the experiment was replicated with cells from 3 tendons of different animals (biological replication) with quadruple repeated measures per

experimental condition (technical replication). Cell viability was measured using a viability/cytotoxicity assay (LIVE/DEAD® Viability/Cytotoxicity Kit, Molecular Probes, Life Technologies, Paisley, UK) according to manufacturer recommendations. Briefly viability was measured as relative fluorescence ($\lambda_{ex}/\lambda_{em}$: 485nm/530nm) in a micro-plate reader (SpectraMAX Gemini XS, Molecular Devices, Sunnyvale, CA) compared to controls. Similarly, cell metabolic activity was measured as fluorescence ($\lambda_{ex}/\lambda_{em:}$ 560nm/590nm) after 8 hours of incubation in 10% alamarBlue (alamarBlue assay, Invitrogen) as previously described [26]. Control experiment showed negligible influence of GEN light absorption characteristics on these assays. Finally, intensities were converted to cell viability [%] and metabolic activity [%]:

$$\frac{F(\lambda_{em})_{GEN} - F(\lambda_{em})_{C-}}{F(\lambda_{em})_{C+} - F(\lambda_{em})_{C-}} \quad (1)$$

, where $F(\lambda_{em})$ represents the average fluorescence intensity of technical replications for each experimental condition at the wavelength (λ_{em}) for each test, with C+ and C- representing the positive and negative controls, respectively.

1.6. Cell motility

Cell motility was determined using a standard scratch assay. Briefly, confluent cell mono-layers from 3 different horses were treated in GEN or control medium for 3 days. The mono-layers were then scored with a sterile pipette microtip to leave a scratch of approximately 0.5 mm width. The scratch widths were monitored under an inverted microscope (Zeiss Observer Z1) over 9h collecting digital images at 3h intervals. The scratch width was then measured (Image J software 1.44p, National Institute of Health, USA). Cell migration speed [µm/h] was calculated at 3h, 6h, 9h after the scratch, and average cell velocity was determined as the rate of scratch width closure divided by 2 to account for cell movement on each side of scratch.

1.7. Cell viability in explants

Equally sized tendon explants from 5 horses were weighed (116±12.3mg) and GEN treated. After 3 days the cells were isolated from the tendon explants using enzymatic digestion as described above and surviving cells were counted twice in a Neubauer chamber using Trypan blue

(Molecular Probes, Life Technologies). Viable cell-density was calculated by normalizing the average number of living cells to the initial wet weight of the explants.

1.8. Differential scanning calorimetry in explants

Explants from 5 horses were treated over the full range of concentrations and incubation times. After briefly blotting with paper to remove excess moisture, they were wet-weighed and placed with the largest flat area onto the bottom of stainless steel pans to guarantee optimal heat transfer. After calibration of the differential scanning calorimeter (DSC; 2920 calorimeter, TA Instruments, Delaware US) the pans were sealed and ramped at a constant heat rate of 10C/min from 0-150°C using an empty pan as reference. Denaturation was determined as the temperature at the peak heat flow in the endotherm (T_m). Following DSC measurements the pans were opened and samples were dried on an oven for 16h at 130°C to determine the dry weight which was then considered with the wet-weight to determine water content [27].

1.9. Gene expression analysis

Cells from 8 horses were cultured in a reduced range of concentrations (C_{GEN}: 0mM, 0.1mM and 1mM Gen) for 3 days at 37C and 5% CO_2. At harvesting, cell cultures were resuspended in RNA-Bee™ (TEL-TEST, Friendswood, TX, USA). RNA was precipitated and reverse-transcribed into cDNA using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). As markers of matrix metabolism, we assayed the genes for Collagen type 1 (Col1A1) and matrix metalloproteinase 1 (MMP1), as well as the apoptotic marker caspase 3 (CASP 3) (Table 2). As internal control, the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used after verification that it was stably expressed across sample conditions (data not shown). Amplifications were performed in duplicates for the tested genes and quadruple for internal controls using Power Sybr® green PCR master mix (Molecular Probes, Life Technologies) according to standard manufacturer guidelines. Quantitative Real-Time PCR was performed (StepOnePlus™, Applied Biosystems, Life Technologies, Paisley, UK) and analyzed using the device software. Averaged gene expressions relative to the housekeeping gene were calculated according to $2^{-\Delta CT}$ method and used to discriminate between groups [28].

1.10. Mechanical testing

Mechanical testing was performed with small modifications to our previously described protocols [29-31], where we have reported the power and effectiveness of the employed experimental setup [6, 31]. Due to the large quantities of tissue needed for mechanical tests, tendons were collected and frozen until the day of testing/treatment. At start of the experiment tendons were thawed for 1h at room temperature (RT). Single strips of tendon were sub-sectioned into either triplets or pairs (triplet=13, pairs=11) each of approximately 1x3x50mm^3 in size. Samples of each triplet/pair were pseudo-randomly allocated either to control or GEN treatment groups. The cross-sectional area (CSA) of each specimen was assessed by averaging triplicate readings from a CCD-based custom linear laser scanner (accuracy: -3.52±1.89% and precision: 0.83%) adapted from Vergari *et al.* [32]. Samples were pre-conditioned ten times up to 6MPa followed by a relaxation of 300 seconds at a strain levels corresponding to 6MPa. After a recovery time of 300s, samples were ramped to failure at a constant strain-rate of 0.5%L0s^{-1} (Figure 1). Engineering stress was calculated based on initial CSA and displacement measurements were normalized to engineering strain based on the initial length (L0) at pre-load corresponding to 0.05 MPa. Stress–strain curves were parameterized according to failure (maximal stress and corresponding strain), yield point (stress and strain values determined with a 0.2% offset method, based on the regression line used to determine elasticity in the linear range). Further, elasticity was determined using linear regression in the linear range and strain-energy up to yield was determined [27].

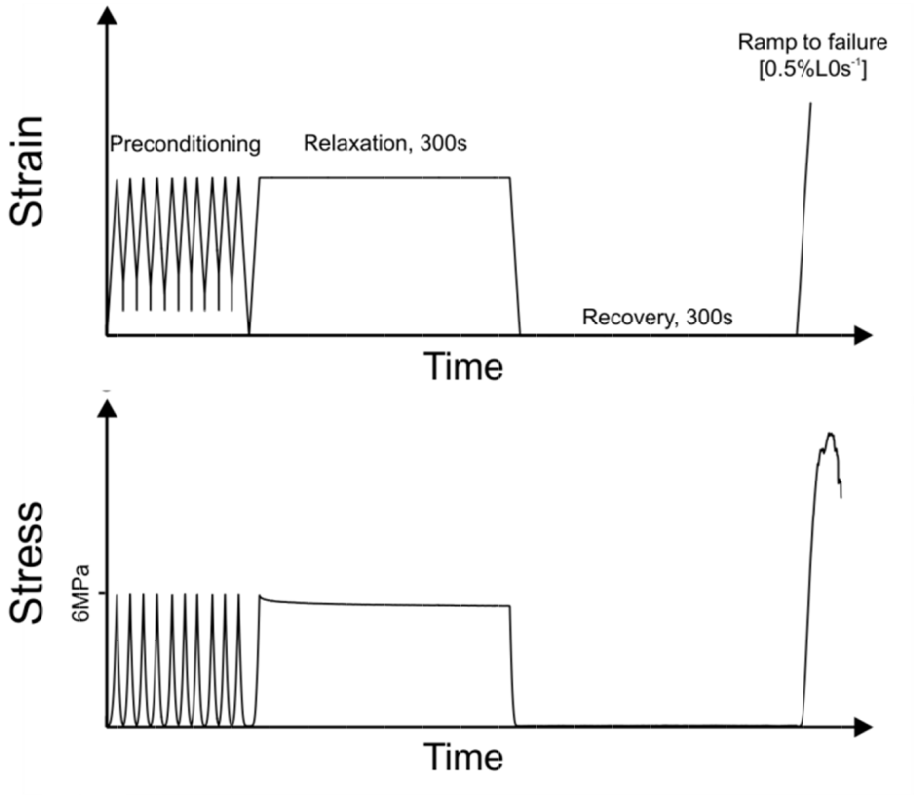

Figure 1: Sketch of the viscoelastic test on an arbitrary scale.

Statistical analysis

Normalized outcome from cytotoxicity experiments (here: % cell viability / metabolism / motility of controls) were analyzed using probit regression (factor: treatment time, covariate: dose), since this model well fits the sigmoidal dose-response curves of toxicity experiments. The probit model was used to estimate toxicity (potency) together with 95% confidence intervals of the GEN treatment in terms of relative effects on cells (similar as e.g. LD_{50}). In case there was a common slope for the regression lines for all treatment times (χ^2-parallelism test, $p<0.05$), axes intercepts and relative median potencies were used to assess the effect of treatment duration on cells, after having assessed an adequate model fit (Pearson Goodness of Fit χ^2-tests: $p>0.15$). The response of the DSC (T_m) was assessed by analysis of covariance (ANCOVA, fixed factor: treatment time, covariate: concentration) yielding regression lines for treatment time. In case there was no significant difference of the interaction term (indicating differences of the slopes of regression lines/covariate) the model was additionally reduced to an analysis of variance to assess

significant effects of concentration (ANOVA, fixed factor: concentration). Post hoc pairwise comparison was performed using Bonferroni correction. Cell motility and mechanical properties were assessed by two-way ANOVAs for randomized block designs (random factor: horse; fixed factor: treatment) and post hoc pairwise comparison was used to discriminate between treatment group effects using Bonferroni correction, or in some cases Dunnett's test to compare test groups against controls. In case model assumptions (sphericity) were not met, Greenhouse-Geisser adjustment or the MANOVA approach was used [6, 27]. Gene expression was assessed by Friedman's test, the non-parametric alternative to the blocked ANOVA. In all statistical tests, differences were deemed significant for p-values ≤ 0.05 and trends for ≤ 0.1. In all cases, two-sided tests were performed. Results are reported as means with standard deviations, if not stated otherwise. All statistics were performed using SPSS v21.0 (IBM Corporation, New York, NY) and/or Matlab R2013a (MathWorks, Natick, Massachusetts).

2. Results

With increasing GEN concentration and time of incubation, the color of the explants changed from light to dark blue, indicating reaction of GEN with primary amines (Figure 2**Error! Reference source not found.**, top). Consistent with the dose and time dependent discoloration, homogeneous cross-linking was indicated by uniform fluorescence throughout the cut sections (Figure 2, bottom).

The probit model indicated that cell viability was clearly concentration dependent and that cell viability was reduced in the 144h treatment group, or stated otherwise, the 144h GEN treatment was more potent. This can be seen by a significant leftwards shift (toward lower concentrations) of the regression line and the increased relative median potency for the 144h group (Figure 4 left, Table 2 and 3). While cell rounding was noted at intermediate concentrations, the two highest tested GEN concentrations (10 and 20mM) apparently fixed (dead) cell morphology in an elongated state. Fluorescence observed throughout the cells may have indicated GEN cross-linking of cellular and intra-cellular proteins (Figure 4).

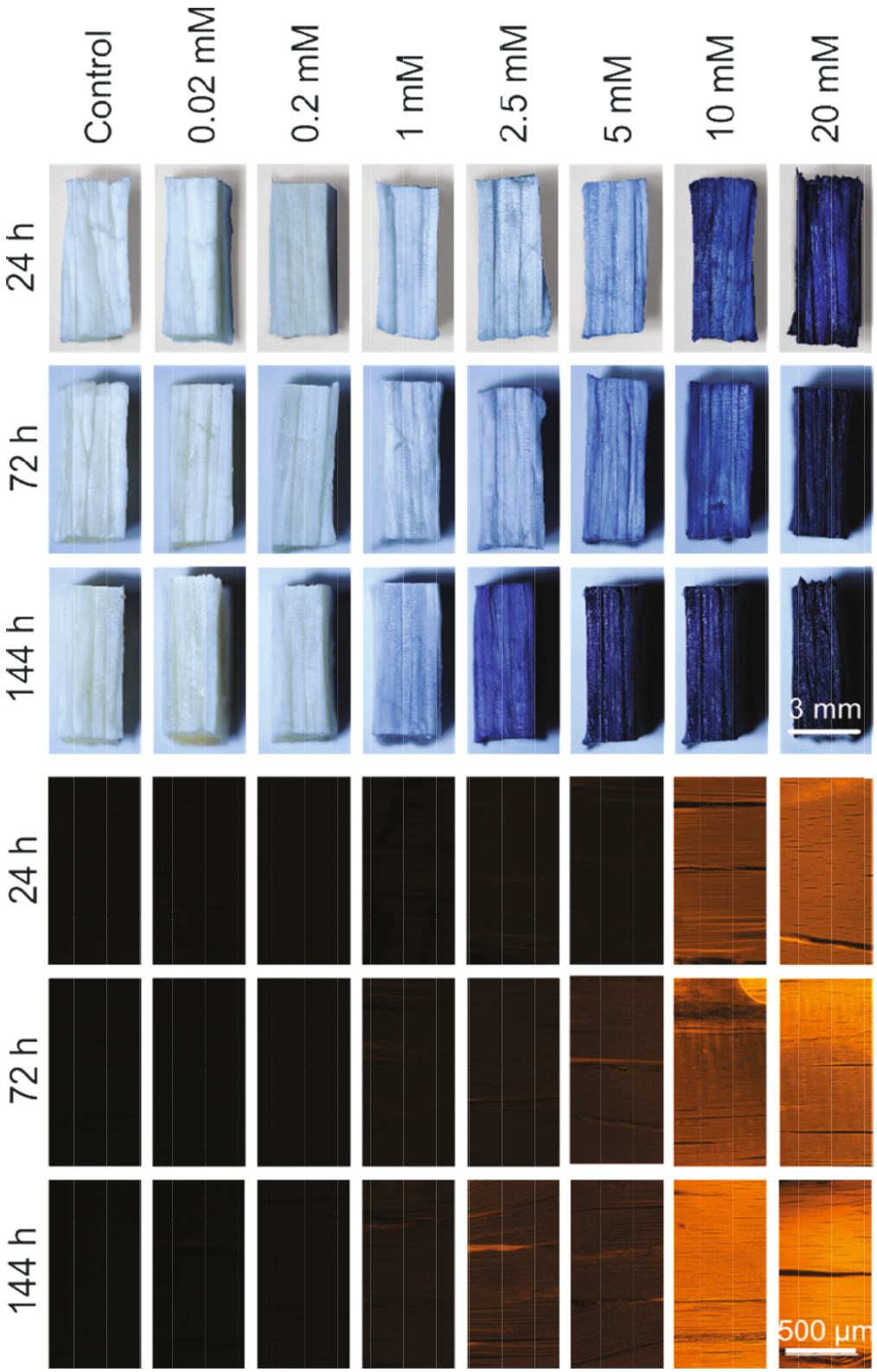

Figure 2: Top: Discoloration of incubated tendon explants. A blue pigment is formed when GEN cross-links primary amines. **Bottom:** Inherent fluorescence indicates cross-links (λ_{ex}: 510-560nm, λ_{em}: 590nm) formed homogenously throughout tendon explants.

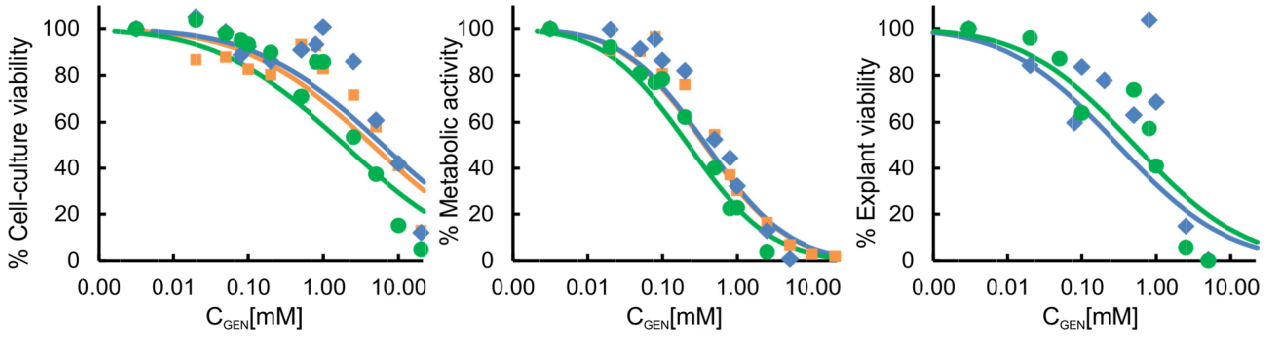

Figure 3: Left) Normalized cell viability measured by Live/Dead Assay. Data points are averages of tendons from different horses (n_r=3) and technical repetitions (n_m=4) of each experimental condition. The probit model (goodness of fit: p=1, common slope: p=0.219) indicated significant effects of dose and time (dose: p<0.0001, reduced axes intercept for 144h group (horizontal location of the curve shifted to lower concentrations): p<0.05). Since $\log_{10}$(0mM) is not defined, this data point was plotted at twice the distance on the $\log_{10}$ scale of the two lowest non-zero concentrations: here 0.003mM. Lines represent the probit models. ■ 24h GEN treatment, ◆ 72h GEN treatment, ● 144h GEN treatment. **Middle)** Averaged (n_r=3 and n_m=4) normalized cell metabolic activity according to Alamar blue assay. The probit model (goodness of fit: p=1, common slope: p=0.907) indicated significant effects of dose (dose: p<0.0001). There was no significant difference in axes intercept (p>0.05). **Right)** Averaged (n_r=3 and n_m=4) normalized cell viability of cells treated within tissue and assessed by Trypan blue. The probit model (goodness of fit: p=1, common slope: p=0.477) indicated significant effects of dose (dose: p=0.03). There was no significant difference in axes intercept (p>0.05).

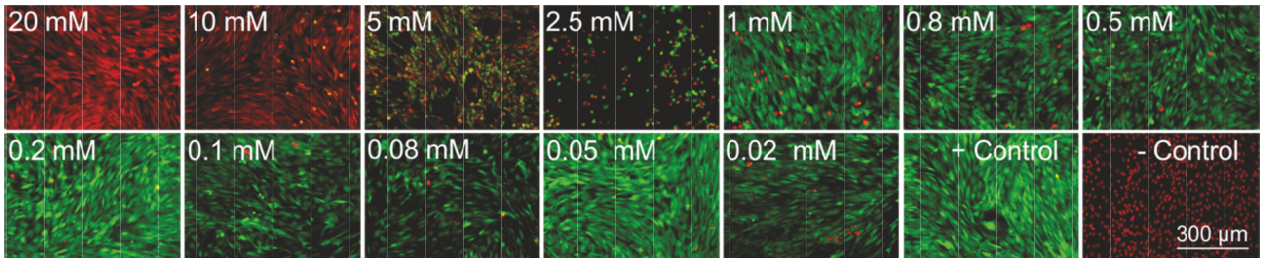

Figure 4: Cell viability tested by Live/Dead assay. Red stained cell-nuclei indicate cell death. Green staining indicates vital cells. The present images were taken after 72h of GEN treatment. Magnification: 10x; C+, positive (sham) control; C-, negative control (ethanol treated cells). Visible cell death is apparent at GEN concentrations starting at 2.5mM, increasing in a dose-dependent manner.

Metabolic activity was also clearly affected in a concentration dependent manner, coupled with a non-significant reduction of metabolic activity in the 144h treatment group. These effects can be seen as a non-significant leftward shift of the regression line, and non-significantly increased relative median potencies (details Figure 4, middle and Table 2 and 3).

		Relative median potency	LCI	UCI
Live / Dead	24 h vs.72 h	0.73	0.22	2.24
	24 h vs. 144 h	2.35	0.78	7.74
	72 h vs. 144 h	**3.23**	**1.01**	**12.13**
Alamar blue	24 h vs.72 h	0.96	0.44	2.11
	24 h vs. 144 h	1.67	0.78	3.92
	72 h vs. 144 h	1.74	0.79	4.18
Trypan blue	72 h vs. 144 h	0.60	0.00	20.95

Table 1: Relative median potencies were used to compare the potencies of the different treatment times. Significant effects are marked by an estimated significant difference from one (upper (UCI) and lower (LCI) do not include one).

Cell viability in tendon explants was similarly concentration dependent according to the probit model. However, cell survival was not reduced in the 144h treatment group with no significantly shifted curves and no significant reduced relative median potency (details Figure 4 left and table 2**Error! Reference source not found.** and 3).

Two-way ANOVA showed a significant effect of C_{GEN} on migration speed, but migration rate did not vary according to time after scratch application (Figure 5 and Table 4). Post hoc pairwise comparison indicated significantly reduced migration speeds in 1mM and higher C_{GEN}.

Relative effect	Cell culture viability (Live/dead)			Metabolic activity (Alamar Blue)			Explants viability (Trypan Blue)			Cell motility (Scratch wound)		
	Estimated dose [mM]	LCI [mM]	UCI [mM]	Estimate dose [mM]	LCI [mM]	UCI [mM]	Estimate dose [mM]	LCI [mM]	UCI [mM]	Estimate dose [mM]	LCI [mM]	UCI [mM]
10%	303.9	85.9	1986.9	5.1	2.6	12.4	9.3	0.3	3.8E+26	3.6	1.0	9.8E+81
20%	79.7	27.2	370.4	2.1	1.1	4.4	2.9	0.1	7.5E+20	2.6	0.7	7.5E+63
30%	30.4	11.6	112.7	1.1	0.6	2.1	1.2	0.1	6.3E+16	2.0	0.5	3.3E+49
40%	13.3	5.5	41.7	0.6	0.4	1.2	0.6	0.03	2.3E+13	1.6	0.31	1.8E+37
50%	6.2	2.7	16.9	0.4	0.2	0.7	0.3	0.01	1.6E+10	1.3	0.17	2.2E+26
60%	2.8	1.2	7.1	0.2	0.13	0.4	0.2	0.004	1.6E+07	1.1	0.05	3.1E+16
70%	1.2	0.5	2.9	0.13	0.07	0.2	0.1	0.001	18307.8	0.9	0.002	1.0E+08
80%	0.5	0.2	1.1	0.07	0.03	0.1	0.03	1.2E-05	40.8	0.74	0	766
90%	0.12	0.04	0.31	0.03	0.01	0.06	0.01	7.8E-10	0.51	0.62	0	18.47

Table 2: Selected potencies of GEN treatment at 72h: Estimated concentrations from the probit models of relative effects on tendon cells. Relative effects refer to sham treated or 0mM GEN controls as 100%. LCI: lower 95% confidence interval, UCI: upper 95% confidence intervals.

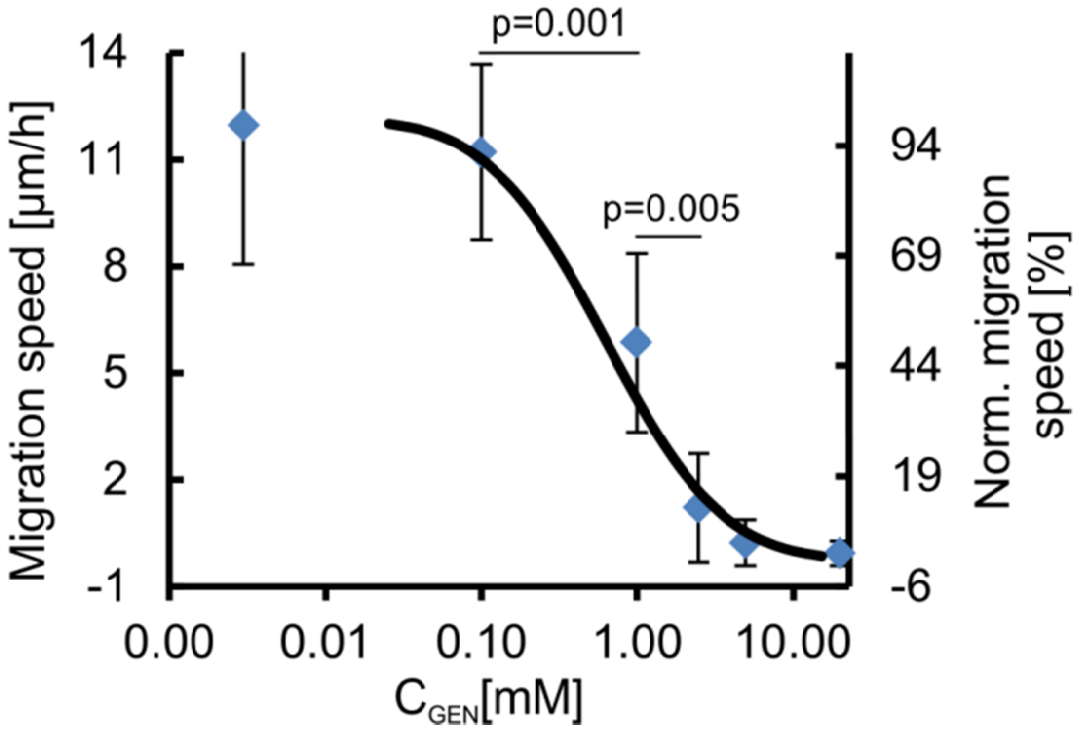

Figure 5: Left axes: Cell migration speed (µm/h) averaged over time of image acquisition after scratch application (nm=3) and over experiments in different horses (nr=3). Horizontal bars indicate significant differences. Only significant difference between adjacent groups were plotted as all other pairwise comparison were significant different. Right axes: Probit fit of normalized cell migration speeds. 0mM GEN is plotted at 0.003mM. 72h GEN treatment.

Analysis of covariance revealed a common slope, independent of treatment duration, of the regression model fitted on denaturation temperature (interaction term, p=0.16). The reduced

ANOVA, model depending on dose only, sowed a significant effect of concentration with increasing denaturation temperature at 5mM (Figure 6 and Table 4).

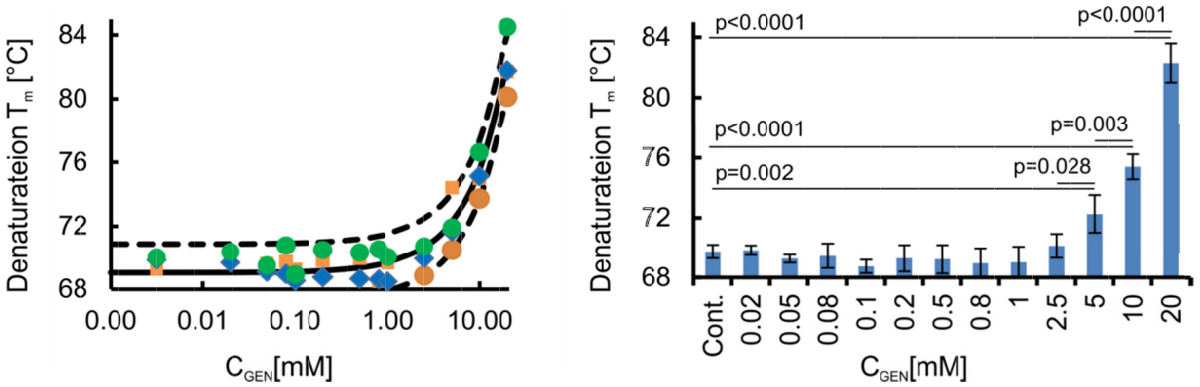

Figure 5: Left: Averaged (n_r=5) denaturation temperatures as measured by DSC. Fit: linear model (T_m=0.66°C/mM x [GEN] mM + 69.26°C, R^2=0.96) with Log_{10} transformed concentrations and 95% confidence intervalls. 0mM GEN is plotted at 0.003mM. ■ 24h GEN treatment, ◆ 72h GEN treatment, ● 144h GEN treatment. Right: Bars indicated significant differences from post hoc pairwise comparison at a significant overall effect. Only significant difference between adjacent groups or to controls were plotted as all other pairwise comparison were significantly different, except below 1 mM for which there was no effect.

Friedman's test revealed a significant effect of the GEN treatment on collagen type 1 gene expression, with 83% smaller relative gene expression ($2^{-\Delta CT}$) in the 1mM GEN group compared to the 0mM GEN controls and a trend of 23% decreased relative expression in the 0.1mM GEN group (Figure 6 and Table 4). Cell apoptosis marker caspase 3 and MMP 1were both unaffected over the range of tested concentrations (Figure: 7 and Table: 4).

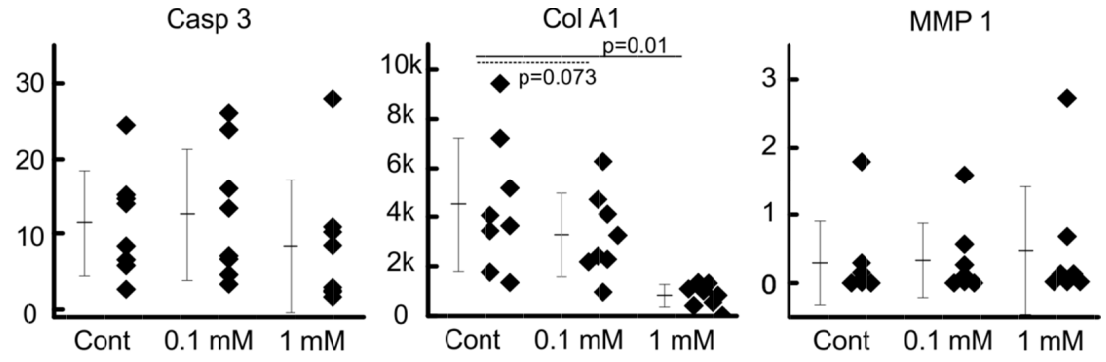

Figure 6: Relative gene expression ($2^{-\Delta CT}$) normalized to an internal standard (housekeeping gene: GADHP): Due to non-normal data distribution, the non-parametric counterpart (Friedman's Test) of a two way blocked ANOVA was run with appropriated post hoc pairwise comparison.

One triplet failed during the preconditioning and was therefore excluded from further analysis. Elastic modulus increased significantly by 34% after 20 mM GEN treatment as assessed by the paired T-test (Figure 8 and Table 4 and 5). MANOVA for blocked design (ANOVA for complete randomized block design was replaced due to violation of model assumptions) also showed a significant treatment effect on elastic modulus, with a significant 23% increase in the 5 mM treatment group, and a non-significant 14% increased elastic modulus in the 0.1 mM group compared to controls using post hoc pairwise comparison. All other parameters assessed by two-way ANOVAs for concentrations 1 mM and 5 mM, as well according to T-test on the 20 mM group, revealed non-significant effects (Table 5). Finally, treatments had a no statistical effect on tissue swelling (6.0±6.7%, p-value=0.948) from an initial water content of 75.2±4.8% before treatment. Further, initial lengths were also very similar between groups (23.7±1.2mm, p=0.796).

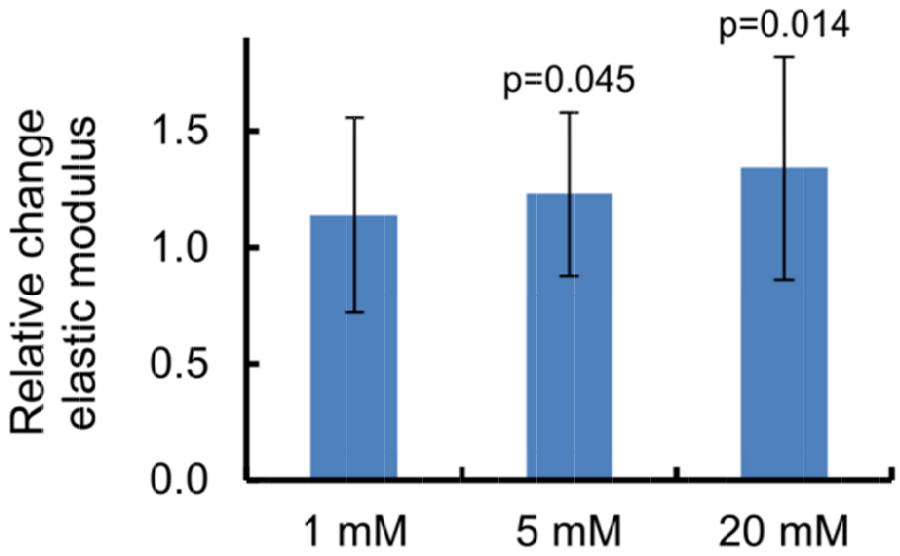

Figure 7: Relative changes of elastic modulus compared to sham treated controls. The 20 mM group was separately assessed with own matched controls: use of samples dissected into quadruplicates resulted in large inter-quadruple variability, which precluded the blocked experimental setup necessary to block variability of the tendon strips caused by dissection. Stars: * significant different from controls (p=0.014).

3. Discussion

Cross-linking of collagen-based implants has been widely used to augment their strength, elasticity, resistance to fatigue-induced mechanical damage and against premature degradation by the host system [4]. Cross-linking can improve implant survival within challenging mechanical environments such as the cardiovascular and musculoskeletal systems. Genipin is a plant-derived collagen crosslinking agent that has been demonstrated to be highly mechanically effective yet substantially less cytotoxic than traditional chemical cross-linking agents like glutaraldehyde. Given the efficacy and relatively low cytotoxicity of GEN, it has emerged as a candidate for *in vivo* application, with various studies demonstrating proof of principle for *in situ* biomechanical efficacy in treating keratoconus of the eye [33] and ruptures of the intervertebral disc annulus [5, 34]. Our own work has shown it be capable of arresting damage propagation in torn tendon and restoring normal levels of tissue strains [4].

Gene	Accession nr.	Product length [bp]	Primers Sequences (5‘ → 3‘)
Casp 3 (Caspase 3)	*NM_001163961*	127	F: TCA-GGC-CTG-CCG-AGG-TAC-AGA
			R: ACC-AGG-TGC-TGT-GGA-ATA-CGC-A
Col1A1 (Collagen IA1)	*XM_001499586*	164	F: TGC-CAT-CAA-AGT-CTT-CTG-CAA
			R: CGC-CAT-ACT-CGA-ACT-GGA-ATC
MMP-1 (Matrix Metalloproteinase-1)	*NM_001081847*	92	F: CGA-AGG-GAA-CCC-TCG-GTG-GGA
			R: TGG-CCT-GGT-CCA-CAT-CTG-CTC
Gapdh (house-keeping gene)	*NM_001163856*	141	F: GTC-AAC-GGA-TTT-GGT-CGT-ATT-GGG
			R: TGC-CAT-GGG-TGG-AAT-CAT-ATT-GG

Table 3: Horse specific primers: nucleotide sequences of the tested genes.

While genipin offers promise as an *in vivo* collagen cross-linking agent, it has been reported to be cytotoxic at moderate concentrations that vary depending on cell type (typically 0.5 to 5mM). Until now it has been unclear whether the dense collagen matrix of connective tissues like those in tendon could be biomechanically augmented *in situ* without adverse consequences for resident cell populations. The present study sought to define limits of cytotoxicity for tendon cells, and then determine whether functionally relevant changes in tissue mechanics could be attained at or below these levels. The overall goal was to better delineate the range of potential *in situ* applications that GEN may have, and provide dosing guidance for the development of delivery strategies.

To provide a basis for effective *in vivo* dosing, we used probit regression to fit our experimental data. For instance, the model predicts that approximately 70% of cells would remain viable at genipin concentrations of 6.2 mM applied for 72h (Table 3), with decreasing cell viability after more prolonged incubation (Figures 3 and Table 2). While many cells remain alive at concentrations of 5mM or less for the time spans we studied, effects on cell metabolism occurred at substantially lower concentrations. The probit model predicts a 50% drop in metabolic activity at a concentration of 0.13 mM after 72 hours with a trend to additional decreases in cell metabolism after 144 h of treatment (Figure 3, Table 2 and 3). These findings were consistent with reduced cell motility at similar concentrations (Figure 5 and Table 3). While reduced collagen I expression was also consistent with reduced metabolic activity, apoptosis markers and matrix degradation markers were not affected –a favorable finding regarding potential of GEN for *in vivo* application .
Functional, physical effects on the matrix were statistically significant only at the tested concentration of 5mM (Figure 6 and 8, Table: 5). We attribute these effects to homogenous cross-link formation throughout the explants, with increasing fluorescence according to both time and dose (Figure 1). Interestingly, already at 1mM elasticity increased variation in the mechanical test data for concentrations indicating altered mechanics in some samples. It may be relevant for eventual clinical application that the model predicts that *in situ* collagen cross-linking can achieved physical effects at concentrations of 2mM, while leaving 20% of cells alive and metabolically active.

Although the degree of cytotoxicity that can be tolerated *in vivo* will vary according to the targeted tissue and clinical indication, properly balancing the need for rapid improvement in tissue function against the long-term consequence of altered cell and matrix metabolism will be imperative. No less important will be the need to develop effective approaches for targeted cross-link delivery.

Although a main aim of the work was to identify “cell-safe” and “matrix-effective” GEN dosing guidelines, it is clear that these objectives may be mutually exclusive to some extent. It appears that 5 mM (and even slightly lower concentrations) may be able to induce relatively rapid cross-linking while leaving subpopulations of resident cells viable. Toward clinical application of *in situ* cross-linking to arrest tendon tear propagation, we believe a dosage of 5 mM or slightly lower with a dosing duration of 72 h would be a reasonable starting point for *in vivo* study.

Measure	Statistical test	Repeated measure / Block	C_{GEN}
Cell motility			
Migration speed	Two-way ANOVA	0.371	<0.0001
DSC			
Denaturation temperature	One-way ANOVA	-	**<0.0001**
Gen expression			
Casp 3 ($2^{-\Delta CT)}$	Friedman's test	-	0.882
Col A1 ($2^{-\Delta CT)}$	Friedman's test	-	**0.01**
MMP 1 ($2^{-\Delta CT)}$	Friedman's test	-	0.607
Mechanical test			
Relaxation	Two-way ANOVA	**0.01**	0.231
Elastic modulus	Two-way MANOVA	**<0.0001**	**0.011**
	T-test		**0.014**
Yield stress	Two-way ANOVA	**<0.0001**	0.395
	T-test		0.227
Yield strain	Two-way ANOVA	**<0.0001**	0.384
	T-test		0.156
Max. Stress	Two-way ANOVA	**<0.0001**	0.387
	T-test		0.187
Strain at max. Stress	Two-way ANOVA	**0.01**	0.906
	T-test		0.397
Energy up to yield	Two-way ANOVA	**0.01**	0.638
	T-test		0.444

Table 4: Summary of 2-way ANOVA analysis of the main experimental outcomes, along with P-values. Statistical significance ($p<0.05$) is noted by bold text. 2-way ANOVAs were only used when assumptions were met, else as indicated appropriated alternative tests were used. For the mechanical tests a separate paired group was used to assess the effects of 20mM.

While robust augmentation of elasticity at 5 mM GEN concentrations and higher are in line with our previous work [6], the mechanical effects in the present study were less dramatic. Further in contrast to our previous studies, we did not observe differences in failure or yield behavior. We attribute this difference to the present use of medium-incubated controls, which differed from our previous comparison to native tissue. We verified the effect of sham incubation in baseline experiments showing that incubation (necessary to create equivalent conditions across our various experiments) increased elasticity, maximal stress, and strain at maximum stress in controls by 10%-15% over the native tissue (data not shown).

When comparing our studies to other investigations of GEN on living tissues and cells, our observed lack of time-dependent effects on cytotoxicity for culture periods up to 72 h echoes previous studies on osteoblastic cells [17]. This study reported no observable cytotoxic effects in 0.044 mM GEN solution, while 0.44 mM GEN induced a 50% reduction in metabolic activity [18]. These findings are in close agreement with our model prediction of 50% reduction in cell metabolism at 0.38mM (95% confidence intervals: 0.22mM and 0.69mM). These results somewhat contradict other reports of limited cytotoxic effects on chondrocytes within explants treated up to 42 days at concentrations of 0.22mM, and this while attaining mechanical enhancement of the tissue [10]. They also stand in contrast with another study reporting little cytotoxicity after crosslinking porcine heart valves in 8mM GEN while obtaining a near doubling of stiffness [20].

While our study was intended to be fairly comprehensive, several limitations must be noted. First, focused investigation in narrower windows was deemed out of scope for this study. Such investigations will be necessary according to the clinical indication and eventual GEN delivery approach that is utilized – aspects that were not explicitly addressed in the current study. Further, whether the effects we observed prove generally applicable to other dense connective tissues and clinical applications remains to be investigated. Nonetheless, the current study provides a useful baseline for dosage guidance in the design of future *in vivo* studies, and will be helpful in interpreting their outcome. As a further limitation, we did observe slight increases in pH (maximally: 0.2) that were attributable to the addition of low GEN concentrations up to 2.5mM. While confounding effects of these pH differences cannot be excluded, we suspect they had only marginal effect on cell viability and metabolism. Finally, we note that the occasionally large variability within the various experiments we performed can be attributed to similarly large variability across horses, and across specimens from a single donor tissue. Inter-specimen

variability can be attributed to handling factors like anatomical sampling location, and challenges in assessing wet-weight in small samples (hydration and dehydration effects). A further limitation is that we focused on relatively rapid cross-linking effects, and did not investigate whether long-term administration of GEN at lower doses (1mM or less) could possibly achieve a cumulative functional effect with less pronounced cytotoxicity. Finally on a more basic level, we did not investigate how GEN cross-links are actually formed and stabilized within tissue. While this information may be helpful to interpreting our results, we note that others have reported that the stabilizing effects of GEN are comparable to glutaraldehyde [35].

		Treatment				
		0 mM[a]	1 mM[a]	5 mM[a]	20 mM[b]	0 mM[b]
Elastic modulus	[Mpa]	419±145	436±121	**483±127[1]**	**434±70[2]**	342±75
Yield stress	[Mpa]	23±13	32±13	34±12	25±6	20±7
Yield strain	[%L0]	8±2	8±1	8±1	7±1	8±2
Maximal stress	[Mpa]	40±18	43±18	45±19	28±6	24±7
Strain at max. stress	[%L0]	15±4	15±3	15±4	10±3	11±2

Table 5: Main results from the mechanical tests. A) Samples were tested in triplets (n_r=13) dissected from a single tendon strip and analyzed by two way ANOVA for a complete randomized block designs. B) Samples were tested in pairs (n_r=11) and analyzed with a paired student T-test. **1)** Post hoc pairwise comparison to control: p=0.45, **2)** Paired T-test: p=0.14.

Conclusions:

The crosslinking agent genipin is increasingly invoked for the mechanical augmentation of collagen tissues and implants, and has been demonstrated to arrest mechanically-driven tissue degeneration. In this study we established an *in vitro* dose-response baseline for the effects of genipin treatment on tendon cells and their matrix, with a view to *in vivo* application for the repair of partial tendon tears. Regression models based on a broad range of experimental data were used to delineate the range of concentrations that are likely to achieve functionally effective cross-linking, and predict the corresponding degree of cell loss and diminished metabolic activity that can be expected. We conclude that rapid mechanical augmentation of tissue properties can only be achieved by accepting some degree of cytotoxicity, yet that post-treatment cell survival may be adequate to eventually repopulate and stabilize the tissue. On this basis, we conclude that development of delivery strategies and subsequent *in vivo* study is warranted.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Chapter 6

Is the cell population from the peritenon rather than the population from the tendon core the driving force of healing in tendon repair?

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Abstract:

The role of intrinsic and extrinsic healing in injured tendons is still debated. In this study we characterized cell plasticity, proliferative capacity, and migration characteristics as proxy measures of healing potential in cells derived from the peritenon (extrinsic healing) and compared these to cell populations from the tendon core (intrinsic healing). Horse superficial digital flexor tendon cells were extracted from these tissues and characterized for tenogenic markers and tested for their migration and replication rates. Furthermore, colony-forming unit assays, multipotency assays, and real-time quantitative polymerase chain reaction analyses of differentiation markers were performed. Finally, cellular capacity for differentiation towards a myofibroblastic phenotype was also assessed. Our results showed that cells from the peritenon migrated faster, replicated more quickly, and showed a higher differentiation potential toward both osteogenic and myofibroblastic phenotypes when compared to cells from the tendon core. Based on these data, we suggest that extrinsic cells from the peritenon have substantial potential to influence tendon healing outcome, and warrant further scrutiny of their role.

5 keywords: *Tendon, Peritenon, healing, differentiation, myofibroblast*

Introduction:

Injuries to energy-storing tendons are prevalent in athletes as well as in the general population. It has been estimated that tendinopathy accounts for 30% to 50% of all injuries related to sports (1). The most common causes of tendon ailments are acute trauma or repetitive activities that create

an accumulation of micro-injuries in the tendon tissue (2). Tendinopathy is a result of a deficient healing response to these accumulated micro-injuries in the tendon tissues, which for largely unknown reasons are unable to effectively regenerate (3). Although many medical options are available to treat tendon injuries, there is a high recurrence rate and the prognosis for returning to previous performance levels is still poor. A better understanding of the cellular mechanisms involved in the natural healing of tendons could enable improved medical treatment.

It was first suggested that tendons lack the capacity for intrinsic healing and that in-growth of cells from the surrounding tissues is necessary to enable healing of tendon injuries (4; 5). The tendon is surrounded by the paratenon, a loose fibrillar tissue that functions as an elastic sleeve permitting free movement of the tendon against other tissues (6). Under the paratenon, the entire tendon is surrounded by a fine connective tissue sheath called epitenon (6). Later work demonstrated the capacity of tendons to heal intrinsically (7-10), and it is now believed that both intrinsic and extrinsic healing play a synergistic role in tendon regeneration (11; 12). However, the extent of the contribution of each is still not well defined. While intrinsic healing capacity is commonly reported as being inferior (13), it remains unknown whether this could be due to a more limited regenerative capacity of the resident cell population.

Another question that remains unanswered is whether aberrant healing is related to the nature of cells migrating towards the injured area: either cells from the surrounding tissue or cells from the tendon core. Cells with a multi-lineage differentiation potential are credited with the capacity to naturally remodel, repair, and regenerate various tissue types when necessary (14). However the multi-lineage differentiation potential of cells can also underlie pathological processes when differentiation is not in accordance with tissue function (ectopic differentiation) (15). Fat deposition as well as calcification has been observed in clinical cases of tendinopathy (16; 17). Furthermore, during extensive tissue-remodeling, fibroblasts may acquire the phenotype of myofibroblasts. Briefly, myofibroblasts have stress fibers that incorporate alpha smooth muscle actin (α-SMA), a protein that facilitates forces required for wound contraction(18). Myofibroblasts also synthesize abundant amounts of collagen and are believed to be responsible for the formation of persistent scar tissue (fibrosis) and the shrinkage of peritendinous tissue (19; 20)

In this study, we investigated the potential healing capacity of cell populations extracted from the tendon core compared to that of cell populations derived from the peritenon tissues comprising both the paratenon and epitenon. Cells were carefully isolated from either the core of the tendon

or the peritenon of horse superficial digital flexor tendons (SDFT). We first investigated for differences in gene expression of these two cell populations based on tenogenic markers. We then compared their migration and replication rates, as well as their capacity to produce collagen. Additionally, our interest was also to assess their potential to differentiate towards osteogenic, adipogenic and myofibroblastic phenotypes, as this might relate to their potential to adversely affect healing outcome.

Methods:

Isolation of cells from the core of the tendon and from the peritenon:

The horse SDFT has a well characterized peritenon overlying the tendon core. Cells were extracted either from the loose peritenon tissue or from the core of the tendon, leaving 2mm of the edge in order to obtain two cell populations with clearly distinct origins. Tendon cells were extracted by digestion of the tendon matrix using Protease Type XIV (Sigma-Aldrich, St. Louis, MO) for 2h at 37°C and Collagenase B solution (Roche, Burgess Hill, UK) for 16h at 37°C. After matrix digestion, the mixture was filtered and centrifuged at 400g for 8 min at room temperature. The cell pellet was re-suspended and cultured at 37°C, 5% CO_2 in expansion medium (Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS), 50 µg/ml gentamicin and 1.5 µg/ml fungizone (all from Life technologies, Paisley, UK)) unless stated otherwise. Cells were cultured and trypsinized at subconfluency and only cells freshly digested or from the first passage were used for the experiments listed below. For *ex vivo* differentiation experiments, explants of horse SDFT (size 10 x 2 x 2mm) with the peritenon still surrounding the tendon were used.

Migration assay:

Using 6-well plate dishes, cells were seeded at a density of 10,000 cells/cm^2 in each well and were maintained at 37°C and 5% CO_2 for 6 days to permit cell adhesion and the formation of a confluent monolayer. The confluent monolayer was then scored with a sterile pipette microtip to leave a scratch of ~0.4-0.5 mm in width. The scratch areas were monitored by collecting digitized images at various time points after the scratch was performed until closure was complete. Digitized pictures were obtained with an inverted Zeiss microscope (Observer Z1, Oberkochen, Germany). The digitized images were then analyzed using Image J software (National Institute of Health, Bethesda, MD). All scratch assays were performed in duplicate. The migration rate was calculated by normalizing the newly covered area to the initial cell-free area and divided by twice

the width of the lesion in order to determine the average breadth of the gap between edges of the closing wound. This distance was then divided by time to calculate the migration speed in µm/h.

Replication assay:

Tendon and peritenon cells (passage 0) from four different horses were plated at 10,000 cells/cm^2 in 3 flasks each. After 7 days in culture, the cells were trypsinized and counted using Trypan blue to exclude dead cells.

Colony-forming unit assay:

Tendon and peritenon cells were plated at 50 cells/cm^2 in Petri dishes. Nine days into culture, colonies were counted and characterized as previously described by Franken et al. (21). Briefly, cells were rinsed with PBS, fixed in 4% paraformaldehyde, stained with 0.5% crystal violet for 30 min and rinsed twice with water. Size and number of the colonies were evaluated.

Differentiation experiments:

For tenogenic control cultures, the cells were cultivated in expansion medium with a seeding density of 10,000 cells/cm^2. The medium was renewed every 2-3 days and the cells were kept in a 5% CO_2 environment at 37°C. To induce differentiation, we followed protocols established by others (22). Briefly, to induce osteogenic differentiation, the cells were seeded at a density of 3,000 cells/cm^2 and cultured in DMEM High Glucose with Glutamax (Life technologies) with 10% FCS (Life technologies), 0.6 % fungizone (Life technologies), 0.1% gentamicin (Life technologies) and freshly added 10mM glycerol phosphate (Sigma-Aldrich), 0.1µM dexamethasone (Sigma-Aldrich) and 0.1mM L-ascorbic acid 2-phosphate (Sigma-Aldrich). To induce adipogenic differentiation cells were seeded at 20,000 cells/cm^2 and the induction medium consisted of DMEM Glutamax (Life technologies) with 10% FCS (Life technologies), 0.6 % fungizone (Life technologies), 0.1% gentamicin (Life technologies) and freshly added 0.1 µM dexamethasone (Sigma-Aldrich), 0.2 mM indomethacin (Sigma-Aldrich), 0.01 mg/ml insulin (Sigma-Aldrich) and 0.5mM 3 iso-butyl-1-methyl-xanthine (Sigma-Aldrich). The cells were kept for 21 days in a 5% CO_2 environment at 37°C and the differentiation media were refreshed twice a week.

Histological and immunohistochemical stainings:

Pretreatment for staining:

The seeded cells were fixed with 3% formaldehyde for 15 minutes and then washed with PBS before further treatment. The paraffin embedded explants were cut in 6µm sections and those

sections were deparaffinized by dipping them for 5 minutes in Histoclear (National Diagnostics, Atlanta, GA), 100% ethanol, 96% ethanol, 70% ethanol and finally cleared in distilled water before further staining.

Alizarin red S staining:

The cells were stained with Alizarin red S (Sigma-Aldrich), for 2 min and washed with microfiltered and deionized water. The paraffin sections were additionally and briefly immersed in acetone, then in an acetone-xylene (1:1) solution and cleared in xylene. All samples were then counterstained with Mayer's hematoxylin for 5 min and rinsed in distilled water. The stained cells that contained mineral deposits appeared orange-red under the microscope.

Oil red O staining:

The paraffin sections were first briefly dipped in 60% of 2-propanol before starting the staining with Oil red O (Merck, Whitehouse, NJ) for 20 min and then dipped again in 60% 2-propanol and finally washed with distilled water. The seeded cells were directly stained with Oil red O. All samples were then counterstained with Mayer's hematoxylin for 5 min and repeatedly washed with tap water. Stained lipid droplets appeared red under the microscope.

Gene expression analysis:

Real time RT-PCR was used to monitor the differentiation potential of the two cell populations in the three lineage-specific induction media. We used the following primers (Table 1) to determine the gene expression of the seeded cells: collagen type 1, tenomodulin and scleraxis were chosen as tenogenic markers; Runx2 and Sp7 were selected as osteogenic differentiation markers; and PPARG and Fabp4 were used as markers of adipogenic differentiation. GAPDH was used as the internal control (housekeeping gene).

RNA isolation and real time RT-PCR

Medium was removed from the wells and cells were suspended in RNA-Bee (Tel-test, Friendswood, TX). Cell's RNA was then isolated using RNeasy Micro Kit 50 (Qiagen, West Sussex, UK) with on-column DNAase digestion. Total RNA content was determined spectrophotometrically using a NanoDrop (Thermo Fisher Scientific, Waltham, MA). The RNA obtained was further reverse-transcribed into cDNA with a RevertAid First Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific). Finally, real time RT-PCR was performed using a real time PCR system thermal cycling block (StepOnePlus Applied Biosystems, Carlsbad, CA) with Power Sybr green PCR master mix (Applied Biosystems) and standard software (StepOne version V2.1, Applied Biosystems).

RT-PCR data processing

Data were normalized to GAPDH which was stably expressed across sample conditions (data not shown). Fold-change expression was calculated according to the $2^{-\Delta\Delta CT}$ formula (23) using pooled duplicate samples (average of duplicate wells). Statistical analysis was performed using SPSS 11.5 software (SPSS Inc., Chicago, IL). Fold change values are presented as Box-Whisker plots, with the boxes representing the middle two quartiles (25–75) and the whiskers representing the highest and lowest data point values.

Western Blotting

The cells were lysed in sample buffer (0.1g/ml SDS (Biosolve BV, Valkenswaard, NL), 1M Tris-HCl (Biosolve BV), 50% Glycerol (Sigma-Aldrich)). Equal amounts of protein were analyzed by Western blotting using antibodies to Collagen type 1 (Abcam, Cambridge, UK), Scleraxis (Abcam) and vimentin (DAKO, Glostrup, Denmark). The band signal strengths in Western blots were further analysed by optical densitometry and related to loading control vimentin.

Myofibroblast differentiation:

The potential of the two cell populations to differentiate toward a myofibroblastic phenotype was assessed by comparing relative expression of α-SMA in cells in culture with the presence or absence of transforming growth factor (TGF-β1 (2ng/ml)) (R&D systems, Minneapolis, MN). TGF-β1 is known to elicit the expression of α-SMA, the most frequently used marker of myofibroblasts (the anti-α-SMA antibody (mouse IgG2a, clone SM1) was the kind gift of Christine Chaponnier and Giulio Gabbiani, University of Geneva, Switzerland) (24). Vimentin (Dako, Glostrup, Denmark), a cytoskeletal housekeeping protein, was used to semi quantitatively assess relative α-SMA protein expression for each cell population before and after the addition of TGF-β1. Additionally, the differentiation potential of the two cell populations was also tested on different ligands. Peritenon cells as well as cells from the core of the tendon were seeded on 100kPa Polydimethylsiloxane (PDMS; ExCellness Biotech SA, Lausanne, Switzerland) coated with collagen (Sigma-Aldrich) or fibronectin (EMD Millipore, Billerica, MA),. The differentiation potential toward a myofibroblastic phenotype was then determined for both cell populations using the Western blot techniques described above.

Statistical analysis

Related- Samples Wilcoxon Signed Ranks test were used to assess differences among conditions. Significance level was set at $p < 0.05$.

Results:

Comparison of gene expression in both populations of cells for tenogenic markers (scleraxis, tenomodulin and collagen type 1) showed that cells from the core of the tendon expressed higher amounts of scleraxis (14 times; p=0.04) and tenomodulin (2 times; p=0.1) but statistically indistinguishable amounts of collagen type 1 compared to cells from the peritenon (p=0.86; fig. 1). The Western blot results for Scleraxis and Collagen type 1 confirmed the gene expression data (fig. 2).

For testing of the hypothesis that peritenon cell populations would demonstrate enhanced propensity for migration, confluent populations of cells from the tendon core and peritenon were monitored using a scratch assay. Peritenon cells showed significantly faster wound closure (1.2 times) compared to cells from the tendon core (p=0.04; fig. 3). Additionally we evaluated the replication rate of the two cell populations. After 7 days, we counted 33% more cells in the peritenon population compared to the cell population from the core of the tendon (p=0.026; fig. 4).

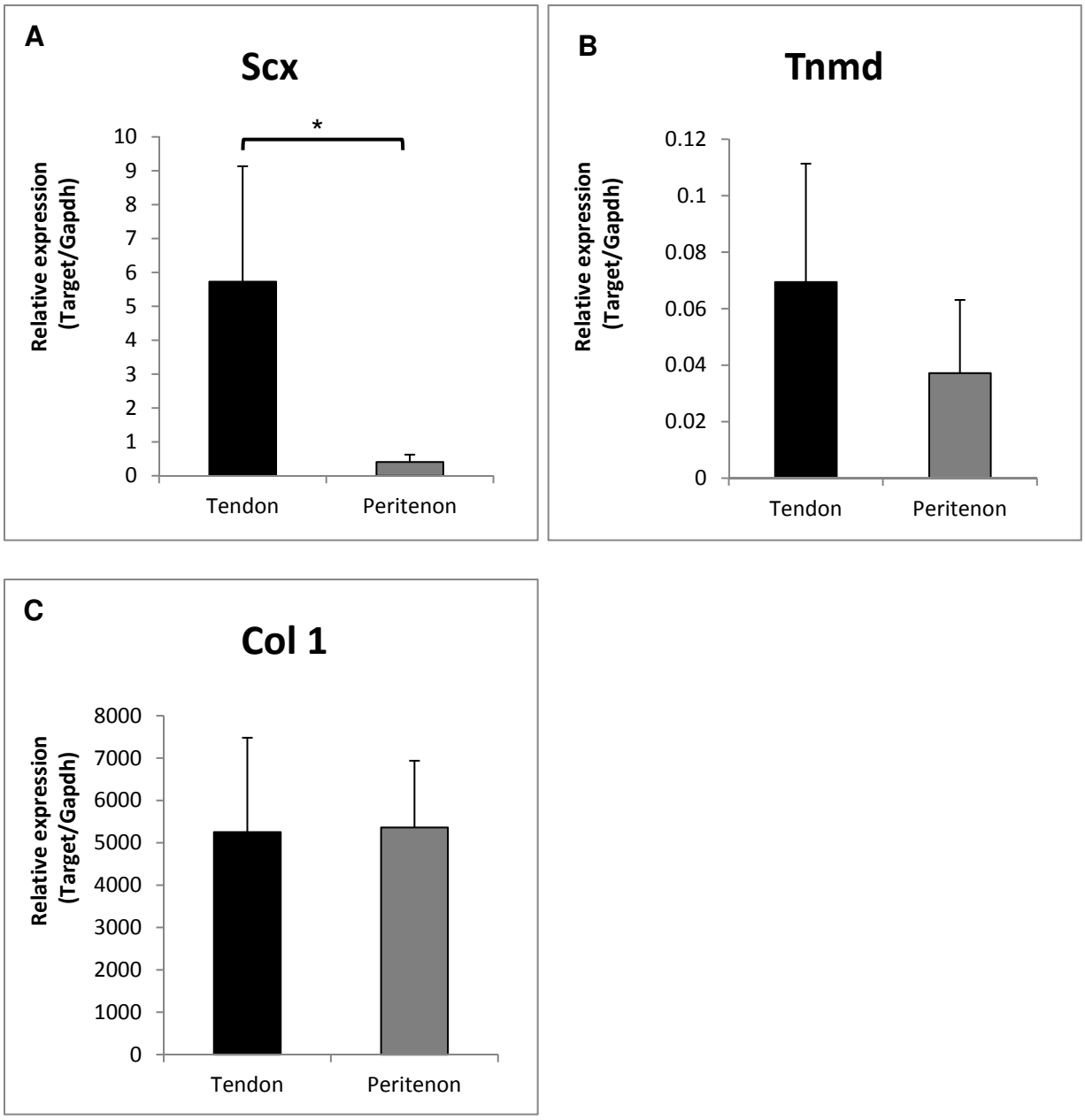

Figure 1: Gene expression levels of tenogenic markers: scleraxis (Scx), tenomodulin (Tnmd) and Collagen type 1 (Col 1) in tendon core cell population and peritenon cell population cultured in expansion medium. Tendon core cell population compared to peritenon cell population demonstrated a 14-fold increase for Scx ($p=0.04$) (A), a not significant 2-fold increase for Tnmd ($p=0.1$) (B) and no difference for Col 1 (C) ($p=0.859$). The error bars are SEM.

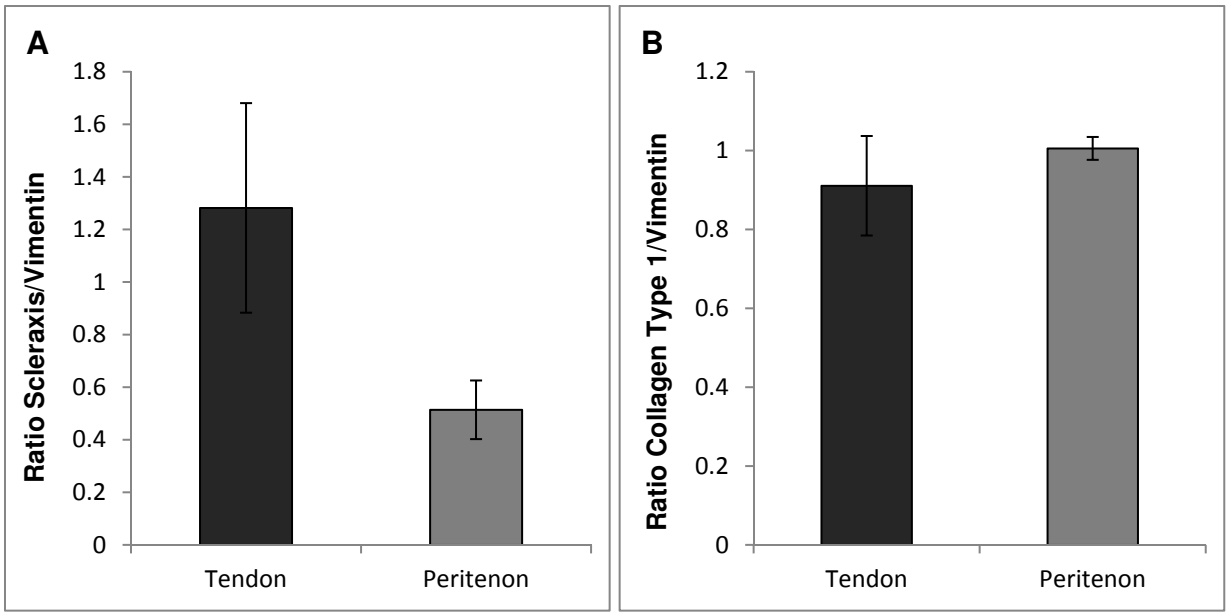

Figure 2: Western blots confirmed the gene expression results. The graph display the relative band signal strength in Western blots analysed by optical densitometry and related to loading control vimentin. Cells from tendon core population demonstrated a higher level of scleraxis protein expression compared to cells from the peritenon when cultured in expansion medium for a week (A). The difference between the two cell populations for collagen protein expression was insignificant (B), (n=2).

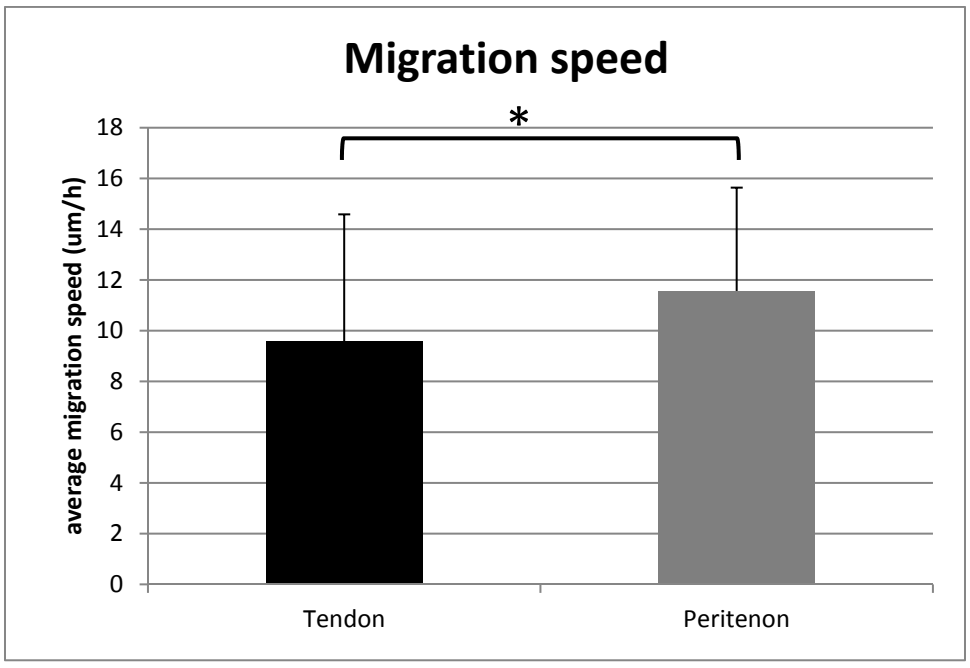

Figure 3: Average migration speed of the cell populations from the core of the tendon and from the peritenon, obtained with a scratch assay monitored over 8 hours. The peritenon cell population was 1.2 times faster than the tendon core population (p =0.04).

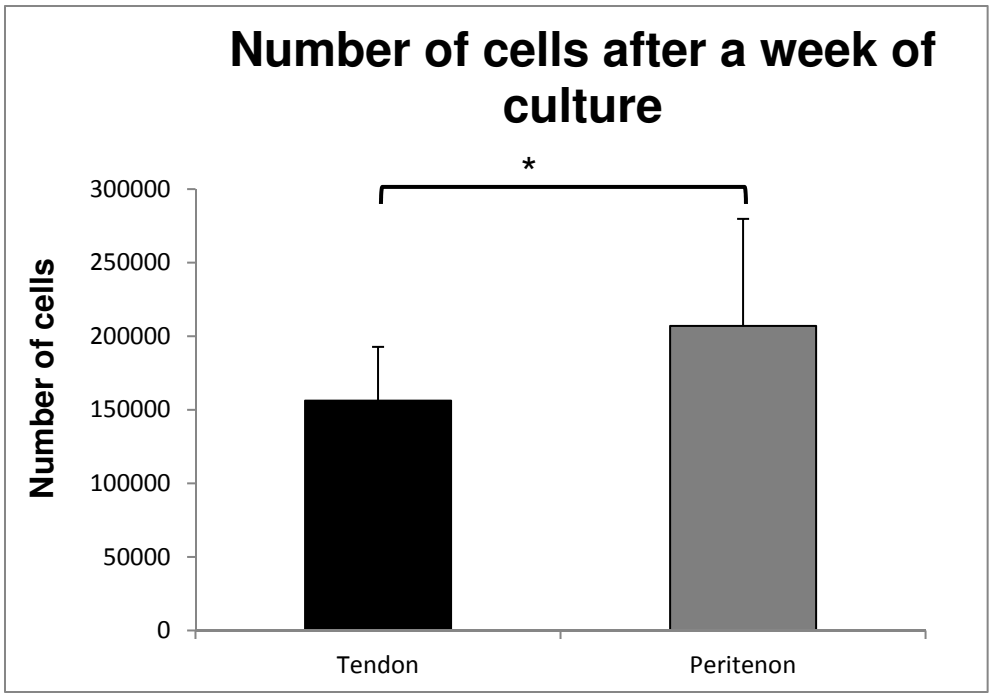

Figure 4: Replication rate: Starting with equivalent numbers of cells, we calculated the number of cells after 7 days in culture in expansion medium. There were 1.33 times more cells in the flasks containing cells isolated from the peritenon compared to the flasks containing cells isolated from the tendon core (p=0.026).

Clonogenicity, multipotency and self-renewal are widely used criteria to define stem cells. To characterize clonogenicity, cells from the tendon core and from the peritenon were seeded at a low density. During the first 2 days, the cells of both populations adhered but stayed quiescent. A small percentage of both populations of cells formed colonies on day 9 (fig. 5a,b). Cells from the tendon core formed 2.6 times more colonies than cells from the peritenon (p=0.018; fig. 5c). Based on the assumption that each observable colony originates from a single progenitor cell, these data indicate a significantly higher proportion of progenitor cells in the tendon core population than in the cell population from the peritenon. The size of the colonies observed varied within one population, however the colonies formed by cells from the peritenon were overall larger (1.82 mm^2 ± 0.4 mm^2 and 0.74 mm^2± 0.2 mm^2 for peritenon and core cell populations, respectively (= 2.5 fold increase), p=0.018; fig. 5d).

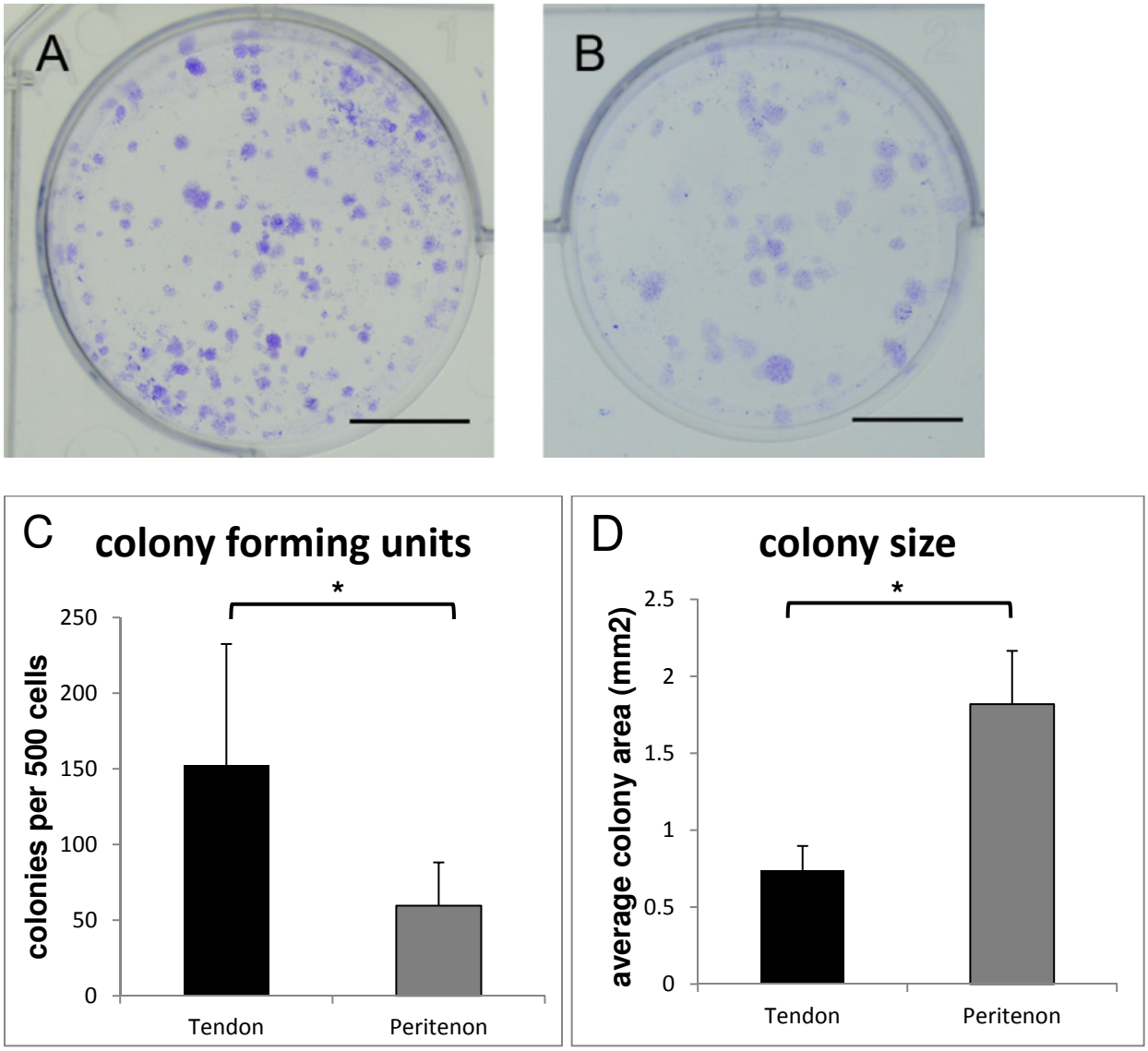

Figure 5: Clonogenicity was tested for the two cell populations. Representative wells with colonies from the tendon core population (A) and from the peritenon population (B) were evaluated (n=14) (scale bar = 0.5 cm). The cells isolated from the tendon core demonstrated 2.6 times more colonies compared to the peritenon cell population (p=0.018) (C).The colonies were 2.5 times larger in the peritenon cell population than in the cells isolated from the tendon core (p=0.018) (D).

As tendons are derived from mesenchymal origins which also give rise to bone and fat, the adipogenic and osteogenic potential of the cells was tested using specific media and cell densities. When cultured in osteogenic medium, both populations showed an ability to differentiate towards bone (fig. 6a-d). Using Alizarin red staining, larger calcium plaques were observed for the population of peritenon cells (fig. 6c). The control conditions were devoid of observable calcium deposits (fig. 6b,d). Like the cell populations from the tendon core, the peritenon cell population showed a heterogeneous differentiation potential toward adipogenesis

and osteogenesis. The expression of the osteogenic markers Runx 2 and SP7 was significantly higher for the peritenon cell population (2.7-fold higher for Runx 2 and 1.8-fold higher for SP7, $p<0.05$ for both; fig. 7a,b). However, there was not a significant increase in baseline expression of these markers when cultured in osteogenic medium compared to control conditions. When cultured in adipogenic medium, cells from both populations exhibited numerous lipid droplets at day 21(fig. 6e-h), indicating their ability to differentiate toward an adipogenic phenotype. Correspondingly, cell morphology drastically changed from a fibroblast-like spindle shape to a round shape. Oil red O staining revealed no clear differences in adipogenic differentiation medium between the two cell populations. No lipid droplets were found in the cells from both populations grown in a control medium lacking adipogenic supplements (fig. 6f,h). The expression of the adipogenic markers FABP4 and PPARG was increased for both populations in adipogenic medium compared to the control medium (FABP4 expression increased by 4265-and 4556-fold for cell populations from the tendon core and from the peritenon, respectively; PPARG showed a 34-fold increase for the cell population from the tendon core and a 20-fold increase for the cell population from the peritenon; $p<0.05$ for all; fig. 7c,d). The cells from the core of the tendon had a small (1.1-fold), but significant, increase in expression for PPARG in the adipogenic condition compared to the cells from the peritenon ($p<0.05$) (fig. 7c). FABP4 expression showed no significant differences between the two cell populations in adipogenic medium (fig. 7d).

Additional explant experiments were undertaken to investigate how cells in their intact matrix would react to adipogenic and osteogenic differentiation media. Here, horse SDFT explants with their intact peritenon sheath were cultured for 21 days. Under adipogenic conditions, Oil red O staining revealed no changes for cells inside the tendon core that remained aligned along the collagen fibers (fig. 8a) In contrast, cells at the edge of the explants as well as those in the endotenon and peritenon showed a response to the differentiation medium. Numerous cells showed an enlarged and rounded shape. Similarly the cells in the dense, intact matrix of the tendon core were not affected by the osteogenic differentiation medium, whereas cells in the endotenon and peritenon showed a positive staining for Alizarin red (fig. 8b). The cell density in these regions also appeared to have dramatically increased.

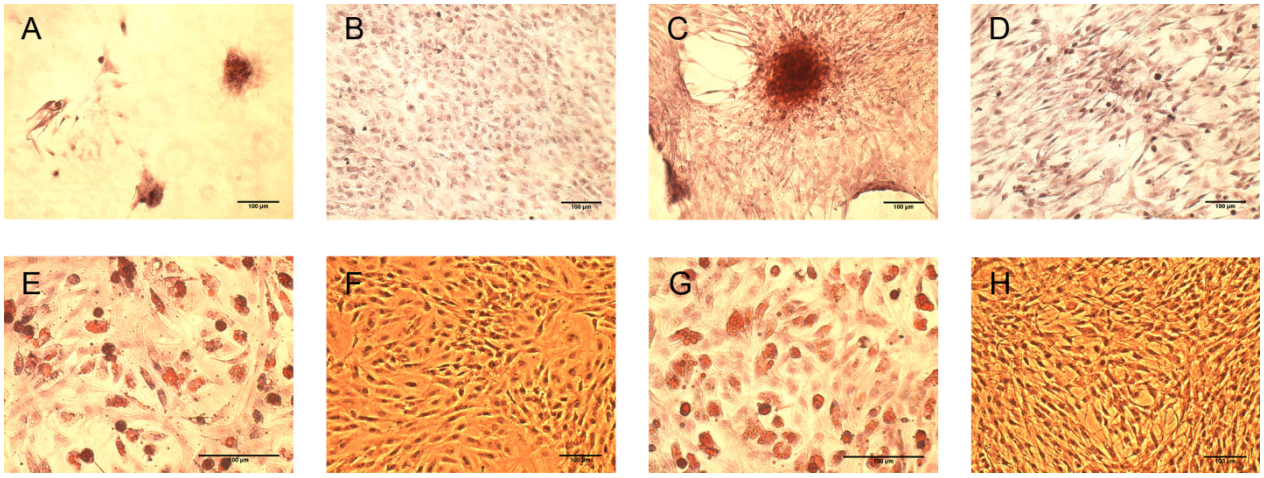

Figure 6: Osteogenic (a-d) and Adipogenic (e-h) differentiation of cells from the peritenon and cells from the core of the tendon: Alizarin red S staining on cells isolated from the tendon core (a,b) or from the peritenon (c,d) and cultured for 21 days in osteogenic medium (a,c) or control medium (b,d). Larger calcifications were observed in the peritenon cell population (c) compared to the cells isolated from the tendon core (a) in osteogenic medium. No calcification was observed in the control condition with expansion medium (b,d). Oil red O staining on cells isolated from the tendon core (e,f) or from the peritenon (g,h) and cultured for 21 days in adipogenic medium (e,g) or control medium (f,h). Oil red O staining revealed lipid vacuoles inside the cells of both populations when cultured in adipogenic medium (e,g), but not in the control condition with expansion medium (f,h).

The propensity for myofibroblast differentiation was investigated by monitoring the level of α-SMA, a protein involved in the heightened contractility that characterizes myofibroblast behavior. In both cell populations the presence of myofibroblasts in the original population could be demonstrated *in vitro*, even prior to stimulation with TGF-β1 (fig. 9a). As expected, the level of α-SMA increased for both populations of cells after the addition of TGF-β1 (α-SMA expression was increased 2.3 times for the cell population of the tendon core and 3.4 times for the cell population of the peritenon (fig. 9b). However, the level of α-SMA was 1.2 times higher in the peritenon cell population, indicating a higher differentiation potential towards myofibroblasts when compared to the cell population from the core (fig. 9b).

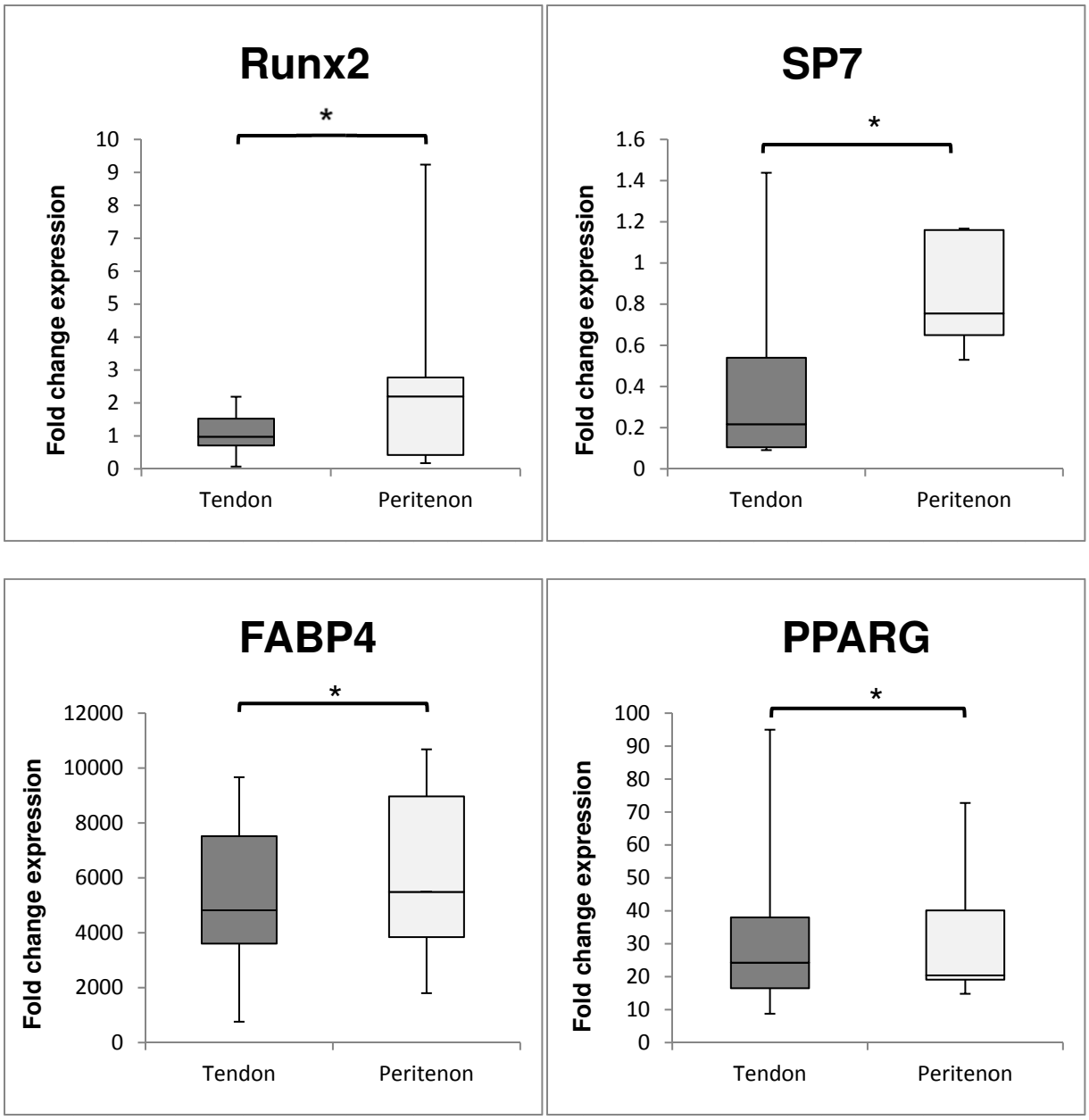

Figure 7: Expression levels of osteogenic and adipogenic markers of cells isolated from the tendon core and from the peritenon cell population. The fold changes were calculated by dividing the relative expression of the gene of interest in the differentiation medium by the relative expression in the control expansion medium. Runx 2 (A) and SP7 (B), both osteogenic markers, were 2.7 times and 1.8 times more expressed in the peritenon cell population compared to the tendon core cell population ($p<0.05$ for both). With respect to the adipogenic markers, no significant difference was found for FABP4 (C); however PPARG (D) was slightly but significantly upregulated (1.1fold) in the cells isolated from the tendon core compared to the cell population from the peritenon ($p< 0.05$).

When seeded in presence of TGF-β1 on PDMS substrates (100kPa) coated with collagen type 1, the cell population from the peritenon showed a higher level of α-SMA (1.9 fold) compared to the cell population from the tendon core (fig. 10a). This tendency was augmented when the cells were seeded on PDMS coated with fibronectin, an ECM protein typical within injured tendons (25). (The cell population from the peritenon showed a 3.6-fold change increase of α-SMA expression on fibronectin compared to the cell population from the tendon core (fig. 10b).

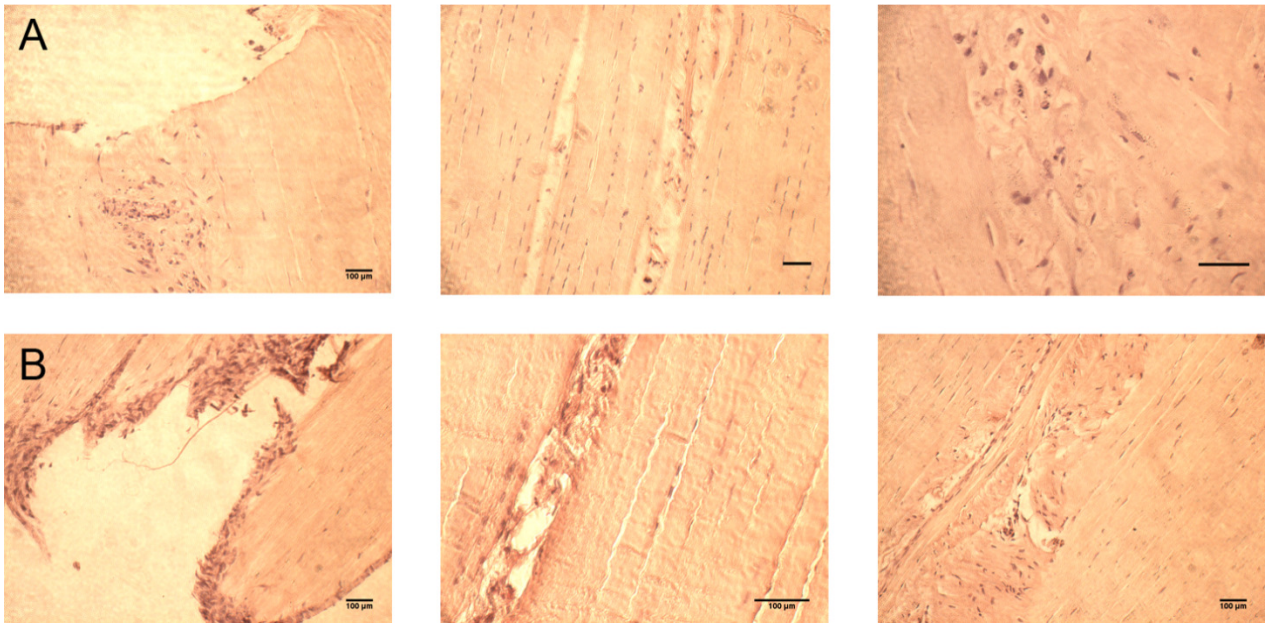

Figure 8: Adipogenic (A) and osteogenic (B) differentiation of SDFT explants. Oil red O staining revealed major changes in the endotenon and peritenon, but not in the tendon core. The cells in the endotenon and peritenon appeared enlarged and positive to Oil red O staining. Alizarin red S staining revealed changes in matrix organization and cell densities in the endotenon and peritenon and again not in the tendon core. Three pictures from different samples were selected for each condition to illustrate more clearly what is happening on the edge of the sample, in the middle of the section and in the endotenon (from left to right).

Discussion:

We have evaluated two cell populations of different origin for proxy measures of their potential healing capacity as well as for their potential for involvement in pathogenesis. One population was directly derived from the core of the tendon (to examine intrinsic healing) and the other came from the surrounding tissues of the tendon, the peritenon (extrinsic healing).

Tenogenic gene expression analysis showed distinctive profiles for each of the two cell populations. The cell population from the tendon core demonstrated a clearly higher expression of tenomodulin and scleraxis compared with the cell population coming from the peritenon. A similar observation was made in murine Achilles tendon (11).

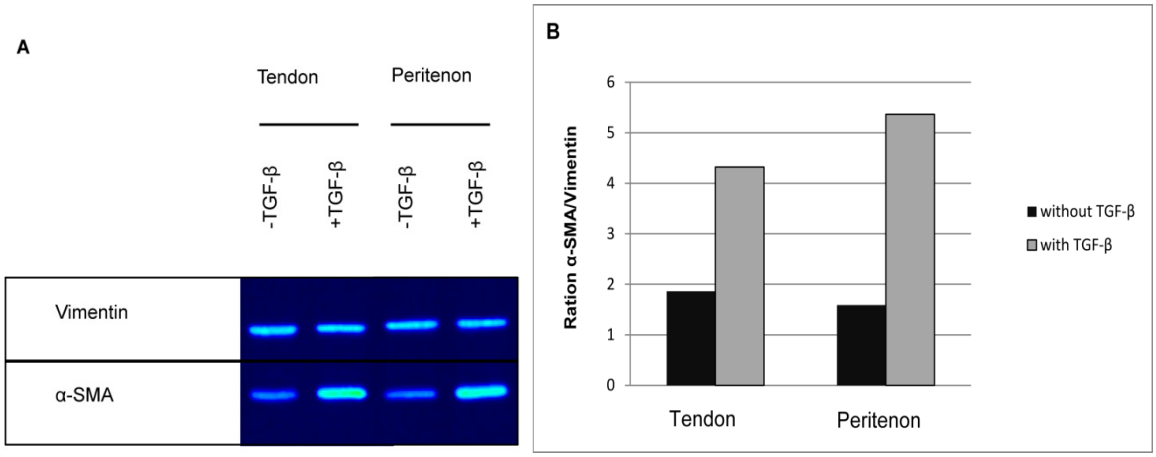

Figure 9: Comparison of the propensity to differentiate into myofibroblasts of the cell population from the peritenon and the cell population from the core of the tendon. (A) Expression of myofibroblast marker α-SMA was assessed together with loading control vimentin, by Western blotting. (B) Band signal strength in Western blots was analysed by optical densitometry and related to loading control vimentin. Following addition of TGF-β1, the level of α-SMA was 2.3 times increased in the cell population from the tendon core and 3.4 times increased in the cell population from the peritenon. The cell population from the peritenon showed a 1.2 increased expression of α-SMA compared to the cell population from the tendon core when cultured with TGF-β1.

Measurements of replication rate, migration rate and collagen production all indicated a faster reactive potential for cells from the peritenon compared with cells from the tendon core. These results are in accordance with functional *in vivo* studies in which cells from the epitenon have been observed to proliferate and migrate towards and into the wound (26-28). The finding of similar levels of expression for collagen type 1 mRNA (also confirmed at protein level) in the peritenon cell population compared to the tendon core population is consistent with the concept of peritenon-mediated extrinsic repair of tendon collagen structures, for instance after tendon suturing (26). We were able to confirm these characteristics with controlled parameters *in vitro.* Taken together, these results suggest a dual healing process with a possible predominance of extrinsic healing.

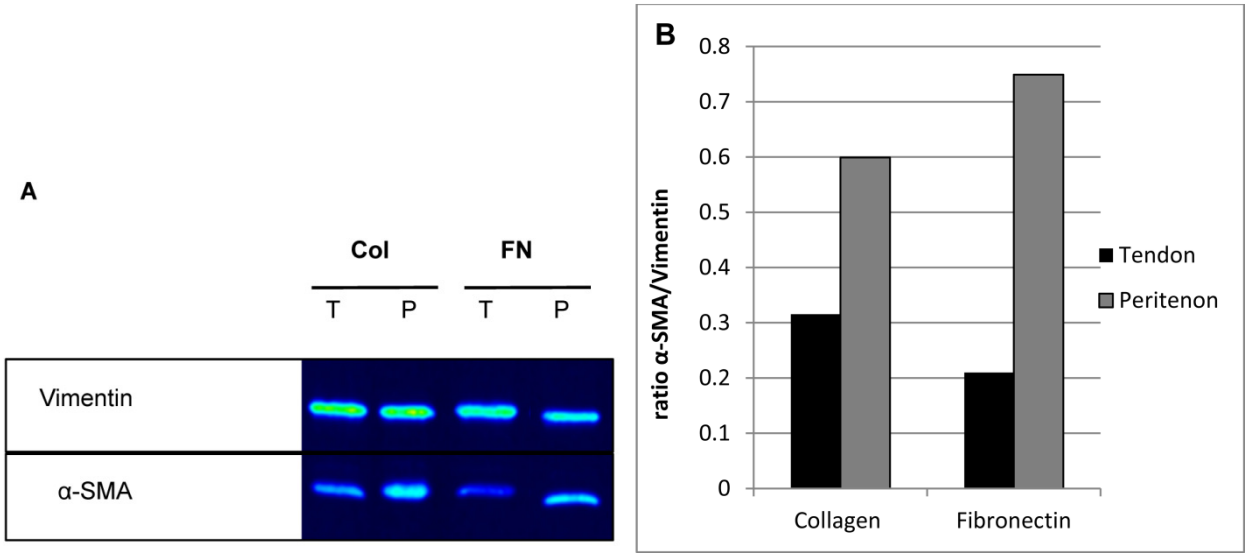

Figure 10: The effects of substrate stiffness and ligands on the differentiation propensity of cells isolated from the peritenon and cells isolated from the tendon core. (A) Expression of myofibroblast marker α-SMA was assessed together with loading control vimentin, by Western blotting. (B) Band signal strength in Western blots was analysed by optical densitometry and related to loading control vimentin. The cell population from the peritenon showed a 1.9-fold increase of α-SMA expression on collagen compared to the cell population of the tendon core and a 3.6-fold increase of α-SMA expression on fibronectin.

While stem cells are heavily involved in homeostasis, growth and repair of many tissues (29), they may also contribute to some forms of tendinopathy by creating ectopic tissue inside the tendon (30). Our assays of clonogenicity indicated a higher number of stem/progenitor cells in the cell population from the tendon core compared with the cell population from the peritenon. However the colonies were larger for the latter. This suggests a potentially higher replication rate of stem/progenitor cells in the peritenon cell population. Our results also indicated an increased formation of calcium deposits after differentiation in osteogenic media for peritenon cell populations compared to populations extracted from the tendon core. A similar differentiation potential toward an adipogenic lineage was observed for both cell populations. Collectively, these data suggest a lower number of stem/progenitor cells in the cell populations extracted from the peritenon. Nonetheless, when these stem/progenitor subpopulations within the peritenon were exposed to a stimulation medium, they indicated greater potential for differentiation, consistent with the concept that peritenon cells are more reactive and replicate faster than those from the tendon core. These findings are consistent with those of Mienaltowski et al., who also found that the number of colonies formed by progenitor cells from the tendon core was higher than from the peritenon (11). As in the present study, these authors also did not observe any clear difference regarding adipogenic differentiation between the two cell populations. One interesting distinction between our results and those of Mienaltowski et al. is that they did not observe any calcium

deposits in the cell population from the peritenon whereas we observed clear calcification in this cell population. The highest amount of calcium deposition was even found in the differentiated peritenon cell population. Supporting RT-PCR data confirmed these histological observations. In contrast to one other report (31), we did not detect adipogenic markers in cells cultured in control medium. One plausible explanation for this discrepancy could be that equine tendon cells display a different behavior from those of rodents, paralleling similar differences in adipogenic and osteogenic potential of tendon/stem progenitor cells in mice and humans (32). Species-related specificity in differentiation could also explain why, unlike in human tendons, clinical observations of lipid accumulation in diseased or injured equine tendons have not yet been reported (33).

We observed that cells at the margins of a tendon explant, but not those inside the more densely packed, well-organized intact matrix, show propensity for differentiation to non-tendinous phenotypes. This suggests that a well-structured matrix may play a key role in regulating the fate of stem/progenitor cells, with possible implications for adipogenesis in tendinopathy, and ectopic calcifications. Previous *in vitro* studies using cross-linked collagen matrices and gels have also shown a clear influence of matrix compliance in directing the stem cell lineage specifications (34; 35). To our knowledge, our study is the first to show the influence of the matrix in tendons *ex vivo*.

Myofibroblasts are key players in the classic connective tissue wound healing paradigm but are also associated with excessive scar formation (36; 37). Little is known of the role of myofibroblasts in healing tendons (38). Our results reveal the presence of α-SMA in native cell populations of both tissue compartments *in vitro*, with higher levels observed in cells from the tendon core. In theory, these observations might be explained in part by the presence of pericytes (contractile cells lining blood capillaries) within the sampled populations and not necessarily by a differentiation of fibroblastic cells, such as tenocytes, into myofibroblasts. However, tendons are poorly vascularized, as confirmed by De Mos et al. who found that 98.5 ±0.7% of the cells isolated from tendons were negative for CD34, an endothelial cell marker (15). More importantly for the present investigation, α-SMA expression increased in both cell populations after the addition of TGF-β1, with the peritenon-derived cell population showing a greater potential to differentiate into myofibroblasts. Furthermore, our findings indicated increased α-SMA levels in cells seeded on fibronectin-coated substrates compared to collagen-coated ones, suggesting a high affinity to fibronectin, which is abundantly found in healing tendons (25). These results

indicate again that the cells from the peritenon are highly reactive cells, of which it is very plausible that they are implicated in tendon healing processes.

Aside from careful anatomical extraction, we did not localize the origin of either the cells that showed a clear tenogenic phenotype or the stem/progenitor cells. Consequently, we were not able to evaluate the proportion of those cells in the population from the core of the tendon that had come from the endotenon. We were also not able to distinguish between individual cells with multilineage potential and more or less strongly committed cells. We did find that not all of the cells showed a clear differentiation towards other lineages (osteogenic, adipogenic and myofibroblastic), which indicates that there were different subpopulations of cells inside our two main populations. While surface markers analysis of the cells (fluorescence activated cell sorting (FACS)) could provide additional clarification in future studies, FACS analysis of mature tendon cells and their progenitors is currently limited, and established surface markers are only now emerging (15). Still, the functional data we provide illuminate other studies where stem/progenitor cells have been reported to originate from the tendon core (15; 31; 32; 39) and from the peritenon (11). Our data also confirm the reportedly higher reactivity of stem/progenitor cells from the peritenon compared with those from the tendon core (11). Beyond previous studies, our study is the first to indicate a higher differentiation potential towards a myofibroblastic phenotype for the cells from the peritenon in comparison with cells from the core of the tendon. This suggests that there may be a greater potential of cells from the peritenon to be involved in formation of scarring and adhesions during tendon healing. We also investigated for the first time the role of a native (explanted) 3D tendon matrix in influencing cell differentiation towards adipogenic or osteogenic lineage and we showed a clear protective effect of an intact matrix. More importantly, our results show that both the cells from the peritenon and the cells from the core of the tendon were able to express and produce large amounts of collagen type 1, indicating a possible dual mechanism (intrinsic and extrinsic) for tendon healing.

Taken together these findings highlight an unexpectedly large plasticity of both tendon and peritendinous cells – with potentially important implications to tissue repair mechanisms. These mechanisms are central to our understanding of the onset and development of tissue pathologies such as tendinopathy, and the eventual development of improved therapeutic strategies.

Acknowledgements:

The authors would like to acknowledge the scientific contribution of Prof. B. Hinz to this study. The funding was provided in part by the Bonizzi-Theler Stiftung and in part by internal funds of Utrecht University, NL.

Table 1: Designed primers

Primer	Gene	product length (bp)	primer sequence (5' ->3') F: forward primer R: reverse primer
Tenogenic markers			
COL1A1 XM_001499586.3	Collagen type 1	164	F: TGCCATCAAAGTCTTCTGCAA R: CGCCATACTCGAACTGGAATC
TNMD NM_001081822.1	Tenomodulin	142	F: AGAAGACCCGTCGCGCCAGA R: CGGCAGTAGCGGTTGCCTCG
SCX	Scleraxis	81	F: GCCGGTCACATCCCTCGCCA

NM_001105150.1			R: TCCTCCGACAGCGGGCTCAC
differentiation markers			
Runx2 XM_001502519.3	runt-related transcription factor 2	81	F: CCCACGGCCCTCCCTGAACT R: TGTGCCTGCCTGGGGTCTGT
Sp7 XM_001494930.3	transcription factor osterix, transcript variant1	130	F: GATGGCGTCCTCCCTGCTTGA R: GCCTGCTTTGCCCAGTGTCGT
PPARG XM_001492430.1	peroxisome prolifertor-activated receptor gamma-like, transcript variant 2 (LOC100051258)	185	F: AGGGGCCTTTACCTCTGCTGGT R: TGGGCCAAAATGGCATCTCCGT
FABP4 XM_001490771.3	fatty acid-binding protein, adipocyte-like (LOC100057425)	195	F: ACACCAGAGGGTCAGACACCT R: GGTTTGGCCATGCCAGCCAC
House-keeping gene			
GAPDH NM_001163856.1	glyceraldehyde-3-phosphate dehydrogenase	141	F: GTCAACGGATTTGGTTATTGGG R: TGCCATGGGTGAATCATATTGG

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Chapter 7

Extracellular-matrix tethering regulates differentiation of tenocytes into myofibroblasts and TGF-β1 is necessary to facilitate contraction.

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Running title: Tenocytes differentiation into myofibroblasts and their contraction

Abstract:

Differentiation of fibroblasts into myofibroblasts is an important step in wound healing and tissue repair. They exert high contractile forces that facilitate wound closure, but in excess this contractility can lead to hypertrophic scarring and impairment of function. Tenocytes are of fibroblast lineage, yet the potential role of myofibroblasts in tendon repair is poorly understood. In this study we investigated physical and molecular drivers of tenocyte differentiation into myofibroblasts and how contractility of the latter is regulated. By seeding tenocytes on polymers of different stiffness and topology we could observe that the compliance of polydimethylsiloxane (PDMS) substrate alone did not affect strongly the level of alpha smooth muscle actin (α-SMA). However, there was a correlation between less compliant, tighter pored polyacrylamide (PAA) substrate (with consequently anchoring sites of collagen ligands that are located more closely to each other) with increased levels of α-SMA. Use of increased ligand cross-linker density to increase the tethering of the collagen further enhanced α-SMA levels. These results indicate that the differentiation of tenocytes to myofibroblasts is less influenced by substrate stiffness and by topology than by ligand tethering. Supporting experiments at the tissue level revealed that rat tail tendon fascicles contracted when cultured free-floating for 5 days either with or without TGF-β1, but the effect was enhanced by the addition of 5 and 10ng/ml of TGF-β1. Inhibition of TGF-β1receptors prevented fascicle contraction entirely; inhibition of matrix metalloproteinase (MMP) activity had a similar effect though still some contraction was seen after adding TGF-β. Collagen Inhibition of TGF-β1receptors prevented fascicle full contraction, as did inhibition of matrix metalloproteinase (MMP) activity. Collagen gels seeded with tenocytes or myofibroblasts did not show differences between the two cell types in contraction regardless of the addition of TGF-β1. Taken together these results indicate the necessity of TGF-β1 for tendon myofibroblast contraction. We thus conclude that tenocytes exert a mechanical force on collagen fibres and gauge the feed-back to make cell-fate decisions and that TGF-β1 signaling appears to be a key mediator of tendon myofibroblast contraction. This information provides novel insights into potentially important aspects of tendon cell plasticity, and may have relevance for the understanding of tendon regeneration and fibrosis.

Keywords: tenocytes, myofibroblasts, contraction, TGF-β1, stiffness

Introduction:

It is generally accepted that fibroblasts can differentiate into myofibroblasts, which are known to be key players in the physiological repair of connective tissue after injury (1; 2). Myofibroblasts have stress fibers containing *α*-smooth muscle actin (α-SMA) that are capable of creating forces required for wound contraction and for the organization of newly synthesized extracellular matrix (ECM) (2). They are also known to synthesize large quantities of ECM and are therefore often associated with excessive scarring and fibrosis (1). It has been shown in various connective tissues, that differentiation of fibroblasts into myofibroblasts can be induced by mechanical tension of the ECM (2; 3). The threshold tissue stiffness for *de novo* expression of α-SMA or its maintenance reportedly ranges around 20 kPa, as demonstrated in contractile wound granulation tissue and for myofibroblasts cultured on elastic substrates (4). Lower matrix stiffness, as in early wounds (~10-100 Pa), is not sufficient to elicit differentiation of fibroblasts into myofibroblasts (5). Transforming growth factor beta one (TGF-β1) is considered to be the major growth factor directly promoting myofibroblast differentiation by inducing expression of α-SMA (6; 7). TGF-β1 is believed to promote the morphological and functional differentiation of myofibroblasts by first enhancing the expression of α-SMA, followed by an increase in contractile force generation, with tissue contraction being an integral part of wound closure (7).

In injured tendons, the presence of myofibroblasts has been shown in the reparative scar tissue that forms following partial rupture (8; 9). After intense treadmill running exercise, numerous myofibroblasts have also been observed in mouse patellar tendons (10). Recently, Gardner et al. found that myofibroblasts were able to re-establish the cytoskeletal tensional homeostasis in lax tendons and thus prevent the production of collagen proteases (11). The presence of TGF-β1 has also been reported in injured tendons (12), which would be consistent with functional contraction of myofibroblasts in tendon repair. However, while the TGF-β1 secreted by infiltrating macrophages and platelets is known to stimulate proliferation and matrix organization (12-15), to our knowledge no investigation has been performed regarding TGF-β1 regulation of tendon cell contraction. Based on the roles myofibroblasts play in other connective tissues, we hypothesized that these cells could underlie the dysfunctional scarring and fibrosis that often occurs after tendon injury (16) or in tendinopathy (19).

We investigate here the differentiation of tendon cells into myofibroblasts, as well as the regulation of their subsequent contraction. While these two processes have been shown to play an important role in healing of other connective tissues, they still need to be studied in tendon tissue. To our knowledge, we demonstrate for the first time that tendon cells can differentiate into myofibroblasts depending on the topology of the matrix and on the tethering of ligands. We have found also that substrate stiffness alone did not influence tendon cell differentiation into myofibroblasts. Further, we show that TGF-β1 is a key player in tendon contraction. We conclude that tendon myofibroblasts could play an important role in tendon healing and, as in other tissues, may be suspects in the aberrant ECM modelling and remodelling processes that may be a sequel to tendon injury.

Methods:

Isolation of cells

Tendon cells were extracted from the core of the SDFT by digestion of the tendon matrix using Protease Type XIV (Sigma-Aldrich, St. Louis, MO) for 2h at 37°C and Collagenase B solution (Roche, Burgess Hill, UK) for 16h at 37°C. After this period of digestion, the mixture was filtered and centrifuged at 400g for 8 min at room temperature. The cell pellet was then re-suspended and cultured at 37°C, 5% CO_2 in expansion medium (Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FCS), 50µg/ml gentamicin and 1.5µg/ml fungizone (all from Life technologies, Paisley, UK)). Cells were cultured and trypsinized at subconfluency and only those cells that had been freshly digested or which were from the first passage were used for the following experiments.

Preparation of Polydimethylsiloxane (PDMS) and polyacrylamide (PAA) substrates

The PDMS substrate were purchased from ExCellness Biotech SA (Lausanne, Switzerland) and coated according to manufacturer's protocol with 2µg/cm^2 Collagen type 1(Sigma-Aldrich).

For the PAA substrates, NuPage 4-12% electrophoresis gels (Life technologies) were used as a mechanovariant substrate. The gels were composed of 4-12% acrylamide and 3.8-5% bisacrylamide. The mechanical properties were previously determined in our laboratory with a material testing machine under tension, as well as under compression using spherical indentation

testing (Zwick 1456, Ulm, Germany)(17). We used gels of three different compliance ranges (Soft= 0.5-20kPa Middle=90-110kPa Hard=170-190kPa).

Gels were washed in MilliQ water twice at 2h intervals at room temperature before soaking overnight at 4°C to remove excess acrylamide and sodium azide. The substrates were removed and strips that were approximately 1 cm in width and 2.5cm in length were sectioned and placed in a 6-well plate (Nunc, Roskilde, Denmark). The substrates were functionalized using procedures as described by others (18). Substrates were overlaid with sulfo-SANPAH (Pierce Biotech, Thermo Fischer Scientific, Rockford, IL), a light sensitive cross-linker, at 0.1 mg/ml or 0.02mg/ml in 50 mM HEPES buffer (Sigma-Aldrich). The substrates were then placed in a Stratalinker 2400 ultraviolet light cross-linker (Life Technologies) for 10 min. This cross-linker was then removed and the substrates were overlaid with additional fresh cross-linker and exposed to ultraviolet light. At this point, the substrates were sterilized and then brought back to the sterile field for further processing. After washing twice with PBS, reactive substrates were overlaid with 50µg/ml collagen I protein (Sigma-Aldrich) and left overnight at 4°C. The substrates were finally washed three times in PBS and used immediately for cell culture. The cells were kept in culture for 5 days in expansion medium before they were scraped off in sample buffer for Western blotting.

Preparation of collagen gel and contraction assay

Collagen gels were prepared in small Petri dishes (Ø40mm; Techno Plastic Products AG, Trasadingen, Switzerland) warmed to 37°C for 1h. Collagen gels were prepared by mixing ice-cold type I rat tail collagen solution (BD Biosciences, Bedford, MA) with 10× PBS (Life Technologies), adjusting the pH to 7.4 with NaOH and then immediately mixing with freshly trypsinized cells. Before starting the contraction assay, passage 1 tenocytes were cultured in expansion medium with or without the addition of 5ng/ml TGF-β1 (R&D systems, Minneapolis, MN) for five days and then trypsinized and mixed with the collagen gel solutions. In this way, the gels contained 2mg/ml collagen type I and 200,000 cells/ml. After mixing, 200µl of the final collagen mix was added to each pre-warmed Petri dish and gels were allowed to polymerize at 37°C for 1h, before 2ml culture medium was added to each gel. The gels were left in culture with or without 5ng/ml of TGF-β1 (R&D systems) for 5 days before they were released from the surface of the petri dishes. Photographs were taken with a Nikon camera (D5100, Nikon, Tokyo, Japan) just before release and 30min later. Images of the gels were analyzed with ImageJ (http://rsb.info.nih.gov/ij; National Institutes of Health, Bethesda, MD) to compute contraction,

which was determined by dividing the area of the gel at the final time point by its initial area. This procedure was carried out in triplicate to give a total of 6 assays per treatment per time point. At the conclusion of the experiment, gels were snap frozen and ground for Western blotting.

Western Blotting

Tenocytes were lysed in sample buffer (0.1g/ml SDS (Biosolve BV, Valkenswaard, NL), 1M Tris-HCl (Biosolve BV), 50% Glycerol (Sigma-Aldrich)). Equal amounts of protein were analyzed by Western blotting using antibodies to α-SMA ((mouse IgG2a, clone SM1) was the kind gift of Christine Chaponnier and Giulio Gabbiani, University of Geneva, Switzerland) (19) and vimentin (DAKO, Glostrup, Denmark).

Isolation of rat tail tendon

Rat tail tendons were extracted from mature rats (over 13 weeks old) which were sacrificed for other experiments. Once physically removed from the tails, four tendons were directly cultured in separate wells of 6-well plates in culture medium (DMEM, 10% FCS, 50µg/ml gentamicin and 1.5µg/ml fungizone (all from Life technologies) at 37°C, 5%CO_2. Tendon fascicles were incubated in 0, 5 or 10ng/ml of TGF-β1 (R&D Systems). For appropriate groups, 1µM SD208 (Tocris Bioscience, Ellisville, MO), or 50µM Ilomastat (US Biological, Salem, MA) were added to the culture medium to investigate the effects of TGF-β1 stimulation. Contraction was observed using macroscopic photographs taken using a digital camera (DMC-FS16, Panasonic, Tokyo, Japan) after 5 days in culture.

Immunohistochemical staining

Rat tail tendons were fixed in 10% formalin (Sigma-Aldrich) for 48 hours, dehydrated and then embedded in paraffin. The samples were cut into 6µm sections. After de-paraffinization and re-hydration, the sections were blocked with 2% bovine serum albumin (Sigma-Aldrich) in PBS for 30min at room temperature and stained with α-SMA antibody and DAPI (Vectashield, Vector Laboratories Inc, Burlingame, CA).

Results:

Based on reported rat embryonic fibroblasts response to altered matrix stiffness (myofibroblast differentiation enhanced on substrates above 20kPa (4)), we expected that varying PDMS cell substrate compliance from 5 to 100kPa would similarly affect tenocyte myofibroblast differentiation. This was not confidently supported by Western-blots using antibodies against α-SMA (Fig.1), as near to equivalent levels of α-SMA were observed for the four tested compliances.

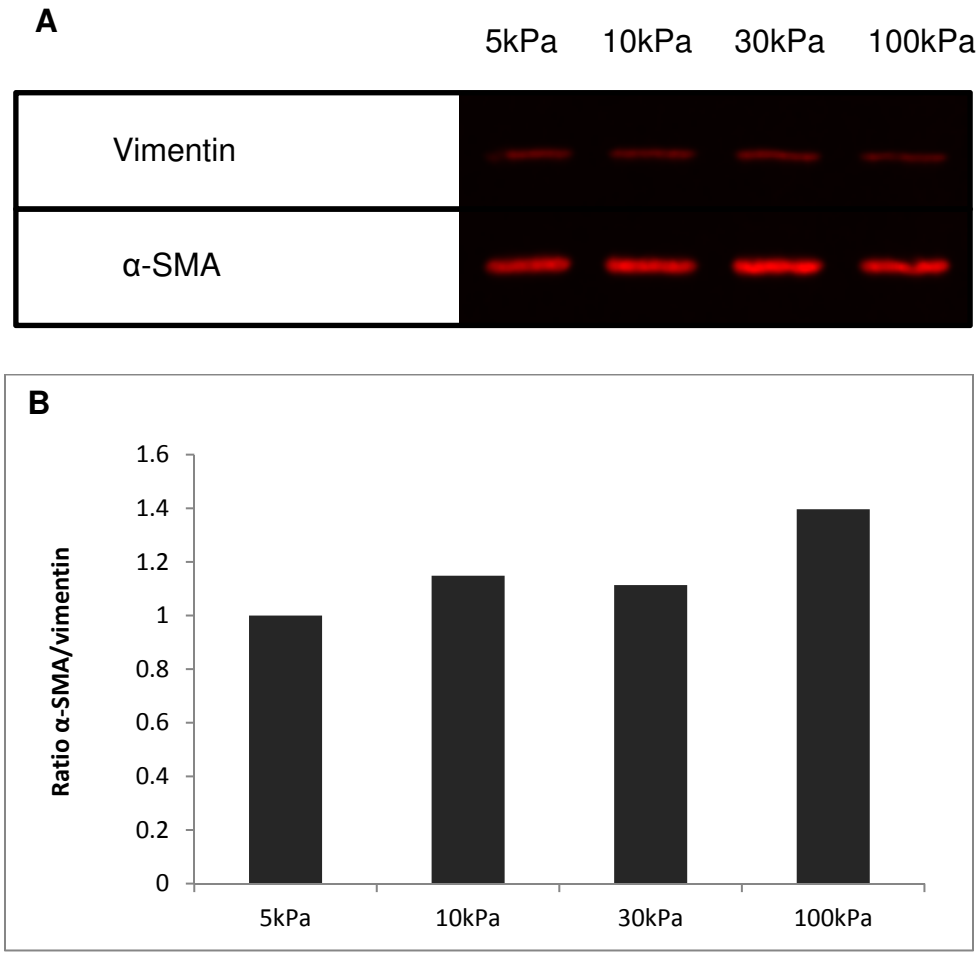

Figure 1: PDMS substrate stiffness (5, 10, 30 and 100 kPa) doesn't influence strongly differentiation of tenocytes to myofibroblasts. (A) Expression of myofibroblast marker α-SMA was assessed together with loading control vimentin, by Western blotting. (B) Band signal strength in Western blots was analysed by optical densitometry and related to loading control Vimentin.

To assess sensitivity to substrate topology, we utilized mechanovariant PAA hydrogel substrates with varying pore size (decreasing pore size with increasing gel stiffness). We observed an increased level of α-SMA for tenocytes that had been cultured on smaller pore sizes, where the collagen anchoring points are also consequently closer to each other (Fig.2).

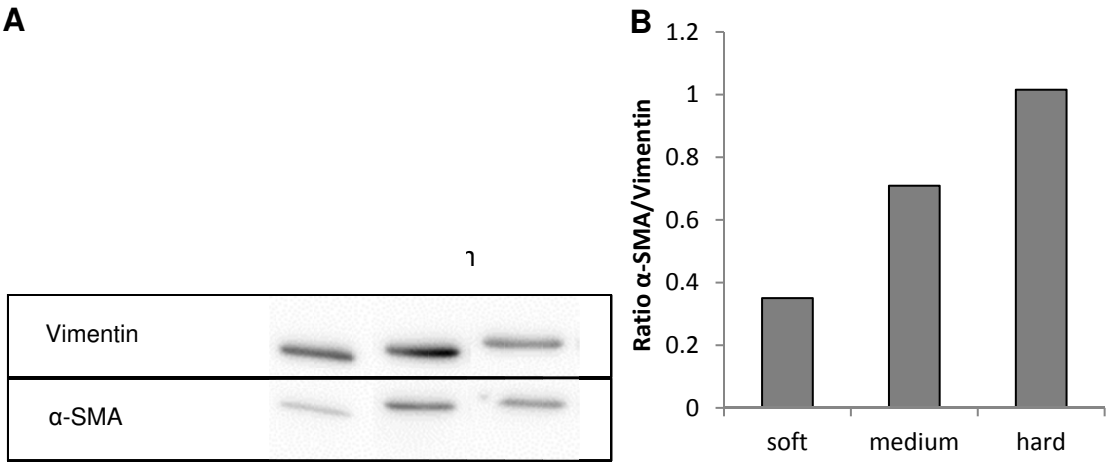

Figure 2: PAA substrate stiffness (soft (0-20kPa), medium (90-110kPa) and hard (170-190kPa)) influences the differentiation of tenocytes to myofibroblasts. (A) Expression of myofibroblast marker α-SMA was assessed together with loading control Vimentin, by Western blotting. (B) Band signal strength in Western blots was analysed by optical densitometry and related to loading control Vimentin

As tenocytes bind indirectly to the PAA gel through the interposed collagen ligand, we also directly investigated the role of the covalently coupled collagen. By varying the concentration of sulfo-SANPAH (a cross-linker of collagen to the PAA gel), the density of the collagen anchoring sites and hence the distance between them varies accordingly. We observed increased levels of α-SMA with increasing concentrations of sulfo-SANPAH when tenocytes were cultured on the softest gels with the largest pore size (Fig.3). This increase of α-SMA was however not observed when culturing on stiffer gels with smaller pore size, perhaps suggesting a saturation phenomenon.

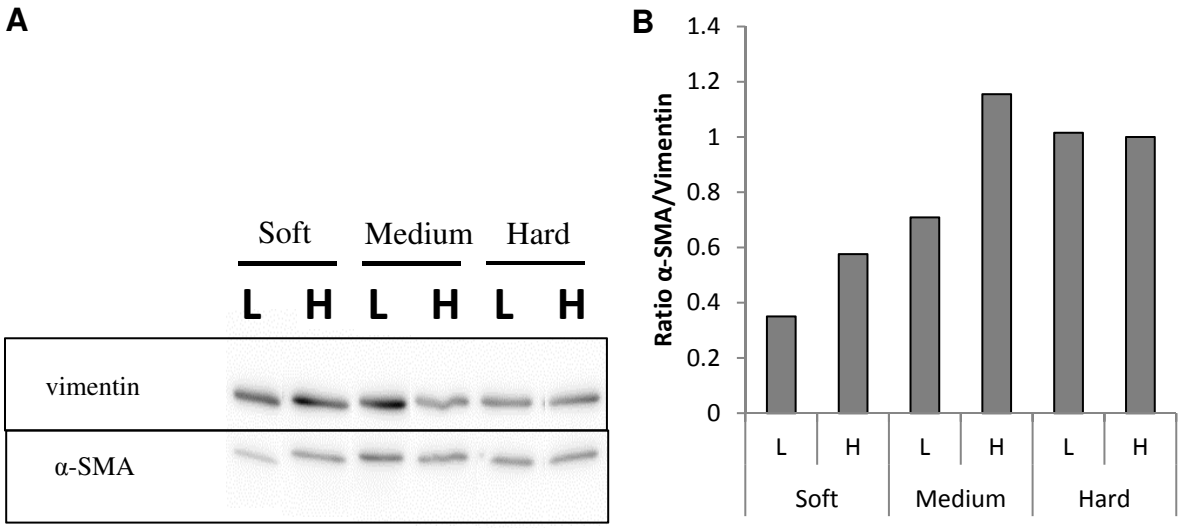

Figure 3: The concentration of sulfo-SANPAH, the collagen cross-linker influences the differentiation of tenocytes to myofibroblasts. Cells were seeded on PAA substrate of differing stiffness (soft (0-20kPa), medium (90-110kPa) and hard (170-190kPa)) and two concentrations of sulfo-SANPAH were used (L=0.02mg/ml and H=0.1mg/ml).(A) Expression of myofibroblast marker α-SMA was assessed together with loading control vimentin, by Western blotting. (B) Band signal strength in Western blots was analysed by optical densitometry and related to loading control Vimentin.

Rat tail tendons showed clear contraction when cultured free floating for 5 days (Fig.4A-C). The effect was enhanced with the addition of 5 and 10ng/ml of TGF-β1 (Fig.4B-C). Interestingly, this contraction was prevented by adding SD208, a widely employed inhibitor of TGF-β receptor 1 (Fig. 4C-E). With SD208, the tendon rat fascicles showed no contraction regardless of whether TGF-β1 had been added. Immunofluorescent probing of α-SMA in tendon fascicles that had been cultured for 5 days free floating in 5 ng/ml of TGF-β1 highlighted the presence of α-SMA positive cells at very localized regions (Fig.5). Although DAPI staining showed the presence of numerous nuclei inside the tendon fascicles, most α-SMA positive cells were observed at the regions of contracture. The addition of Ilomastat, an established matrix metalloproteinase (MMP) inhibitor, also limited the contraction of the tendon fascicles compared to non-treated tendon fascicles (Fig. 6). However, even in the presence of the MMP inhibitor contraction was enhanced by addition of 5 and 10ng/ml of TGF-β1 (Fig.6B,C). An increase of α-SMA expression was revealed by immunohistochemistry with increasing TGF-β1 concentration (Fig.6D-E). In order to investigate whether TGF-β1 was necessary for myofibroblasts to develop contraction, we compared the contraction of collagen gels containing either tenocytes or tenocytes that had previously differentiated into myofibroblasts and measured the effect of the presence or absence of TGF-β1. The gels were kept in culture for 5 days before performing the test for contraction.

Confirming the tissue level response in tendon fascicles, addition of TGF-β1 to the culture medium enhanced gel contraction (Fig.7A). However, in this case no clear differences could be observed between gels containing tenocytes and gels containing myofibroblasts in either condition (i.e. with or without TGF-β1). Western blot analysis confirmed that myofibroblasts did not dedifferentiate into tenocytes in the absence of TGF-β1 as the level of α-SMA was still higher in gels containing tenocytes that had been previously differentiated towards myofibroblasts than in gels containing tenocytes only (Fig.7B). The level of α-SMA expression was however similar between tenocytes and previously differentiated myofibroblasts when seeded in collagen gels with TGF-β1 supplemented to the culture medium over five days.

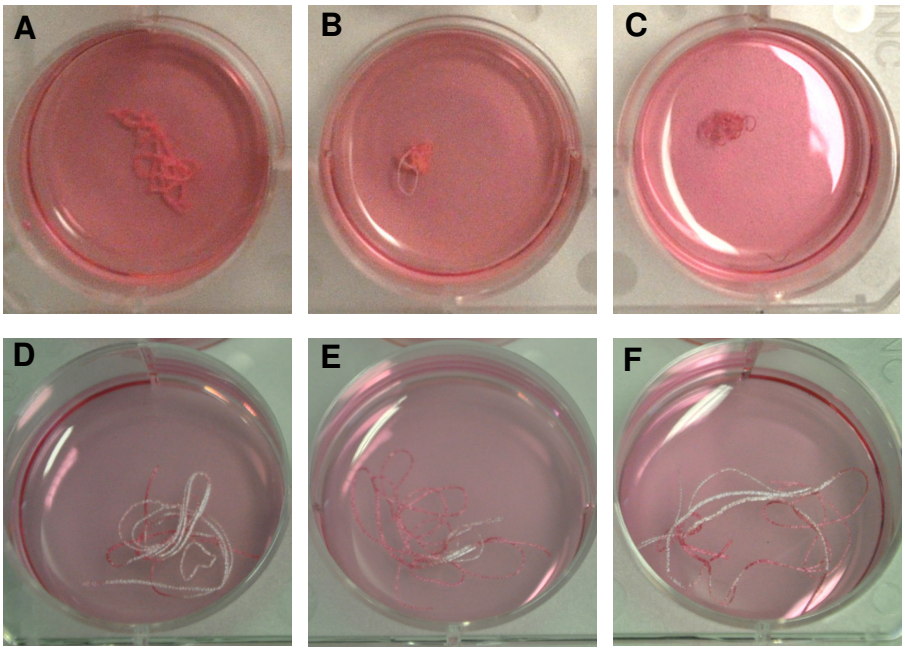

Figure 4: Macroscopic pictures comparing RTTFs contraction in medium containing increasing dose of TGF-β1 and either 1μM of TGF-β receptor 1 inhibitor (SD208) or no SD208. The pictures are showing the following: RTTFs in medium containing no TGF-β1 (A and D), 5ng/ml TGF-β1 (B and E) or 10ng/ml TGF-β1 (C and F) and the upper row (A-C) contains no SD208 while the lower row (D-E) contains 1μM of SD208. The RTTFs were kept free-floating in the different medium for five days. As reference, the diameter of the wells equals 3.5cm.

Discussion:

While myofibroblasts are widely recognized as key mediators of soft connective tissue healing and fibrosis, their potential role in tendon repair is largely unknown. In this study, we investigated tenocyte differentiation into myofibroblasts and their subsequent contraction. In the

established paradigm of myofibroblast differentiation from less contractile fibroblastic cells, progressive post-injury recovery of matrix tension in conjunction with the action of TGF-β1 is believed to be a prerequisite for *de novo* expression of α-SMA (2; 6; 20). The results of our studies on PDMS substrates indicated that increasing two-dimensional substrate stiffness alone was insufficient to provoke myofibroblastic differentiation of tenocytes, as no clear difference in α-SMA expression was observed on substrates between 5 and 100kPa. *In vivo*, Goffin et al. showed that in rat wound granulation tissue, there was a positive correlation between an increasing matrix elastic modulus and increased presence of α-SMA positive cells (4). While numerous differences between *in vivo* and *in vitro* study likely play a role, in vitro studies by these same authors interestingly showed that 90% of differentiated rat embryonic fibroblast (REF-52) myofibroblasts that had been cultured on PDMS substrates of stiffness above ~20kPa maintained their expression of α-SMA whereas 97% of the cells plated on substrate below 20kPa had lost their expression of α-SMA (4). Taken together those results suggest that myofibroblasts may de-differentiate according to substrate stiffness, but that substrate stiffness alone is not sufficient to drive *in vitro* differentiation of tenocytes into myofibroblasts. This latter finding supports our own observations.

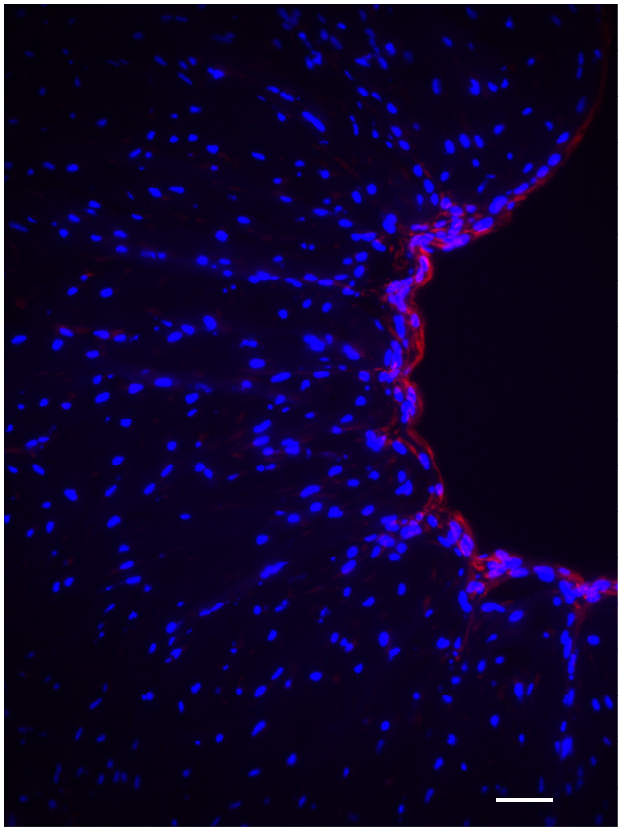

Figure 5: Representative photomicrograph demonstrating the immunohistochemical staining for α-SMA proteins in the cells inside the bent area of an RTTF cultured for five days free-floating in expansion medium containing 5ng/ml TGF-β1. α-SMA (red); nuclei (blue); Scale bar = 20µm.

Goffin and colleagues also reported that the size of cellular focal adhesions apparently controlled tension-dependent recruitment of α-SMA to the cytoskeletal stress fibers. They were able to show that cells plated on islets that promoted large focal adhesions (10 and 20μm long), which enabled the generation of higher intracellular tensions, maintained α-SMA expression compared to cells plated on islets permitting only smaller focal adhesions (2, 4 and 6μm), which effectively lost their α-SMA expression. These results point to the topology of the substrate as having a strong influence on myofibroblastic differentiation, which is in agreement with our findings.

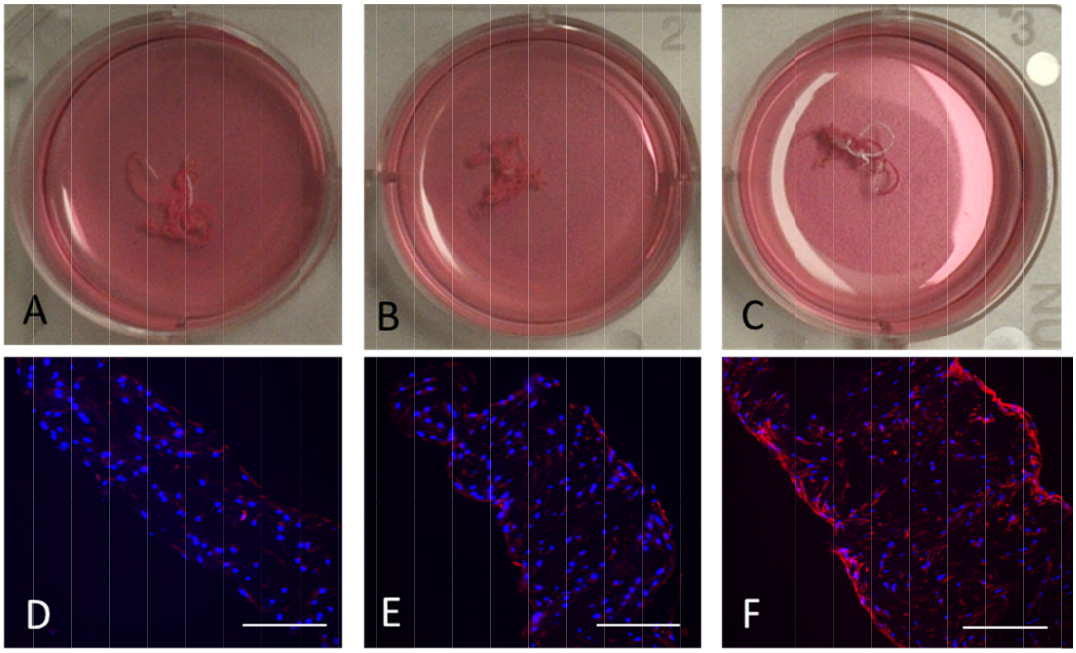

Figure 6: Images of RTTFS cultured free-floating in medium containing 50μM of Ilomastat and either no TGF-β1 (A and D), 5ng/ml TGF-β1 (B and E) or 10ng/ml TGF-β1(C and F). After 5 days of culture, those samples were fixed and immunostained for α-SMA (red) and dapi (blue) (scale bar = 200 μm).

In the present study, we utilized PDMS and PAA substrates to explore the effects of topology, in a manner somewhat decoupled from substrate compliance. Unlike PDMS substrates that have a uniform, glass-like topology regardless of stiffness, PAA substrates demonstrate a decrease in pore size with increasing stiffness. In our study, we were able to observe that the expression of α-SMA on PAA substrate increased with increasing stiffness, hence with decreasing pore size. The different responses to PDMS and PAA substrates indicate that substrate topology is an important factor in tenocyte differentiation into myofibroblasts. Further experiments will be required to determine the degree to which topological effects interact with substrate mechanics. In any case, the interface between the cell and the substrate seems to play an important role in tenocyte differentiation. With increasing pore size, the anchoring sites of the collagen ligands become

relatively more distant. As collagen is a semi-flexible polymer, the strength of adhesion and the mechanical feedback that cells sense, decreases rapidly with increasing lengths of non-anchored collagen (21). In this study, we modified the concentration of sulfo-SANPAH permitting control over ligand anchoring and mobility without affecting the amount of substrate bound collagen, as described by Trappmann (21). Our results showed that the differentiation of tenocytes into myofibroblasts was positively influenced when the collagen ligands were more tightly anchored to the substrate. This indicates that the effect of the density of tethering sites can heavily interact with effects of substrate stiffness and pore size. We thus conclude that tenocytes exert a mechanical force on collagen fibers and gauge the feedback to make cell-fate decisions.

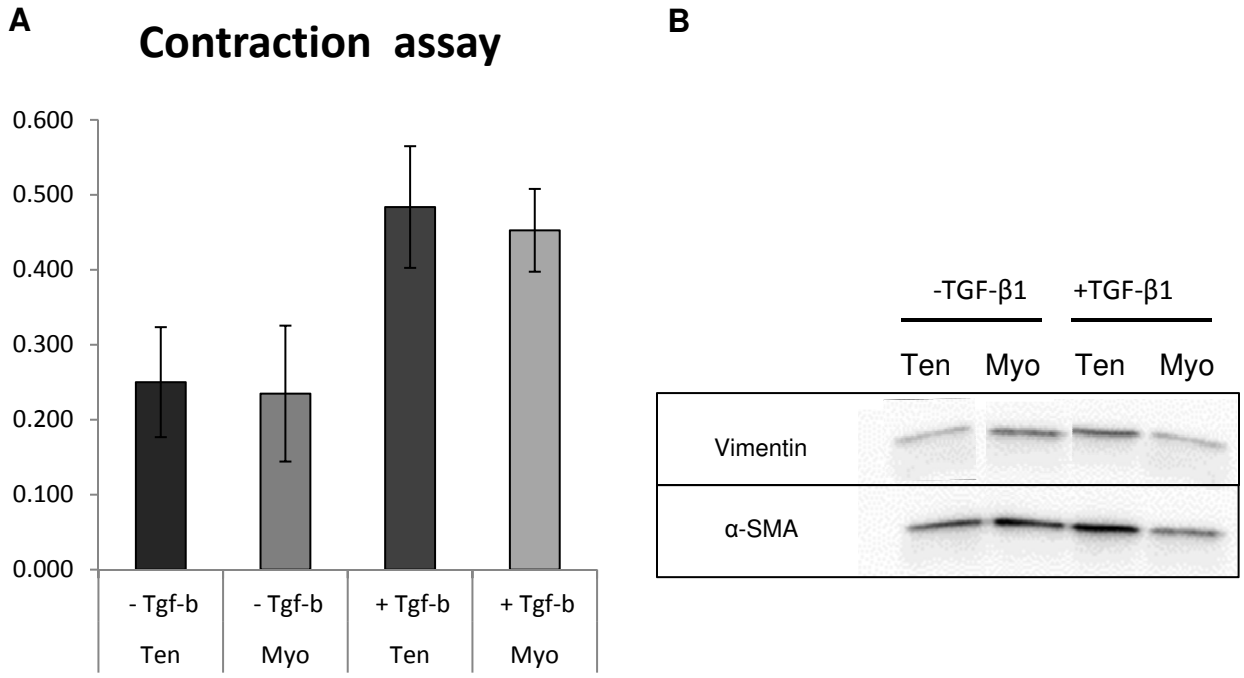

Figure 7: (A) Graph illustrating the contraction of tenocytes (Ten) or tenocytes previously differentiated into myofibroblasts (Myo) seeded in collagen gels for 5 days and cultured in expansion medium containing or not 5ng/ml of TGF-β1. The values were obtained by dividing the area of the gels after contraction by the area before release (n=6). (B) After the contraction assay was performed, expression of myofibroblast marker α-SMA was assessed together with loading control vimentin, by Western blotting.

After characterizing the effects of substrate mechanical tension on the differentiation of tenocytes into myofibroblasts, we were interested in elucidating the role of TGF-β1 in promoting the morphological and functional differentiation of tenocytes into myofibroblasts. Our data showed increasing tissue (RTTF) contraction with increasing concentration of supplemented TGF-β1, supporting the hypothesis that TGF-β1 is involved in the functional differentiation of tenocytes. We also demonstrated that adding TGF-β receptor 1 inhibitor to the medium effectively eliminated observable RTTF contraction, thereby confirming the role of TGF-β1 in tendon tissue contraction. Consistent with these results, immunohistochemical staining of the tendon fascicles revealed the presence of numerous myofibroblasts in the locally contracted zones of the fascicles, also indicating a potential morphological differentiation of the tenocytes into myofibroblasts. Interestingly, the addition of an MMP inhibitor (Ilomastat) limited the contraction of the RTTFs, although immunohistochemical staining still revealed the presence of α-SMA positive cells. *In vitro*, MMP-3 has been shown to mobilize TGF-β from the extracellular matrix by proteolytic cleavage of the latent TGF-β binding protein (22). However, the limited contraction we observed in our MMP-inhibition experiments cannot be argued as being due to limited release of TGF-β from the matrix, as TGF-β1 was supplemented directly to the medium. Thus MMP inhibition reduced contractility independently of latent TGF-β1 release. MMPs are mostly known for their ability to degrade a wide range of ECM molecules (23; 24). Arnoczky et al. showed that Ilomastat prevents loss of pericelullar matrix integrity when compared to non-treated free-floating RTTFs (25). The tensile modulus of RTTFs treated with Ilomastat was comparable to that of fresh RTTFs while it was significantly higher than that of non-treated free-floating RTTFs after 7 days in culture (25).We propose that these results suggest that Ilomastat could hinder tissue contraction simply by maintaining a baseline mechanical integrity of the ECM that prevents its folding under cellular contraction. While little α-SMA expression itself may provide only limited ability to contract intact tendon tissue matrix, our results indicate that this ability is nonetheless augmented with increasing TGF-β1 concentration, which consequently increases α-SMA expression. This conclusion is consistent with previous studies showing that TGF-β1 induces an increased expression of α-SMA (6), and that α-SMA expression upregulates fibroblast contractile activity (26). Taken together, these results indicate that TGF-β1-promotes functional differentiation of myofibroblasts, and that this occurs in a dose-dependent manner.

We also investigated whether tenocytes that had been pre-differentiated into myofibroblasts would maintain their phenotype, and exhibit higher contractile abilities than non-differentiated tenocytes. While Western-blot analysis of α-SMA expression confirmed that pre-differentiated

tendon myofibroblasts did not de-differentiate, collagen gel contraction assays revealed no differences in contractility between the two cell types. These results are in accordance with those of Hinz et al., who showed that there was no difference in contraction between α-SMA positive cells and α-SMA negative cells cultured on substrates of medium stiffness (26). Only on substrates of high stiffness could a clear difference be observed. In our study, adding TGF-β1 to the culture medium increased the contraction of the gels for both populations, but no clear difference could be observed between the two cell populations. Western blots showed no difference in α-SMA expression between the two cell populations, suggesting that previously undifferentiated tenocytes had differentiated into a myofibroblastic phenotype as well. While experiments on higher stiffness gels and tissue explants may provide additional confirmation, our results strongly underline the role of TGF-β1 in promoting the functional and morphological differentiation of tenocytes into myofibroblasts.

In conclusion, we have shown in several experimental systems (i.e. 2D and 3D culture, tissue explants) that *de novo* expression of α-SMA in cultured tenocytes can be elicited by increasing the mechanical tension of collagen ligands (by increasing the density of their tethering sites) and by the addition of TGF-β1 to the culture medium. Myofibroblasts showed no increased contractility on medium stiffness substrates compared to tenocytes. An intact pericellular matrix in *ex vivo* tendon explants due to the influence of Ilomastat also limited their capacity to contract. These findings provide an important baseline for further elucidation of the potentially important role that tendon myofibroblasts may play in healing and tissue degeneration – an aspect that has until now been widely ignored.

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Chapter 8

General discussion

Tendons transmit force from muscle to bone, thereby permitting movement of the whole body. Consequently, any damage to tendons will rapidly have an invalidating effect. Tendon problems account for 30-50% of all sporting injuries [1]. However, tendinopathy affects not only athletes but also the general population. Repetitive movements at work may cause injuries due to tendon overuse. Hundreds of thousands of employees are affected by this pathology which causes significant occupational disability and substantial economic losses. An abundant variety of treatments is currently available, but scientific evidence for their effectiveness is often still lacking [2]. Tendinopathy remains therefore a major frustration for patients as well as for their physicians and conservative treatment in the form of rest is often argued as still being the best treatment after tendon injury [2].

The situation in equine medicine is similar to that of humans. No efficacious treatments are available that achieve full functional recovery of injured tendons and this causes enormous wastage of athletic and often expensive horses, leading to a similar degree of frustration [3-5].

Tendinopathy is believed to be a problem of defective healing. The work in this thesis aimed at improving our knowledge of the fundamental mechanisms of successful tendon healing so as to be able to understand better the reasons for defective healing.

The model

The first step that we have taken for understanding tendon healing has been to create standardized lesions in tendons. The establishment of an appropriate animal model is therefore essential. In chapter 2, we have characterized an existing animal model for tendinopathy. Several attempts had already been made to develop a good animal model for tendinopathy. However, the ideal situation, which would be the creation of a highly standardized animal model which demonstrates the clinical characteristics of natural tendon lesions and which, at the same time, reflects their pathogenesis, is quite difficult to attain in case of tendinopathy. The choice of the appropriate species already raises questions. Many groups have been using rodents [6-14], as they are easy and cheap to keep. However, no naturally-occurring tendinopathy has been observed in rodents. Some other groups have been working with sheep or cows [15; 16]. But here again, these species

are not known to suffer from any form of clinical tendinopathy. The two species that are known to have naturally-occurring tendinopathy that is similar to that observed in humans are dogs (racing grey hounds) and horses [17; 18]. We have opted for the horse, as our research group had previous expertise in tendinopathy in this species and several papers had already shown strong similarities with human tendinopathy [17; 19; 20]. Selecting the means of creating standardized tendon injuries was the second challenge. In order to mimic overuse injuries, challenging the tendons by an intense running protocol on a treadmill would have been the preferred choice. However, this way of creating lesions, even if more etiologically relevant, produces a high degree of variation between animals with regard to the extent of their injuries [21]. Jelinsky et al. have established a running protocol for rats that provoked the occurrence of tendon lesions, but here a second problem arose. The injury was found to heal by itself, giving no opportunity to investigate the efficacy of prospective treatments [13]. Other groups have created irreversible injuries using enzymes such as collagenase [22-26]. Those protocols result in lesions with promising signs of similarity to clinical cases of tendinopathy, but the extent of the injuries is insufficiently uniform and often lesions extend to outside the paratenon [21; 27]. In the end, the model originally developed by Schramme et al. [28; 29], in which a lesion in the tendon core is created artificially by use of an arthroscopic burr (Ø 3.5mm), was selected for our study as it offered the best means of creating highly uniform lesions. The major drawback of this model is the etiological discrepancy with natural tendinopathy. However, it is difficult to elaborate a model which encompasses all of the etiological factors that might play a role in tendon degeneration. For instance, tendinopathy is not always solely the result of overuse and there are also intrinsic and/or extrinsic factors that can have additional effects that lead to defective healing [30-33].

In order to avoid wastage of horses, it is of course important to provide evidence that any model is truly mimicking natural occurring tendinopathy. The important criterion of a good tendinopathic model is that the injuries should comply with the parameters observed in clinical cases. These consist namely of imaging, and compositional, organizational and functional parameters and were verified for the abovementioned model against high standards [21]. Based on our results we were able to conclude that the model is creating injuries similar to those observed in clinical cases [34-36]. Computerised ultrasonographic tissue characterisation revealed a strong hypoechogenicity and an altered longitudinal fibre pattern in the centre of the tendons. This is one of the hallmarks of naturally occurring tendinopathy in horses [37]. At a microscopic scale, hypercellularity, hypervascularity, loss of matrix organisation and rounding of cell nuclei were clearly observed. Furthermore, increased levels of MMp activity as well as an

accumulation of GAGs (glycosaminoglycans) were observed in the core of the injuries. This is similar to what has been characterized in clinical cases of tendinopathy [34; 38]. For instance, the level of collagen and the HP crosslinking in the core of the lesion had not yet reached the level of the adjacent tissue after 6 weeks. Taken together, albeit still being imperfect, all these results indicate that this model demonstrates many hallmarks of naturally-occurring tendinopathy. The main limitation of our study however, lies in the small number of time points of sample harvesting.

The model also appeared to create irreversible injuries, but further investigations into the long-term healing status of these artificially created lesions would be of interest to establish if the injury model is truly irreversible. Mechanical results obtained from previous work in our group from samples harvested 24 weeks after surgery provides encouraging evidence that this might well be the case [39]. This model for tendinopathy, in comparison with other existing models, appears to be the one that most satisfactorily fulfills all the basic requirements that a model for tendinopathy should fulfill. In fact, our attempt to create highly uniform lesions, comparable to those observed in cases of naturally-occurring tendinopathy has been successful and lays the groundwork for further research aiming at a better understanding of the healing mechanism of tendons.

The effect of immobilization

As mentioned earlier, rest is often the prescribed treatment option after tendon injury. In Chapter 3, we have investigated whether forced rest by early cast-immobilization would influence injury propagation in equine tendons. Mechanically induced progressive tearing of fibre bundles can be expected under the influence of weight bearing, although proteolytic enzymes secreted by the cells after the initial injury probably play a more important role in the enlargement of the initial lesion [40-42]. As it is generally accepted that the smaller the lesion, the better the prognosis for good healing will be, prevention of such lesion propagation would be beneficial [43; 44]. Whether early immobilization influences the dispersion of enzymes and prevents tearing of fibres and thus limits propagation of the injury, had not been answered by previous studies. For the study in Chapter 3, the same model that was characterized in Chapter 2 was used again with the lesions in the tendons being monitored using computerized ultrasonography on a weekly basis. In both lower front limbs lesions were created, one was immobilized using a cast which prevented hyperextension of the metacarpophalangeal joint while the other was only bandaged. Tendon

samples were harvested 6 weeks after surgery, at which moment also the size of the lesion was evaluated macroscopically. Our data demonstrated a significant difference in the size of the injury 6 weeks after surgery between the cast limb and the bandaged one. Immobilization with the cast had a stronger protective effect on propagation width (57%) than on length (19%). One possible explanation for this observation could be that the bandage might have loosened over time while the cast maintained a stable pressure on the width of the injury and hence limited the lateral propagation of the injury.

In the control leg the injury enlarged in both directions until 35 days after the surgery, but after that time point the injury size started to diminish. The injury increased relatively more in length than in width, suggesting a prominent effect of mechanical loading most probably combined with proteolytic enzymatic activity. This enlargement might have been influenced by the horses shifting weight onto the bandaged leg, as they were apparently more comfortable putting weight on that leg than on the cast leg. A limitation of this study is the lack of information regarding the healing progress at a cellular or tissue level. Stress deprivation of the tendon resulting from cast-immobilization could potentially have detrimental effects on the cells inside the tendon. Stress-deprivation in tendon fascicles has been shown to lead to apoptosis and increased catabolic activities [45; 46]. Therefore, the potential detrimental effects of immobilization need to be investigated before clinical use can be advised. Nonetheless, this study confirmed our hypothesis that cast-immobilization would significantly reduce lesion propagation in the SDFT of horses and hence might potentially improve the prognosis of tendon injuries.

PRP: a promising treatment for tendinopathy

In Chapter 4, the model described above was used to assess the effects of a possible new therapy for tendon injuries; namely Platelet Rich Plasma (PRP). PRP is an autologous concentrate of blood platelets which are known to play a crucial role in the cascade of tissue healing by delivering growth factors to the site of injury [47]. Upon activation, platelets release growth factors from their α-granules such as platelet-derived growth factor (PDGF), transforming growth factor (TGF-β), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF)-1 [48]. In the study that we undertook, either PRP or saline solution was injected into the lesion that had been created in the front legs of 6 horses, at 7 days after surgery. Twenty-four weeks later, the tendon samples were harvested for biochemical, biomechanical and histological evaluations. Compared to the saline treated tendons, those that had been treated with PRP showed

a better healing trend with a better structural organization of the collagen network, as well as signs of increased metabolic activity with a higher DNA content and a higher content of GAGs and collagen. Importantly, the strength at the point of mechanical failure and the Elastic Modulus were also significantly increased in the PRP-treated tendons compared with the placebo-treated samples (although still remaining inferior to healthy tendon tissue). The exact mechanism of action of PRP remains complex, as it is a cocktail of many growth factors which might have direct effects on cells and/or indirect effects on other growth factors. The levels of TGF-β and PDGF were clearly elevated in the PRP solution and both have known positive effects on tendon healing [49; 50]. The most noticeable change in PRP-treated samples was the increased DNA content, as GAG and collagen content expressed per DNA was similar in PRP-treated and placebo-treated limbs, indicating a lasting effect of PRP on cell proliferation and/or migration. The cells in the tendon had restructured the newly synthesized collagen and GAGs leading to a better structural organization of the injured tendon. The effects of the cells on structural integrity might explain the better mechanical values obtained for the PRP treated samples compared with the placebo treated samples. Taken together, these results suggest a promising future for PRP treatment and suggest that other treatments promoting cell proliferation may be beneficial as well, as the cells are the key players in tendon injury. It remains to be seen if the creation of a compartment is necessary to retain PRP solution inside the lesion. Tendons are naturally dense tissues and inserting any additional volume can not only be painful, but the solution may also easily leak out through the puncture site. Further, the moment of treatment might also play an important role. In our study, the horses were treated one week after surgical injury. However, in many clinical cases, patients are visiting medical centers more than a week after injury, at a stage when tendinopathy has reached a chronic phase. Both factors may have played a role in the study by de Vos et al., in which PRP treatment was found to have no additional effect to eccentric training in chronic human Achilles tendinopathy [51].

Rescue from traditional Chinese medicine?

In chapter 5, we wanted to investigate the potential of another therapeutic drug, a naturally occurring organic compound derived from the fruit of the Gardenia plant (Jasminoides, Rubiceae) [52] which is frequently used in Chinese medicine, namely Genipin. Genipin is a natural cross-linker which has already shown promising results in a range of clinical applications including heart valves [53], pericardial patches [54], conduits for nerve growth guidance [55], scaffolds for

tissue-engineered cartilage [56], and decellularized tracheal transplantation [57]. More generally, the compound augments the strength and degradation resistance of collagen-based gels [55; 58-60]. Previous work from our group had showed that Genipin could effectively improve the mechanical properties of severely injured tendon samples [61]. In our study, we wanted to evaluate the cytotoxicity of Genipin at concentrations where mechanical improvements were induced. To assess cell toxicity and possible changes in metabolic activity, live/dead Alamar blue staining and migration assays were performed, as well as real time PCR. The effects of Genipin on tendon cross-linking were measured by colorimetry, differential scanning calorimetry and ordinary mechanical testing. Based on our results, we could say that concentrations above 1mM of Genipin are toxic to tenocytes in culture and lead to decellularization of the explants. Those results indicate that tenocytes have similar tolerance to Genipin as other cells like chondrocytes and osteocytes [62; 63]. Concentrations above 5mM appeared to be necessary to attain mechanical improvements; however at those concentrations most of the cells died. Our results in chapter 4 (PRP study) clearly illustrated the role and importance of cells in injured tendons and it is hard to believe that a decellularized tendon will be able to heal. As the migration of the cells is also inhibited at high Genipin concentrations, chances of cells migrating from the periphery to the injured site are few. Although not promising, the results of our study do not fully discredit the potential use of Genipin in tendon healing. Mechanical testing of horse tendon samples is highly dependent on the cutting of the sample and therefore variation among samples is high. Small mechanical changes might have been obscured by this high variability and further testing on tendon fascicles with less variability in mechanical properties might demonstrate an effect of Genipin at concentrations that are not toxic for the cells. Another avenue would be investigating the mechanical effects of multiple low dosages of Genipin. However, there may be other unwanted effects of Genipin, as the compound might not only cross-link the collagen molecules but also some other molecules that are important for tendon healing, such as growth factors like TGF-β. Our RT-PCR data already showed that with increasing concentrations of Genipin, the cells were producing more matrix metalloproteinases (MMPs). MMPs are important in matrix remodeling, but also involved in TGF-β release from the matrix [64]. On the other side, TGF-β is an inhibitor of MMPs [65]. Cross-linking important molecules in a healing tendon might lead to imbalance of the healing process. Furthermore, changing the extracellular environment of tendon cells may have serious consequences, as previous work has shown that stiffness or compliance of the matrix can cause differentiation of the cells towards another lineage than tendons [66]. Taken together, the potential for Genipin as a therapeutic drug for tendon injuries seems limited.

Intrinsic healing or extrinsic healing, that is the question

As demonstrated in chapter 4, the cells are key players in tendon healing. Tendon cells are able to remodel the tendon matrix, synthesize new collagen and improve overall structural integrity of the tendon after injury, leading to better mechanical properties. However, as mentioned earlier, tendinopathy is believed to be principally a problem of defective healing. This means that for some reason the cells are not, or insufficiently, responding to the new situation in a proper manner. In Chapter 6, we wanted to investigate if the cell population from the tendon core (intrinsic healing) had better healing potential than cells from the peritenon (extrinsic healing). We also wanted to investigate the potential role of these cells of different origin in defective healing. In order to do so we performed RT-PCR, Western-Blotting, histology, migration assays, replication assays, colony assays and differentiation studies.

We observed higher tenogenic marker expression in the cell population from the tendon core compared to the cell population from the peritenon, but measurements of replication rate, migration rate and collagen production all indicated a faster reactive potential for cells from the peritenon. These results suggest that the peritenon cell population has a higher healing potential compared to the cell population from the tendon core. The results of the differentiation studies indicated that there were more stem/progenitors cells in the cell population from the tendon core compared to the cell population from the peritenon. However, the cells from the peritenon were clearly more reactive, formed larger colonies and demonstrated a higher differentiation potential towards osteogenic and adipogenic lineages. Highly reactive cells are needed to realize fast and appropriate healing. The downside is that those cells seem to have also non-negligible abilities to provoke defective healing through ectopic differentiation. In our experiment using *ex vivo* tendon explants, we demonstrated the importance of a well-structured matrix in preventing adverse differentiation. As myofibroblasts are known to play an important role in wound healing, but also in permanent scarring, myofibroblast differentiation was investigated as well. Here it was again the cell population from the peritenon that showed a stronger potential to differentiate towards myofibroblasts. This difference was even more outspoken on fibronectin substrate compared to collagen substrate, indicating a high plasticity and sensitivity to the extracellular environment of these peritenon-derived cells.

For all experiments, cells were used that had been isolated from clearly distinct locations. However, the differentiation studies showed that not all the cells differentiated to the same extent, indicating the presence of subpopulations inside our samples. The cell population from the tendon core most probably contained cells from the endotenon and also the peritenon-derived population will not have been entirely homogeneous. This is a weakness of the study and further cell characterization, for instance by cell sorting, would be necessary in possible follow-up studies. Knowing whether the stem/progenitor cells are coming principally from the endotenon or the tendon core would be relevant for further studies. Unfortunately the number of tenogenic markers is still limited and the most frequently used markers scleraxis and tenomodulin have been detected in other tissues as well [67; 68].

Overall, the study demonstrates an unexpectedly large plasticity of both tendon-derived and peritendinous cells and shows clear differences between the two cell populations. As the peritendinous cells (extrinsic healing) are more reactive, they might be the driving force in tendon healing but can also play a more prominent role in aberrations in tendon healing.

Myofibroblasts: another fate for tenocytes.

The plasticity of tendon derived cells has been demonstrated in Chapter 6. Tendon cells are able to differentiate into osteocytes, adipocytes and also myofibroblasts. With their strong contractile abilities, myofibroblasts are known to play a substantial role in wound healing, but in excess this contractility can lead to hypertrophic scarring and impairment of function. In tendons, myofibroblast presence has been noted after partial rupture [69; 70] and after intense treadmill running exercise [71]. In Chapter 4, the importance of TGF-β1 in tendon healing was discussed. TGF-β1 is often found in healing tendons up to 28 days after injury [72]. The resulting increase in matrix tension after injury in conjunction with the action of TGF-β1 is believed to be a prerequisite for *de novo* expression of α-SMA [73-75]. The tendon matrix is a complex structure which, as shown in chapter 6, may prevent differentiation of tenocytes into other lineages. In Chapter 7, we have attempted to investigate how the mechanical environment of the cells is able to elicit or prevent differentiation of tenocytes into myofibroblasts. Secondly, we were also interested in understanding the role of TGF-β1 in promoting the morphological and functional differentiation of tenocytes into myofibroblasts. For this study, we seeded tenocytes on polydimethylsiloxane (PDMS) or polyacrylamide (PAA) substrates having differing stiffness. We also varied the density of anchoring sites of the collagen ligands by increasing the concentration

of sulfo-SANPAH, the cross-linker of collagen to the substrate. Next, we investigated the role of TGF-β1 in contracting rat tail tendon fascicles (RTTfs) by using different concentrations of TGF-β1 and inhibitors of TGF-β1 receptors. Finally, we tested the contractile abilities of myofibroblasts compared to tenocytes in a preserved matrix of RTTFs and in collagen gels of medium stiffness. Our results were in concordance with published results from other studies [76; 77] and showed that also for differentiation of tenocytes to myofibroblasts, mechanical tension of the ligands is required. Our results showed that varying the stiffness of the PDMS substrate did not increase the expression of α-SMA on its own. However, increasing the stiffness of PAA substrate, which in turn decreases the pore size of the substrate, led to an increased expression of α-SMA. Furthermore by increasing the density of the anchoring sites of the collagen ligands on a PAA substrate of a similarly large pore size, an increased expression of α-SMA was observed. Taken together these results indicate that the tight tethering of collagen ligands overrules substrate stiffness and porosity in affecting the differentiation of tenocytes into myofibroblasts. Myofibroblasts are specialized contractile cells but without any hard structure to adhere and pull on, they have no purpose. This might explain why cells do not differentiate into myofibroblasts when these conditions are not met.

Our results on the role of TGF-β1 were also in concordance with previous work [75] and we were able to confirm that TGF-β1 promotes the functional and morphological differentiation of tenocytes into myofibroblasts. Our results demonstrated that there was increased contraction of the RTTfs with increasing TGF-β1 concentration. These contractions were prevented by the addition of TGF-β receptor 1 inhibitor to the culture medium. Immunohistochemical staining of the samples treated with 5ng/ml of TGF-β1 revealed the presence of numerous myofibroblasts in the bent area of the RTTfs, indicating a clear role of myofibroblasts in the contraction of the RTTFs. Interestingly, the addition of Ilomastat, an inhibitor of MMp activity, limited the contraction of the RTTFs. When the extracellular matrix is protected from digestion by the addition of Ilomastat [78], the efforts needed from the myofibroblasts to contract the tissue are considerably larger. It was interesting to note that increasing the concentration of TGF-β1 in the medium increased the level of contraction of the RTTfs. This confirms previous work [74; 79] showing that increasing the concentration of TGF-β1 increases the expression of α-SMA which in turn is directly linked to the contractile abilities of the cells. To further test the contractile abilities of myofibroblasts in comparison with tenocytes, the contraction levels were measured for collagen gels seeded with either tenocytes or tenocytes that had previously differentiated into myofibroblasts. No clear differences were observed. These results could not be attributed to

dedifferentiation of the myofibroblasts as the level of α-SMA was clearly higher for the previously differentiated myofibroblasts on Western-blots. Hinz et al. have also shown that myofibroblasts and fibroblasts had no noticeably different contractile abilities on substrates of medium stiffness [79]. Overall, this study indicates a clear role for TGF-β1 in promoting functional and morphological differentiation of tenocytes into myofibroblasts. However, the mechanical properties of the matrix may play a predominant role in controlling the contraction of myofibroblast. These results bring interesting insights for tissue engineering. If the ideal level of TGF-β1 for differentiation cannot be decided upon, the mechanical properties of engineered material can be easily optimized to prevent the differentiation of tenocytes into myofibroblasts, thereby preventing the formation of extensive scarring and thus the consequential detrimental effect of making the mechanical properties of the tendon much more heterogeneous.

Overall conclusion:

Tendinopathy is a complex problem that needs to be tackled from different angles. In this dissertation an attempt has been made to clarify, explain, compare and characterize some of the processes that accompany the pathology and healing mechanisms of tendons. The major overall conclusion is that the cells are the uncontested key players in these processes. The cells residing in the tendon or around it in the peritenon have enormous potential to adapt to different situations. These residing cells are playing key roles, both in maintaining normal tendon homeostasis and in the normal and sometimes abnormal healing processes of tendons. The structure, composition and mechanical properties of the ECM are also important in the overall function of the tendon. However, if one wants to understand why tendinopathy exists, the focus should definitely be on the cells.

Tendon research in perspective:

Research on tendons started at a very slow pace with less than 10 publications per year until 1945 (Fig.1). Like other fields of orthopedic medicine, interest in tendon research increased after World War II (Fig.2). The perspective of life after the war had changed. Average life expectancy

in the US was up to 70 years by 1960 from only 63 in 1940. As most infectious and parasitic diseases were under control at that stage in developed countries, attention could shift to the less acutely lethal chronic/degenerative diseases [80], explaining the increase in publications on tendons from 1950 onwards. However, unlike cancer or cardiovascular disease, tendon disorders are not life-threatening for humans and therefore investigations in this field remained relatively limited for a long period. A sudden acceleration in publication rate on tendons came in the late 1960s. With increased recreational time, as well as increasing numbers of workers involved in repetitive mass production, the number of patients suffering from tendon problems increased. This societal demand led to a four-fold increase of the number of publications on tendons between 1980 and 2010.

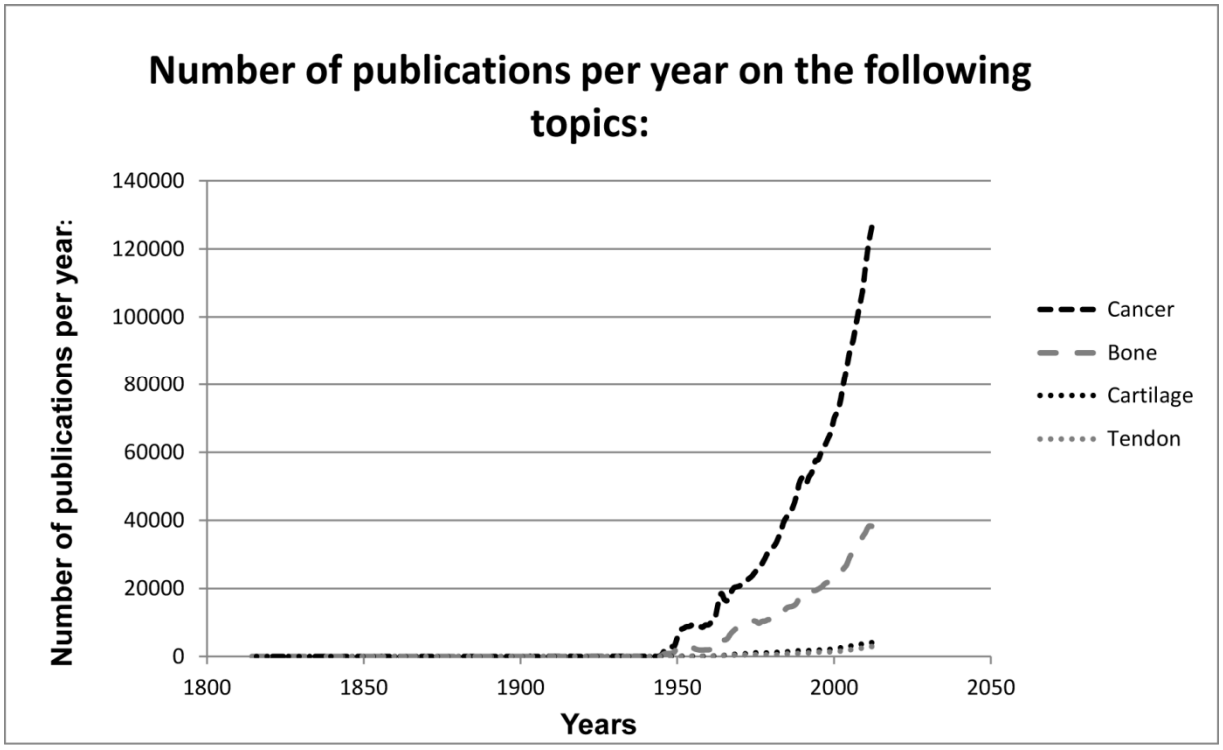

Figure 1: A comparison of the number of publications overtime in tendon, cancer, cartilage and bone research. The number of publications for these topics started to rise around 1950. Publications on tendon are limited compared to publications on cancer and bone.

As no fully satisfying treatment options have been discovered so far, the exponential rise in tendon publications is expected to continue for the coming years. Another important factor is the dramatically and world-wide increasing incidence of diabetes, one of the etiological factors leading to tendon disorders (Fig. 2). Tendinopathy can therefore indeed become a major concern

worldwide. If the current exponential trend in tendon publications is maintained, there could be over 40,000 scientific publications on tendons released in the year 2050. We are not that far yet. At present, tendon research is still considered as a niche subject by many scientists and the pharmaceutical industry. The total number of published studies (from 1841 to 2012) in tendon research was equal to 2% of that in cancer research (55,061 versus 2,716,730). There is a nice side to this "underdog position" for the scientific community. Much basic knowledge on tendon biology is still lacking. Consequently, the possibility to make major discoveries or breakthroughs with a high impact still exists. Furthermore, the clinical relevance of tendinopathies has now been well recognized and the number of current and future patients is starting to justify investments in this area by pharmaceutical companies. Therefore, from the scientist's perspective tendon research may have a very bright future.

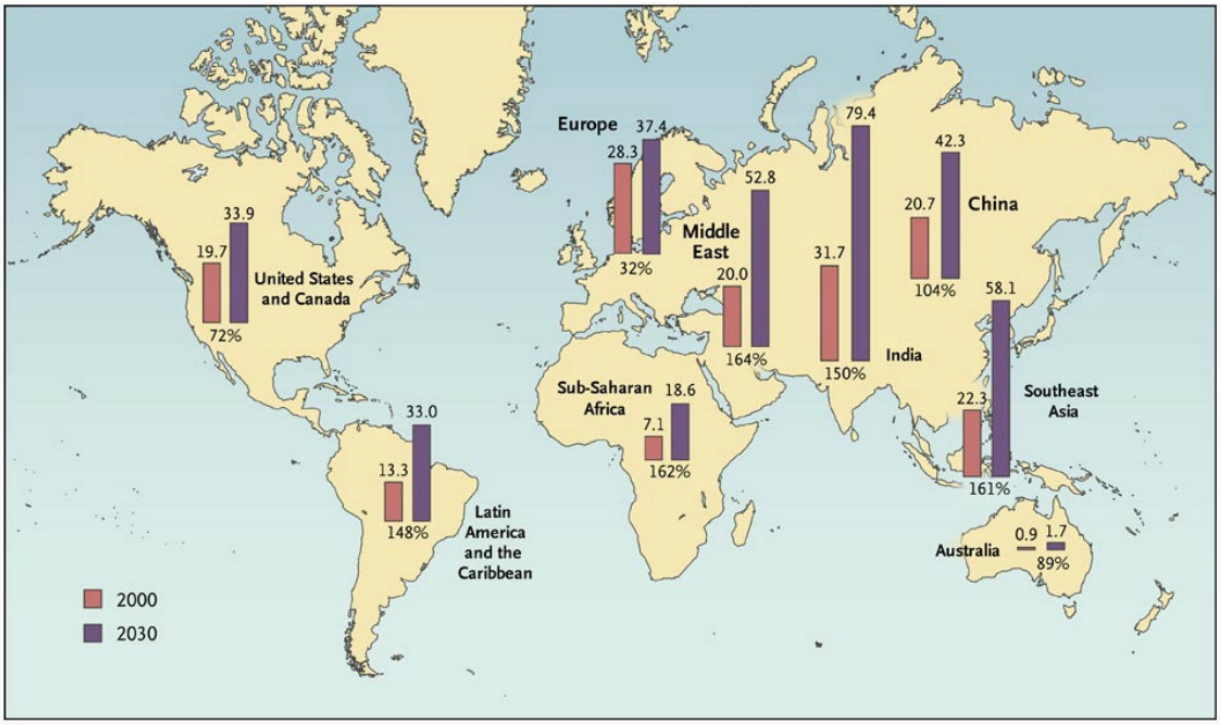

Figure 2: Worldwide prevalence of diabetes in 2000 and estimates for the year 2030 (in millions). (Source: International Chair on cardiometabolic Risk adapted from Hossain P et al. N Engl J Med 2007[81])

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Summary

Tendons are connecting bones to muscles and are therefore important for the movement of the whole body. Tendon problems are frequent among horses and humans as they both have athletic abilities. Repetitive exercise frequently leads to overuse injuries in tendons but other factors like excess bodyweight, diabetes and age are also known to contribute to tendinopathy. Although tendon injuries are rarely lethal, they may become rapidly invalidating. Currently, many treatment options exist for tendon disease. These vary widely and range from cooling to acupuncture, which in itself is probably indicative of the absence of really effective treatments on the market today. The number of reported cases of tendon injuries has increased enormously during the last decades and will most probably keep growing, as obesity, diabetes and aging establish themselves further as critical health issues for the coming generations. If the number of people concerned by tendinopathy has increased strongly, we note with satisfaction that the number of people investigating tendon problems and making important contributions is also rising.

In this thesis, we have tried to tackle the subject of tendon injuries through different angles. In the first study, we describe a model for tendinopathy in the equine superficial digital flexor tendon; a tendon which is often injured in race horses and which for many points is comparable to the human Achilles tendon. In order to test the efficacy of different treatment modalities it is of foremost importance to have a well characterized model which mimics the clinical signs of naturally occurring tendinopathy and which creates standardized lesions to the tendon. This equine model met all the important criteria and therefore has interesting potential for further studies.

In chapter 3, we investigated whether casting and hence preventing loading of an injured tendon would be beneficial. It is widely accepted in veterinary sciences that the smaller the lesion the better will be the healing prognosis. By casting only one of the two front legs of horses that had equivalent tendon injuries in both front legs, we could monitor the lesion propagation and thereby obtain clear indications of the effect of casting. We found that the lesion in the cast-protected leg was smaller. This interesting result might have some influences on how veterinarians treat tendon injuries in horses in the future.

In chapter 4, we used the model described above to test the efficacy of a new and popular treatment, namely PRP. PRP stands for platelet rich plasma and it is made directly from the patient's own blood. This has evident advantages. By centrifuging the blood, it is possible to isolate the platelets which contain many growth factors. Growth factors are known to improve

healing of different tissues throughout the body. A concentrate of one's own healing molecules sounds like a promising treatment for tendon injuries. The repair tissue from the tendon treated with PRP did indeed show better structure and better mechanical properties than that from the tendon treated with placebo. A similar study was performed blinded in humans suffering from long-term tendinopathy but unfortunately similar improvements were not observed. The different results might be explained by the fact that tendon lesions in the horse were acute and not chronic, as were those in the human subjects. The artificial lesions in the horse may have been creating cavities inside the tendon where the PRP solution could be easily injected and maintained, unlike in the human cases.

In chapter 5, we have investigated the potential for genipin, a Chinese medicine, to heal injured tendons. Genipin is a natural cross-linker which means that it may restore the mechanical properties of injured tendons and hence enable them to support higher loads. One should, however, not forget that the elastic properties of tendons are at least as important as their load-supporting properties. The intention of this study was to find a concentration of genipin at which a majority of cells would survive and at which evident enhancements of the mechanical properties could be observed. Genipin is a strong cross-linker and the cells were only able to survive its effects at very low concentrations. Unfortunately, at these low concentrations no clear mechanical improvements could be observed. Our results on the effects of genepin on tendon cells are in accordance with what had already been observed on cartilage and bone cells.

In chapter 6, instead of looking at the effects of different external drugs that could be applied on injured tendons, we focused our attention on the healing capacities of the cells themselves. To this end, we compared the healing capacities of two cell populations; one from the tendon core (intrinsic healing) and one from the thin sheaths surrounding the tendons; namely the peritenon (extrinsic healing). The cells from the peritenon showed *in vitro* a higher reactive potential for tendon healing than the cells from the tendon core. The cells from the peritenon on the other hand showed an increased propensity to differentiate towards bone cells or myofibroblasts; cells that are associated with permanent scarring. In this study, we have also illustrated the importance of a well-structured matrix in preventing undesirable differentiation of the cells inside tendon explants.

In chapter 7, we have been looking in closer detail to the differentiation of tendon cells into myofibroblasts and their subsequent contraction. In this study, we could demonstrate that the differentiation of tendon cells into myofibroblasts was influenced by the matrix stiffness and by

the tethering of the collagen ligands on the matrix. The different contraction assays that we performed also revealed the stimulating effect of transforming growth factor beta 1 on cell contraction.

Tendon problems are a result of defective healing. In this thesis, we have tried through different angles to improve tendon healing (immobilization, PRP and Genipin) and also we have attempted to increase our understanding of what could be the causes of this defective healing.

Samenvatting

Pezen vormen de verbinding tussen de benige delen van het skelet en de spieren en zijn daarom van cruciaal belang voor de voortbeweging van het lichaam. Peesproblemen komen frequent voor bij zowel paarden als mensen, vooral wanneer er sprake is van athletische activiteiten. Vaak herhaalde bewegingen kunnen leiden tot laesies tengevolge van overbelasting, maar andere factoren zoals overgewicht, suikerziekte en veroudering zijn ook van belang bij het ontstaan van peesaandoeningen. Hoewel peesaandoeningen zelden of nooit fataal zijn kunnen ze wel tot ernstige invalidering leiden. Momenteel bestaan er vele behandelingen voor peesproblemen. Het palet aan gangbare behandelingen is groot en zeer divers, variërend van sterke afkoeling tot de toepassing van acupunctuur. Deze grote verscheidenheid in therapieën geeft feitelijk al aan dat er op het moment geen werkelijk effectieve behandelingen zijn. De incidentie van peesaandoeningen is enorm toegenomen gedurende de afgelopen decennia en het valt te verwachten dat deze groei verder door zal zetten, gezien de wereldwijde toename van het belang van overgewicht, diabetes en veroudering in de gezondheidszorg. Gelukkig neemt, samen met het aantal mensen dat lijdt aan peesaandoeningen, het aantal peesonderzoekers ook sterk toe.

In dit proefschrift wordt een poging ondernomen om het probleem van peesaandoeningen vanuit verschillende hoeken te benaderen. In het eerste hoofdstuk beschrijven we een model voor peesaandoeningen dat gebruik maakt van de oppervlakkige buigpees van het paard. Dit is een pees waarin frequent laesies voorkomen, vooral bij renpaarden, en die in veel opzichten goed vergelijkbaar is met de menselijke Achillespees. Voor het testen van de effectiviteit van verschillende behandelingsmogelijkheden is het van groot belang de beschikking te hebben over een goedbeschreven model dat sterk lijkt op natuurlijk voorkomende peeslaesies en waarin de laesies die gecreëerd worden goed gestandaardiseerd zijn. Dit specifieke model bij het paard voldeed aan alle belangrijke eisen en is daarom zeer interessant voor verdere studies op het gebied van de behandeling van peesletsels.

In hoofdstuk 3 hebben we onderzocht of het immobiliseren van het onderbeen, en dus het sterk reduceren van de belasting van de aangetaste pees, een gunstig effect zou kunnen hebben. Het is namelijk bekend dat de vooruitzichten op goed herstel van een peesletsel beter zijn naarmate het letsel kleiner is. Door in de pezen van beide voorbenen een laesie aan te brengen en één daarvan te immobiliseren konden we het effect van immobiliseren goed in kaart brengen. De laesie in het geïmmobiliseerde been bleek inderdaad minder te vergroten nadat deze aangebracht was en dus uiteindelijk kleiner te blijven. Dit interessante resultaat kan gevolgen hebben voor de manier waarop dierenartsen acute peesletsels bij paarden in de toekomst behandelen.

In hoofdstuk 4 is het bovenbeschreven model gebruikt om de werkzaamheid van een nieuwe en populaire behandeling te testen, namelijk die van PRP. PRP is de afkorting van *"platelet rich plasma"* en het gaat hier om een product van het eigen bloed van de patiënt waarin het aantal bloedplaatjes (met bijbehorende groeifactoren) sterk verrijkt is. Groeifactoren zijn van groot belang voor het goed verlopen van genezingsprocessen op vele plaatsen in het lichaam. Een sterke concentratie van deze eigen genezingsfactoren van de patiënt klinkt veelbelovend voor de behandeling van peesproblemen. Het bleek inderdaad zo te zijn dat het herstelweefsel van met PRP behandelde pezen een betere structuur had en ook betere biomechanische eigenschappen dan herstelweefsel van met een placebo behandelde pezen. Een vergelijkbare studie uitgevoerd bij mensen die aan chronische peesaandoeningen leden liet helaas niet zo'n positief effect zien. Het verschil kan verklaard worden door het feit dat de peesletsels bij de paarden acuut waren en niet chronisch, zoals in de humane studie. Het is verder waarschijnlijk dat er door het creëren van de laesies bij de paarden compartimenten in de pees ontstaan zijn waar de PRP gemakkelijk ingespoten kon worden en waar het ook enige tijd in aanwezig bleef, terwijl zulke compartimenten bij de chronische humane laesies niet (meer) bestonden.

In hoofdstuk 5 hebben we bekeken of Genipin, een product toegepast in de traditionele Chinese geneeskunde, een positief effect zou kunnen hebben op peesherstel. Genipin is een natuurlijk product dat de vorming van *cross-links* initieert hetgeen betekent dat het potentieel de mechanische eigenschappen van pezen zou kunnen verbeteren en zodoende hun belastbaarheid zou kunnen doen toenemen. Overigens moet men hierbij niet uit het oog verliezen dat de elastische eigenschappen van pezen zeker zo belangrijk zijn als de feitelijke sterkte. Het was de bedoeling van de studie om te zien of er een concentratie van Genipin was die voldoende hoog was om een duidelijke verbetering in de mechanische eigenschappen van peesweefsel te induceren, maar waarbij wel de meerderheid van de cellen zou overleven. Omdat Genipin een krachtige *cross-linker* is kunnen cellen namelijk alleen maar bij lagere concentraties overleven. Helaas bleek het zo te zijn dat er bij deze lagere concentraties geen duidelijk effect op de mechanische eigenschappen van het weefsel was. Onze resultaten betreffende het effect van Genipin op peescellen stemmen overeen met eerder onderzochte effecten op kraakbeen- en botcellen.

Het onderwerp van hoofdstuk 6 was niet het effect van externe factoren zoals medicamenten op peesherstel, maar de herstelcapaciteit van de cellen zelf. In het hoofdstuk worden twee celpopulaties vergeleken: afkomstig uit de kern van de pees (intrinsiek herstel) en afkomstig uit de dunne bindweefselbladen (het epitenon) die de pees omgeven (extrinsiek herstel). De cellen

uit de laatste populatie lieten in een *in vitro* situatie meer potentie voor het bijdragen aan herstel zien. Daarentegen hadden cellen uit de kern van de pees een grotere neiging om te differentiëren tot botcellen of myofibroblasten, cellen die geassocieerd worden met littekenvorming. In deze studie laten we ook het belang zien van een goed gestructureerde matrix voor het voorkomen van ongewenste differentiatie van cellen in pees *explants*.

In hoofdstuk 7 wordt er in detail gekeken naar de differentiatie van peescellen tot myofibroblasten en de door die cellen veroorzaakte wondcontractie. In dit onderzoek hebben we aan kunnen tonen dat de differentiatie van peescellen naar myofibroblasten beïnvloed wordt door de stijfheid van de matrix en de verbinding van het collageen met die matrix. Verder tonen we het stimulerende effect van *Transforming Growth Factor beta 1* aan op de contractie van de cellen.

Peesproblemen zijn het gevolg van onvolledige genezing van peesletsels. In dit proefschrift hebben we geprobeerd vanuit verschillende invalshoeken (immobilisatie, PRP, Genipin) het genezingsproces van peesletsels positief te beïnvloeden en hebben we ook pogingen ondernomen om meer inzicht te krijgen in de mogelijke achterliggende oorzaken van de onvoldoende genezing van peeslaesies.

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Curriculum Vitae

Jennifer Cadby was born in Chênes-Bougeries in Switzerland on the 31st of January 1983. She attended primary and secondary school in that area with the exception of one semester at a specialized school in Normandy, France (Collège équestre de Conches) which provided extracurricular equestrian training leading to her selection for the French dressage team. On returning to Switzerland, she completed her studies and obtained the Swiss Federal Maturity diploma (Section: Science) in 2001. In the same year, she started undergraduate studies at the ETH (Eidgenosische Technische Hochschule, Swiss Federal Institute for Technology) in Zurich in environmental sciences. After having successfully completed the first year (1. Vordiplom Prüfung), she decided to change the focus of her studies towards functional biology at the University of Neuchâtel, Switzerland where she obtained her Bachelor degree in 2005 and her Master degree in 2007 with high distinction. She started her PhD at Utrecht University in the Netherlands in April 2008 completing her thesis research in an interdisciplinary collaboration in the laboratory of Professor Snedeker at the ETH Zurich from March 2010.

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