Response to mechanical loading in healing tendons

Pernilla Eliasson

Division of Orthopaedics
Department of Clinical and Experimental Medicine
Linköping University
Linköping, Sweden

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During the course of the research underlying this thesis, Pernilla Eliasson was enrolled in Forum Scientium, a multidisciplinary graduate school at Linköping University, Sweden.

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“The more you know, the more you realize you know nothing.”

– Socrates
Supervisor
Per Aspenberg
Department of Clinical and Experimental Medicine, Division of Orthopaedics,
Linköping University

Co-supervisor
Anna Fahlgren
Department of Clinical and Experimental Medicine, Division of Orthopaedics,
Linköping University

Faculty opponent
Michael Kjaer
Institute of Sports Medicine Copenhagen, Bispebjerg Hospital, University of
Copenhagen, Denmark

Committee board
Torbjörn Ledin
Department of Clinical and Experimental Medicine, Division of Oto-Rhino-
Laryngologi, Linköping University

Tomas Movin
Department of Clinical Science, Intervention and Technology, Division of
Orthopaedics, Karolinska Institutet

Folke Sjöberg
Department of Clinical and Experimental Medicine, Burn Unit, Linköping
University
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Unloading by botulinium toxin reduced the strength of the healing tendon dramatically... 49
...but short loading episodes increased the strength of the unloaded tendon calluses.  
The loading episodes increased the amount of bleeding in the callus tissue.  
Healing tendons with continuous loading had less expression of inflammatory genes and more of ECM and tendon specific genes than unloaded.  
Similar but different effect on gene expression by one single loading episode.  
The gene expression is regulated up to 24 hours after one single loading episode.  
Unloading by botulinium toxin for 5 days did not have much impact on intact tendons.  
The difference between the healing and the intact tendons, however, comprised more than just strength.  
Myostatin stimulates proliferation.  

Discussion  
Rest between loadings might allow the tendon callus to contract.  
Loading generates more matrix but not necessarily of better quality.  
What is optimal loading?  
Inflammation: good or bad?  
All research has limitations.  

Conclusions and future research  
What's next?  
Take-home message:  

Acknowledgements  
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ABSTRACT

Ruptured tendons heal faster if they are exposed to mechanical loading. Loading creates deformation of the extracellular matrix and cells, which give rise to intracellular signalling, increased gene expression and protein synthesis. The effects of loading have been extensively studied in vitro, and in intact tendons in vivo. However, the response to loading in healing tendons is less known.

The general aim of this thesis was to understand more about the response to mechanical loading during tendon healing. The specific aims were to find out how short daily loading episodes could influence tendon healing, and to understand more about genes involved in tendon healing.

The studies were performed using rat models. Unloading of healing tendons resulted in a weaker callus tissue. This could be reversed to some extent by short daily loading episodes. Loading induced more matrix production, making the tendons thicker and stronger, but there was no improvement in the material properties of the matrix. Lengthening is one potential adversity with early loading, during tendon healing in patients. This was also seen with continuous loading in the rat models. However, short loading episodes did not result in any lengthening, not even when loading was applied during the inflammatory phase of healing. It also appeared as loading once daily was enough to make healing tendons stronger, while loading twice daily with 8 hours interval did not give any additional effect. The strongest gene expression response to one loading episode was seen after 3 hours. The gene expression changes persisted 12 hours after the loading episode but had disappeared by 24 hours. Loading appeared to regulate genes involved in inflammation, wound healing and coagulation, angiogenesis, and production of reactive oxygen species. Inflammation-associated genes were regulated both by continuous loading and by one short loading episode. Inflammation is an important part of the healing response, but too much can be harmful. Loading might therefore have a role in fine-tuning the inflammatory response during healing.

In conclusion, these studies show that short daily loading episodes during early tendon healing could potentially be beneficial for rehabilitation. Loading might have a role in regulating the inflammatory response during healing.

Syftet med denna avhandling har därför varit att öka förståelsen om hur belastningen påverkar den läkande senan, dels genom att studera hur man kan använda korta belastningar som en del av rehabiliteringen, och dels genom att lära sig mer om vad som händer inuti senan vid belastningen.

Vi har studerat hur belastning påverkar senans läkning i en djurmodell. Vi har sett att korta dagliga belastningar på 15 min kan påskynda läkningen och ge en starkare sena. Dessa korta belastningar kunde utföras utan att senan förlängdes. Vi såg också att belastning oftare än en gång per dag inte gav en ytterligare förbättrad läkning. Svaret i senan fanns kvar i mer än 12 timmar efter belastningen, men var borta efter 24 timmar. Vanligtvis anser man att belastning bara stimulerar de senare läkningsfaserna, men vi har funnit att även den tidigaste läkningsfasen påverkas gynnsamt, bl a genom att belastningen inverkar på inflammationen.

Slutsatsen är därför att korta dagliga belastningar kan ge en starkare läkande sena utan att nödvändigtvis riskera komplikationer såsom förlängning av senan. Detta kan potentiellt användas inom rehabiliteringen av senskador för att få en kortare rehabiliteringstid och minskade vårdkostnader.
LIST OF PAPERS

This thesis is based on the following original papers:

I. Andersson T, Eliasson P, Aspenberg P.
   Tissue memory in healing tendons: short loading episodes stimulate healing.
   J Appl Physiol. 2009 Aug; 107(2):417-21

II. Eliasson P*, Andersson T*, Aspenberg P.
    Achilles tendon healing in rats is improved by intermittent mechanical loading during the inflammatory phase.
    Accepted in J Orthop Res

III. Eliasson P, Andersson T, Aspenberg P.
     Rat Achilles tendon healing: mechanical loading and gene expression.

IV. Eliasson P, Fahlgren A, Aspenberg P.
    Mechanical load and BMP signaling during tendon repair: a role for follistatin?

V. Eliasson P, Andersson T, Kulas J, Seemann P, Aspenberg P.
    Myostatin in tendon maintenance and repair.

VI. Eliasson P, Andersson T, Aspenberg P.
    Influence of a single loading episode on gene expression in healing rat Achilles tendons.
    Submitted to J Appl Physiol.

*Equal contribution
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTR</td>
<td>Activin receptor</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>COMP</td>
<td>Cartilage oligomeric matrix protein</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GDF</td>
<td>Growth differentiation factor</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>JAK/STAT</td>
<td>Janus kinase/signal transducer and activator of transcription</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEKK</td>
<td>Mitogen-activated protein/ERK kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>OP-1</td>
<td>Osteogenic protein-1</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>PINP</td>
<td>Procollagen type I N-terminal propeptide</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline and tween-20</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine 5'-triphosphate</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
INTRODUCTION

Sore or painful tendons due to overloading and degeneration are a common cause of morbidity in the general population. The etiology includes lifestyle, loading pattern, biological variables (genetics, age, sex) as well as different pharmacological agents (51). A painful tendon can heal, turn into a chronic degenerative condition or rupture. Tendon ruptures are often preceded by degeneration, even though there are rarely prodromal symptoms (56).

The Achilles tendon has to withstand forces up to 12.5 times the body weight during running (122). Achilles tendon ruptures consequently occur quite frequently. By age and by inactivity, the tendon becomes weaker and may therefore rupture at high loads (51). This is particularly common among middle-aged men who combine a sedentary lifestyle with occasionally intense sporting activities like floorball, badminton, squash etc. Other tendons prone to injuries due to degeneration or high loads are the rotator cuff tendons and the ligaments and tendons in the knee. The population of older individuals is growing, and there is an increased interest in recreational exercise, therefore the incidence of tendon pathology will most likely even continue to increase in the future.

Tendons heal poorly after injuries compared to other connective tissues like skin, muscles and bones. Unloading or immobilization during tendon healing has been shown to be detrimental for the healing process in animal studies (33, 35, 89, 95). A few clinical studies have also shown that early loading can improve the rehabilitation after rupture (26, 59, 88, 110). The effect of mechanical loading in tendon healing is still not fully understood, and the management of tendon injuries is still challenging. Very few studies have investigated the mechanisms behind the improved healing after loading. The purpose of this thesis was therefore to study the mechanism behind the response to loading during tendon healing.
Bundles, cross-links and a few cells

The two main components of the tendon extracellular matrix (ECM) are collagens and proteoglycans (58). The collagen of the tendon is organised in a parallel manner according to the direction of force transmission. The collagen molecules are structurally arranged into fibrils, in an imbricate pattern with cross-links in-between. The cross-links reduce the strain at failure and increase the elastic modulus (122). The fibrils form fibres which creates a strong structure. Fibre bundles are surrounded by a connective tissue, the endotenon, which provides the tendon with blood supply and innervation (58).

Proteoglycans are important for retaining water inside the tendon, but also for the creation of collagen fibrils (61). The water and the proteoglycans may also be important for lubrication and spacing of the tendon. The proportions and the amount of matrix are important for the mechanical properties of the tissue as well as the direction of matrix alignment.

The cells, tenocytes are connected to each other through the matrix and can communicate via gap-junctions (120). The cells are also connected to the ECM and can therefore detect mechanical changes in the surrounding and respond to this. Recent studies have also shown that tendons contain a small amount of tendon stem cells (18). Tenocytes together with tendon stem cells and cells from the surrounding are involved in the healing response in different ways.
Tendons heal by three steps

Tendon healing after rupture is believed to occur through three somewhat overlapping phases (34, 105). First the injury causes bleeding, platelet activation and haematoma formation, which is followed by infiltration of inflammatory cells (e.g. neutrophils and macrophages) (Figure 1). The macrophages remove damaged necrotic tissue and the neutrophils release chemotactic and vasoactive factors. These factors will increase vascular permeability, stimulate angiogenesis, tenocyte proliferation and further recruitment of inflammatory cells. The tendon healing occurs by both extrinsic cells from the blood supply and intrinsic cells from the ruptured tendon and paratenon (57).

The first initial cellular response is followed by a more proliferatory response together with an increased protein synthesis, where fibroblasts proliferate and starts to form new ECM (34, 105). This ECM is of quite poor quality in the beginning, consisting of mainly type III collagen, proteoglycans and water. The callus size increases, and thereby also the mechanical strength of the tissue.

The last phase is dominated by remodelling of the tissue (34, 105). The poor quality matrix is replaced by more organised, better quality matrix, mainly type I collagen. Loading is generally believed to be important during this phase when the thick tendon callus can withstand high strain due to the amount of matrix. The collagen is structurally arranged according to the direction of the forces, and cross-linking increases in the collagen. The tendon callus thereby reduces its size and cellularity, and the material properties start to improve. However, the tendon will most likely never regain the exact same properties as before the injury (122).

Figure 1. Tendons heal by three overlapping phases: the inflammatory phase with cell infiltration (1), the proliferatory phase with matrix production (2), and the remodelling phase for which loading is generally believed to be important (3).
Numerous growth factors synthesized and secreted by cells in the callus are thought to be important during tendon healing. These growth factors include bone morphogenetic proteins (BMPs), connective tissue growth factor (CTGF), epidermal growth factor (EGF), fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), platelet-derived growth factors (PDGFs) and transforming growth factor (TGF)-β \(^{55, 106}\). Some of these growth factors might promote scar-free healing more than others, but this is an ongoing debate.
The strength of the tendon comes from parallel collagen fibres

The tendon fibre bundles are arranged in a crimp pattern in the resting tendons, which protects the fibres from rupture during loading (122). This crimp pattern is stretched out by loading, creating a toe region in a force-distension or stress-strain curve during mechanical testing (Figure 2) (81). The fibres of the tendon can subsequently be stretched out further. Still, the elasticity is limited and at roughly 4% strain of the tendon, micro ruptures starts to occur in the tendon fibres (122). At approximately 8% strain, the tendon ruptures completely. The stretching ability of the tendon differs between species and different tendons (15, 114). The properties of the tendon can be described by a number of mechanical parameters: Force, stiffness, strain, stress, elastic modulus and energy uptake (81). Stiffness describes the relationship between the force and the deformation of the tendon. Strain describes the deformation of the tendon and is depended on the length of the tendon. Stress is the force of the tendon divided by the cross-sectional area and thereby describes the material properties. Also describing the material properties is the elastic modulus, this is the stress divided by the strain. Energy uptake describes how much energy the tendon can store, and is calculated by the area under the force-distension curve.

Figure 2. Tendon force–distension curve (left) and stress–strain curve (right). The curves show the mechanical properties of the tendon, force and stiffness, and the material properties of the tendon, stress and elastic modulus.
INTRODUCTION

Tendons also have viscoelastic properties, allowing them to be more deformable at low strain rates (122). The tendons can thereby absorb more energy but transfer less load at low strain rates compared to high strain rates (122). The viscoelastic properties are important for dynamic interactions between the tendon and the muscle and for energy storage (83). Viscoelasticity is defined by hysteresis, creep and stress-relaxation.
Forces are transferred to the cells though matrix-cytoskeleton connections

Forces generate deformation of the tendon matrix and cells. This initiates an intracellular response with increased transcription of genes and protein synthesis. The response is called mechanotransduction. Cells in different tissues detect load in a similar fashion, however the outcome is depending on the cell type and the mechanical demands of the tissue (16). Stress in the ECM is transferred to the cytoplasm and the cytoskeleton via ion-channels, integrins, receptor tyrosine kinase (RTK), g-proteins, second messengers, mitogen-activated protein kinases (MAPK), janus kinase/signal transducer and activator of transcription (JAK/STAT) and mitogen-activated protein/ERK kinase kinase (MEKK) 3/6 cascades (16, 101, 122, 128). It initiates both rearrangements of the cytoskeleton and intracellular signalling pathways.

The most rapid response to loading probably involves ion-channels (16). These ion-channels can be coupled to the cytoskeleton. Loading activates stress sensitive ion-channels and thereby influx of extracellular Ca\(^{2+}\) and release of intracellular Ca\(^{2+}\) storage to the cytoplasm (54, 119). Deformation of a cell membrane, by for example indentation, shear stress or tension, can induce a rapid increase in intracellular Ca\(^{2+}\) (16). This response also probably involves an increase in inositol trisphosphate (IP\(_3\)) and diacylglycerol (DAG), which are intracellular messengers involved in calcium signalling. The calcium-signalling can be transferred to the surrounding cells (in a 7-10 cells radius) via gap-junctions and IP\(_3\) transfer.

Integrins and cadherins in the cell membrane are linked to both intracellular proteins as well as the ECM and they regulate intracellular signalling pathways (101, 122). Focal adhesion points are clusters of matrix-integrin-cytoskeletal components which contain multiple proteins like focal adhesion kinase, c-Src (a mechanosensitive kinase) and different members of the cytoskeleton (16, 101). These focal adhesions are usually concentrated at cell adherence sites, and loading can rearrange both the shape and distribution of them (16, 98, 101). Conformational changes in the focal adhesions and especially in the kinases of these complexes can induce autophosphorylation and a rapid signalling cascade initiation via for example c-Jun N-terminal kinase (JNK) (16, 101). This cascade can be regulated by modulators like g-proteins, inhibitors or phosphatases (16, 122). Inhibition of the kinases in the
focal adhesion points blocks down-stream events like MAPK activation and cell cycle progression (101). The exact functions of the focal adhesion proteins are not entirely known. However, different types of mechanical stimuli are believed to trigger these proteins. Cadherins might also have a role in mechanotransduction, by adhering cells to each other and to the ECM, and activation of intracellular signalling. There is also probably much more to know about the role of cadherins in mechanotransduction.

The cells respond to loading in everything from rapid changes (milliseconds) to longer changes (minutes-hours-days) (16). The rapid changes include responses like activation of ion channels (Ca2+, Na+, K+, H+), second messengers (IP3, cAMP, cGMP, prostaglandin-E2, DAG), kinases (RTKs, NRTKs), g-proteins etc. The subsequent response includes kinase signalling (SHC, SOS, GRB2, raf-ras, MEK, ERK), transcription (c-fos, jun other transcription factors), translation (fos, jun, other transcription factors, cyclins, CDKs), cytoskeletal changes and rearrangement of focal adhesions. The more long term changes have mainly an effect on the basal stress state, cell division, apoptosis, migration etc.

Cells are also connected to the ECM by tight- and adherens-junctions and they can communicate with each other via gap-junctions (62). The adherence junctions consists of cadherins and catenins (α- and β-) and they are connected to the actin in the cytoskeleton. The gap-junctions are suggested to be involved in the mechanotransduction (79). They mainly consist of connexins and they allow the cells in a tissue to respond in a synchronized way to both chemical and electrical signals (119). Different connexins may have diverse roles. Gap-junctions with connexin 43 have been shown to mediate inhibition of collagen synthesis, while gap-junctions with connexion 32 had a stimulatory role (117). Gap-junctions are co-localised with the actin filaments in the cytoskeleton and the number of connections appear to be regulated by loading (79, 120). The permeability of the gap-junctions can also be regulated by loading with a reduced permeability (79). This indicates that gap-junctions have an important role in the response to loading.

Each tendon cell has also a single primary cilium, a sensory organelle consisting of microtubule (71). The primary cilium is thought to be involved in the mechanotransduction by sensing mechanical signals in the ECM and converting them to changes in gene expression. The exact function of the primary cilium is not entirely known but the length of the cilium as well as its angle to the cell surface appears to be regulated by changes in loading (43, 71).
The response to loading and thereby the adaption of the ECM is also regulated by growth factors and hormones (22). These induce intracellular signalling together with the integrins and the cytoskeleton. Growth factors like BMPs, FGF, IGF-1, interleukins (IL-1 and IL-6), nitric oxide (NO), PDGF, prostaglandin E₂ (PGE₂), TGF-β and vascular endothelial growth factor (VEGF) have all been shown to induce changes in fibroblasts, in vitro and in vivo, in both animals and humans. The effect of growth factors can be modulated by mechanical loading, and vice versa (15, 16, 38). For example addition of PDGF and/or IGF-1 together with load increases the phosphorylation of protein tyrosines in avian flexor cells (15).

![Mechanotransduction in tendon cells](image)

**Figure 3.** Mechanotransduction in tendon cells. Loading generates deformation of the extracellular matrix. The deformation is transferred to the cytoskeleton and the cytoplasm via ion-channels, integrins, second messengers, receptor tyrosine kinase (RTK) etc. This initiates a response with increased transcription of genes and protein synthesis. The most rapid response involves Ca²⁺ influx through ion-channels coupled to the cytoskeleton. This change in intracellular Ca²⁺ levels can be transferred to the surrounding cells via gap-junctions and IP₃ transfer. Integrins in the cell membrane are linked to intracellular proteins in focal adhesion points (FA). FA are clusters of matrix-integrin-cytoskeletal components with multiple proteins. Loading can rearrange both the shape and distribution of them, induce autophosphorylation and initiate a rapid signalling cascade. The response to loading can also be co-regulated by growth factors which induce intracellular signalling. Each tendon cell has also a primary cilium. This is believed to sense mechanical signals in the extracellular matrix and convert them to changes in gene expression.
INTRODUCTION

Tendons are dynamic tissues and adapt to loading and unloading

Like other connective tissues (e.g. bone and muscles), tendons adapt to altered levels of load or physical activity. The mechanical stimulus is crucial for cell survival, growth and tissue specific functions. There are a number of studies on the effect of loading in intact and healing tendons (20, 27, 63, 64, 95, 104, 125). These studies show that tendons can change its mechanical properties and cross-sectional area due to altered loading conditions. Several studies show that the tendon tissue responds to exercise in an anabolic way by an increased collagen production (12, 23, 25, 46, 48, 49, 69, 70, 86, 87, 93, 94, 100, 118). However, some studies also show that tendon tissue can respond to loading in a catabolic way by stimulating the release matrix degrading enzymes like matrix metalloproteinases (MMPs) (12, 48, 65, 78). Overloading is believed to cause tendon micro-damage and disorders like tendinosis and tendinopathy (10). On the other hand, tendon healing is promoted by motion and loading (21, 26, 59, 88, 95, 110, 125). The effect of loading has mostly been studied in vitro in tendon cells or explants, or in vivo in intact tendons. There are only a few studies on the response to loading in healing tendons.

The understanding that prolonged immobilization of the tissue can delay the recovery and influence the surrounding tissues, has lead to a great improvement in the promotion of musculoskeletal tissue healing. However, early motion is not without risks of adverse effects. Loading might create excessive damage to the repair tissue leading to failure of the healing process and scar formation. It is therefore important to understand the interplay between loading and tendon healing.
**Mechanical stress on tendon cells in vitro have given a lot of indications about the response**

Tenocytes are subjected to mechanical loads. They detect and respond to fluid flow, strain and shear stimuli by activating different mechano-transduction pathways. In vitro experiments can tightly control different loading parameters and it is therefore possible to study the effect of each parameter separately. In vitro studies have investigated the effect of different frequencies, strain magnitudes and duration of loading. However, these studies are only on the response of a few genes and they do not say much about the in vivo situation. The response to mechanical stimuli also differs between cells from different anatomical locations and different species (119).

Intracellular adenosine triphosphate (ATP) is a known energy source for cells, but it also functions in the extracellular space. ATP and UTP (uridine 5’-triphosphate) acts as signal transducers via cell surface receptors when released in the extracellular space (44). ATP is released by tenocytes in vitro after loading and is believed to modulate the load response (116). Extracellular signalling by ATP is thought to be regulated by two mechanisms: one is by the ecto-nucleotidases families (ENTPD- and ENPP-family) expressed in tenocytes. These can regulate the ATP levels and signalling by limiting the availability of extracellular ATP in tendons in response to loading (115).

In vitro experiments have shown that proliferation of human tendon fibroblasts increases after stretching (129, 132), but also apoptosis can be induced by stretching (109). Strain, fluid flow and vibrations can all induce mechanical deformation of cells and lead to increased intracellular Ca²⁺ levels (54, 119). Numerous in vitro studies have evaluated how loading regulates the gene expression and protein levels of PGE₂, cyclooxygenase (COX)-1 and -2 as well as collagen-1 and -3 (Table 1). Most of these studies have shown that the levels of collagens and PGE₂ are increased by loading (3, 4, 31, 36, 52, 53, 60, 78, 97, 123, 129, 130). Also MMPs and growth factors like TGF-β and VEGF have been shown to be increased by loading, however not in all studies (6, 7, 32, 37, 77, 78, 81, 96, 97, 114, 116, 128, 130). Other growth factors like PDGFs and FGFs have a more diverse response to loading, depending on the model (37, 77, 107, 108).
INTRODUCTION

Table 1. Changes in gene expression or protein levels in response to altered levels of loading/unloading in different in vitro models of tendon or ligaments. ↑ means increased levels, ↓ means decreased levels and – means unchanged levels by loading or unloading.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Loading/unloading</th>
<th>Response</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACL (rat)</td>
<td>Static: 6 N, 0.5-2 h</td>
<td>↑ exp. of Coll 1 at 1h, ↓ exp. of Coll1 at 2h</td>
<td>(52)</td>
</tr>
<tr>
<td>ACL &amp; MCL fib. (H)</td>
<td>Stretch: 0.05-0.075 strain, 1 Hz, 0.5-24 h</td>
<td>ACL: ↑ exp. of Coll 1, Coll 3 (0.05) ↓ exp. of Coll 3 (0.075) MCL: ↑ exp. of Coll 3 ↓ exp. of Coll 1</td>
<td>(53)</td>
</tr>
<tr>
<td>ACL fib. (H)</td>
<td>Stretch: 10%, 0.17 Hz, 24 h</td>
<td>↑ exp. of Coll 1, Coll 3 and levels of TGF-β1</td>
<td>(60)</td>
</tr>
<tr>
<td>ACL &amp; MCL fib. (canine)</td>
<td>Fluid flow: 25 dynes/cm², 1 min</td>
<td>↑ intracellular Ca²⁺</td>
<td>(54)</td>
</tr>
<tr>
<td>ACL fib. (rab)</td>
<td>Stretch: 4%, 0.1 Hz, 4 h/day, 3 days</td>
<td>↑ activation of c-jun, ATF-2, SAPK</td>
<td>(128)</td>
</tr>
<tr>
<td>AT fib. (rab)</td>
<td>Stretch: 5%, 0.33 Hz, 6 h</td>
<td>↑ exp. of MMP-3 - exp. of MMP-1, COX-2, Coll 1 unchanged</td>
<td>(6)</td>
</tr>
<tr>
<td>AT fib. (rab)</td>
<td>Fluid flow: 1 dyn/cm², 6 h</td>
<td>↑ exp. of IL-1β, COX-2, MMP-1, MMP-3</td>
<td>(7)</td>
</tr>
<tr>
<td>AT fib. (rat)</td>
<td>Stretch: 8%, 0.5-1 Hz, 24 h</td>
<td>↑ levels of VEGF and HIF-1α</td>
<td>(96)</td>
</tr>
<tr>
<td>AT &amp; SST (rat)</td>
<td>Stress deprivation or Cyclic compression: 1 MPa, 0.5 Hz, 1 min/15 min, 4 h</td>
<td>SD: ↑ exp. of MMP-3, MMP-13, TIMP-2 CC: ↑ exp. of MMP-13 in SST.</td>
<td>(114)</td>
</tr>
<tr>
<td>AT (M)</td>
<td>Fluid flow: 0-0.6 dyn/cm²</td>
<td>↑ levels of scleraxis, p-smad2 and release of active TGF-β1</td>
<td>(80)</td>
</tr>
<tr>
<td>Fetal tendon fib. (M)</td>
<td>Fluid flow: 0.1 dyn/cm², 14 h</td>
<td>↑ exp. of genes related to stress response, transport, transcription ↓ exp. of genes related to ECM, apoptosis, cell division, cell signalling</td>
<td>(76)</td>
</tr>
<tr>
<td>FDP tendon (C)</td>
<td>Stretch: 3-12 MPa, 1 Hz, 1 day or 2 h/day for 12 days</td>
<td>↑ collagenase activity, GAG content and PGE₂ production - collagen content unchanged</td>
<td>(31)</td>
</tr>
<tr>
<td>FDP tendon (C)</td>
<td>Stretch: 0.25-12 MPa, 1 Hz, 4-24 h</td>
<td>↑ levels of PGE₂, NO</td>
<td>(36)</td>
</tr>
<tr>
<td>FDP fib. (C)</td>
<td>Stretch: 75 millistrain, 1 Hz, 8 h/day, 4 days</td>
<td>↑ levels of n-cadherin, vinculin, tropomyosin - levels of actin unchanged</td>
<td>(98)</td>
</tr>
<tr>
<td>FDP fib. (H)</td>
<td>Stretch: 3.5%, 1 Hz, 5-120 min</td>
<td>↑ secretion of ATP and ATPase activity</td>
<td>(115)</td>
</tr>
<tr>
<td>FDP fib. (H)</td>
<td>Stretch: 3.5%, 1 Hz, 1 h/day, 1-5 days.</td>
<td>↑ exp. of Coll 1, biglycan, fibronectin, TGF-β1, COX-2, MMP-27, ADAMTS-5</td>
<td>(97)</td>
</tr>
<tr>
<td>FDP fib. (H)</td>
<td>Stretch: 3.5%, 1 Hz, 2 h</td>
<td>↑ exp. of IL-1, COX-2, MMP-3 and ATP secretion - exp. of MMP-1 unchanged</td>
<td>(116)</td>
</tr>
<tr>
<td>Flexor fib. (rat)</td>
<td>Fluid shear stress: 0.41 Pa, 6-12 h.</td>
<td>↑ exp. of TGF-β1, MMPs, BMPs, VEGF ↓ exp. of collagens, TGF-β1, IGFs, FGFs, PDGFs, TIMPs</td>
<td>(37)</td>
</tr>
</tbody>
</table>
Continuation of Table 1:

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Loading/unloading</th>
<th>Response</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT fib. (canine)</td>
<td>Stretch: 1-9%, 0.5-3 Hz, 15-120 min</td>
<td>† activation of JNK1, JNK2</td>
<td>(12)</td>
</tr>
<tr>
<td>PT fib. (H)</td>
<td>Stretch: 4-12%, 0.5 Hz, 4 h</td>
<td>† levels of LTB4, - levels of 5-LO unchanged</td>
<td>(75)</td>
</tr>
<tr>
<td>PT fib. (H)</td>
<td>Stretch: 5%, 1 Hz, 15-60 min</td>
<td>† activation of JNK1, JNK2</td>
<td>(109)</td>
</tr>
<tr>
<td>PT fib. (H)</td>
<td>Stretch: 5%, 1 Hz, 15-60 min</td>
<td>† production of NO</td>
<td>(121)</td>
</tr>
<tr>
<td>PT fib. (H)</td>
<td>Stretch: 8%, 0.1-1 Hz, 4 h</td>
<td>† levels of PLA2, COX-1, COX-2, PGE2</td>
<td>(124)</td>
</tr>
<tr>
<td>PT fib. (H)</td>
<td>Stretch: 4-12%, 0.5 Hz, 4-24 h</td>
<td>† levels of PGE2, COX-1, COX-2</td>
<td>(123)</td>
</tr>
<tr>
<td>PT fib. (H)</td>
<td>Stretch: 4-8%, 0.5 Hz, 4 h</td>
<td>† PGE2 production and exp. of COX-2, MMP-1</td>
<td>(130)</td>
</tr>
<tr>
<td>SDF tendon (E)</td>
<td>Stretch: 5%, 1 Hz, 24 h</td>
<td>† levels and activity of MMP-2 MMP-9 and release of degraded COMP</td>
<td>(32)</td>
</tr>
<tr>
<td>Tendon fib. (H)</td>
<td>Stretch: 0.25 strain, 0.17-1 Hz, 3 h</td>
<td>† secretion of PGE2, - levels of LTB4, LDH unchanged</td>
<td>(3)</td>
</tr>
<tr>
<td>Tendon fib. (H)</td>
<td>Stretch: 5%, 1 Hz, 15-60 min</td>
<td>† secretion of IL-6, - levels of TGF-β, PDGF, bFGF unchanged</td>
<td>(107, 108)</td>
</tr>
<tr>
<td>Tendon fib. (H)</td>
<td>Stretch: 0.25 strain, 1 Hz, 12 h</td>
<td>† secretion of PGE2, IL-6, - levels of IL-1 unchanged, LTB4 undetected</td>
<td>(4)</td>
</tr>
<tr>
<td>TT (rat)</td>
<td>Stress deprived, 24 h</td>
<td>† exp. and levels of MMP-1</td>
<td>(11, 72)</td>
</tr>
<tr>
<td>TT (rat)</td>
<td>Stress deprived 1-3 days</td>
<td>SD: ↓ TIMP-1/MMP-13 ratio</td>
<td>(42)</td>
</tr>
<tr>
<td>TTfsc (rat)</td>
<td>Stress deprived, 0.5-48 h</td>
<td>† exp. of Coll 1, decorin, CatK (early), MMP-2, MMP-3, MMP-13</td>
<td>(74)</td>
</tr>
<tr>
<td>TTfsc (rat)</td>
<td>Stretch: 3% (+2% static strain), 1 Hz, 1-24 h</td>
<td>↓ exp. of Coll 1, decorin, CatK (late)</td>
<td>(77)</td>
</tr>
<tr>
<td>TTfsc (rat)</td>
<td>Stretch: 3%, (+2% static strain), 1 Hz, 10-24 h</td>
<td>↑ exp. of Coll 3, MMP-3, TGF-β, - exp. of Coll 1, biglycan unchanged</td>
<td>(78)</td>
</tr>
<tr>
<td>TTfsc (rat)</td>
<td>Static: 1 N, 10 min-1 h</td>
<td>↑ exp. of connexin 43, - levels and exp. of connexin 43</td>
<td>(79)</td>
</tr>
</tbody>
</table>

The next step is animal models where the tissue is in its normal surrounding

It is easy to select and control different loading parameters during in vitro studies, this is harder in vivo. Most studies on the effect of mechanical loading in different animal models are performed in intact tendons. The response to unloading does not appear to be the exact opposite to the response to loading. The response also differs depending on the loading model, tendon type and for how long the loading has been performed. Some studies have pronounced effects of loading, while others have shown very modest response.

Intact tendons

Most studies on intact tendons have been performed with unloading or different overloading models, thereby studying the response after several weeks of unloading or loading. The gene expression of collagen-1 and -3 have been extensively studied in intact tendons (Table 2). Unloading appears to decrease the collagen levels but not always, the levels are sometimes unaltered (49, 80, 118). The response to loading usually shows the opposite pattern with increased collagen expression (8, 28, 48, 94). Growth factors like CTGF, IGFs, TGF-β and VEGF have also been studied, but with diverse results (9, 48-50, 73, 92, 94, 102). The expression of TGF-β and CTGF are sometimes elevated but this is not a clear-cut response and needs to be further investigated (48, 92). The IGF-system with agonists and binding proteins appears to be mechanosensitive and regulated by both unloading and loading (9, 49, 50, 94, 102).
**INTRODUCTION**

Table 2. Changes in gene expression or protein levels in response to altered levels of loading/unloading in intact tendons, in vivo. ↑ means increased levels, ↓ means decreased levels and – means unchanged levels by loading/unloading.

<table>
<thead>
<tr>
<th>Model</th>
<th>Loading/unloading</th>
<th>Response</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACI &amp; MCL (rab)</td>
<td>Knee immobilization, 1-12 weeks</td>
<td>↑ levels of integrin β1, α, α, α subunits</td>
<td>(2)</td>
</tr>
<tr>
<td>ACI &amp; MCL (rab)</td>
<td>Knee immobilization, 9-12 weeks</td>
<td>↑ levels of integrin β1, α, fibronectin</td>
<td>(1)</td>
</tr>
<tr>
<td>AT (rab)</td>
<td>Chronic loading: 1.25 Hz, 2 h/day, 3 day/week, 11 weeks</td>
<td>↑ exp. of IL-1β, Coll 3</td>
<td>(8)</td>
</tr>
<tr>
<td>AT (rab)</td>
<td>Overloading by kicking, 2 h/day, 1-3 weeks</td>
<td>↑ exp. of IGF-2</td>
<td>(14)</td>
</tr>
<tr>
<td>AT (rat)</td>
<td>Treadmill running, 30-60 min/day, 5 days/week, 8 weeks</td>
<td>↑ rate of collagen deposition</td>
<td>(28)</td>
</tr>
<tr>
<td>AT (rat)</td>
<td>Increased loading, 1-28 days or Treadmill running, 20-60 min/day, 5 days or Decreased load by muscle transection 1-28 days</td>
<td>↑ levels of IGF-1</td>
<td>(45)</td>
</tr>
<tr>
<td>AT (rat)</td>
<td>Hindlimb susp. 7-14 days Reload 2-16 days</td>
<td>↑ rate of collagen deposition</td>
<td>(49)</td>
</tr>
<tr>
<td>AT (rat)</td>
<td>Concentric, eccentric or isometric training, 4 days, 2-4 sets/day of 10x2 s stim.</td>
<td>↑ exp. of TGF-β1, Coll 1, Coll 3, LOX, MMP-2, TIMP-1, TIMP-2</td>
<td>(48)</td>
</tr>
<tr>
<td>AT (rat)</td>
<td>Concentric, eccentric or isometric training, 4 days, 2-4 sets/day of 10x2 s stim.</td>
<td>↑ exp. of IGF-1Ea, MGF</td>
<td>(50)</td>
</tr>
<tr>
<td>AT (rat)</td>
<td>Running, strength or vibration strength training, 12 weeks</td>
<td>↑ exp. of TIMP-1 (run.)</td>
<td>(73)</td>
</tr>
<tr>
<td>AT (M)</td>
<td>Unloading by Botox, 1-4 weeks</td>
<td>↑ exp. of IGF-1</td>
<td>(94)</td>
</tr>
<tr>
<td>FDP (rab)</td>
<td>Electrical stimulation, 2 h/day, 3 day/week, in total 80 h</td>
<td>↓ levels of IGF-1, PCNA and IRS-1 phosphorylation</td>
<td>(102)</td>
</tr>
</tbody>
</table>

INTRODUCTION

**Healing tendons**

The response to loading or unloading on the molecular level has been less studied in healing tendons compared to intact tendons. The majority of the studies done with loading/unloading and healing tendons have focused on the outcome, stronger, stiffer tendons or more organized collagen (33, 89, 95, 125). There are also a few studies on the effect of loading on tendon to bone healing (17, 113), however the response to loading most likely differs between the tendon and the bone-tendon-junction, because this is a very specialised tissue. Most studies on the response to loading or unloading in healing tendons have focused on the expression of ECM molecules like collagens and proteoglycans. There are also a few studies on different neuropeptides or nerve marker (Table 3). It appears as the response to unloading, in collagen and proteoglycan expression, during tendon healing varies in a time-dependent matter (13, 21, 84, 85, 100). There is sometimes an up-regulation and sometimes a down-regulation of these genes. The expression of different neuropeptides appears to be dependent on the type of loading or unloading (19-21, 29).
**Table 3.** Changes in gene expression or protein levels in response to altered levels of loading/unloading in healing tendons and ligaments, in vivo. ↑ means increased levels, ↓ means decreased levels and – means unchanged levels by loading/unloading.

<table>
<thead>
<tr>
<th>Model</th>
<th>Loading/unloading</th>
<th>Response</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACL rupture (rab)</td>
<td>Complete vs partial rupture (with more loading), 1-6 weeks</td>
<td>↑ exp. of Coll 1, Coll 3 (6w), α-SMA, MMP-1 ↓ exp. of Coll 1, Coll 3 (2w)</td>
<td>(13)</td>
</tr>
<tr>
<td>AT rupture (rat)</td>
<td>Cast, 8 and 17 days</td>
<td>↓ exp. of bFGF, BDNF, COX-1, iNOS, HIF-1α - exp. of NGF, IGF-1, COX-2 unchanged</td>
<td>(19)</td>
</tr>
<tr>
<td>AT rupture (rat)</td>
<td>Cast, 8 and 17 days</td>
<td>↓ exp. of Coll 1, Coll 3, versican, decorin, biglycan, NK-1, CRLR, RAMP-1</td>
<td>(21)</td>
</tr>
<tr>
<td>AT rupture (rat)</td>
<td>Cast, 4 weeks Running, 4 weeks</td>
<td>Cast: - levels of CGRP unchanged Run: ↓ levels of CGRP</td>
<td>(20)</td>
</tr>
<tr>
<td>AT rupture (rat)</td>
<td>IPC, 45-52 mmHg, 1 h/day, 2-4 weeks</td>
<td>↑ levels of substance P, CGRP</td>
<td>(29)</td>
</tr>
<tr>
<td>AT rupture (rat)</td>
<td>Cast, 14 days ± IPC 45-52 mmHg, 1h/day</td>
<td>UL: ↓ levels of Coll 3 IPC: ↑ levels of Coll 3</td>
<td>(100)</td>
</tr>
<tr>
<td>MCL rupture (rab), in vitro</td>
<td>Hydrostatic pressure or tensile stress, 1 MPa, 0.5 Hz, 1 min/15 min for 4 h</td>
<td>↑ exp. of Aggrecan, Coll 2 (only HP) - exp. of Collagenase - exp. of Collagenase - exp. of Coll 1, Coll 3, biglycan, decorin, fibromodulin, versican, c-fos, c-jun unchanged</td>
<td>(84)</td>
</tr>
<tr>
<td>MCL rupture (rat)</td>
<td>Hindlimb unloading, 3-7 weeks</td>
<td>↑ exp. of fibronectin, biglycan, decorin (7w), TIMP-1 (7w) ↓ exp. of Coll 1, Coll 3, Coll 5, decorin (3w), MMP-2, TIMP-1 (3w)</td>
<td>(85)</td>
</tr>
</tbody>
</table>

ACL – anterior cruciate ligament, AT – Achilles tendon, MCL – Medial cruciate ligament, rab – rabbit
IPC – intermittent pneumatic compression, α-SMA – α smooth muscle actin,
BDNF – brain-derived neurotrophic factor, NGF – nerve growth factor, NK-1 – neurokinin-1,
CRLR – calcitonin-receptor-like-receptor, RAMP-1 – receptor activity modifying protein-1,
CGRP – calcitonin gene related peptide,
The ultimate goal is studies in humans, however it is usually more limited

Studies in humans are usually more restricted due to ethical concerns, sampling procedures and sample sizes. It has been shown that mechanical loading can increase the tendon cross-sectional area and also stiffness of the tendon can be altered by loading (47). This indicates that collagen synthesis and cross-linking could be influenced by loading. There are a few studies where tendon biopsies have been collected after loading or unloading (30, 86, 87, 111). However the introduction of the microdialysis technique in the peritendinous space opened up for more studies on the effect of mechanical loading in tendons. This technique is done in the close proximity to the tendon, and therefore likely reflects what is going on in the tendon, even though there might be some discrepancy. Microdialysis has mostly been done on healthy men, and there is as far as I know no data on healing tendons in humans. Most of these studies have looked at the collagen synthesis rate after loading and a few studies have studied the growth factor response (Table 4). Collagen production appears to increase after exercise, but not in all studies (23-25, 30, 46, 67, 69, 70, 86, 87, 93, 111). The increase might be dependent on the duration, magnitude or type of exercise. Insulin-like growth factor binding proteins (IGFBPs), IL-6, MMPs, PGE₂, tissue inhibitors of metalloproteinase (TIMPs) and TGF-β have all been showed to have altered levels in humans after exercise (24, 46, 65, 66, 68, 70, 87, 93, 111, 113).
**Table 4.** Changes in gene expression or protein levels in response to altered levels of loading/unloading in humans. ↑ means increased levels, ↓ means decreased levels and – means unchanged levels by loading or unloading.

<table>
<thead>
<tr>
<th>Model</th>
<th>Loading/unloading</th>
<th>Response</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD Peritend. space (AT)</td>
<td>Immobilization, 2 weeks</td>
<td>Im: - levels of PINP unchanged</td>
<td>(25)</td>
</tr>
<tr>
<td></td>
<td>Remobilization, 2 weeks</td>
<td>Rem: ↑ levels of PINP, ICTP</td>
<td></td>
</tr>
<tr>
<td>MD Peritend. space (AT)</td>
<td>Immobilization, 6-10 weeks</td>
<td>Im: ↑ levels of PINP, ICTP</td>
<td>(24)</td>
</tr>
<tr>
<td></td>
<td>Remobilization, –7 weeks</td>
<td>Rem: ↓ levels of PINP, ICTP</td>
<td></td>
</tr>
<tr>
<td>MD Peritend. space (AT) blood samples</td>
<td>Uphill running, 1 h</td>
<td>↑ levels of TGF-β1 (blood), PICP - levels of ICTP unchanged</td>
<td>(46)</td>
</tr>
<tr>
<td>MD Peritend. space (AT)</td>
<td>Uphill running, 1 h</td>
<td>↑ levels of MMP-9, TIMP-1, TIMP-2, lactoferrin - levels of Pro-MMP-2</td>
<td>(65)</td>
</tr>
<tr>
<td>MD Peritend. space (AT)</td>
<td>Eccentric training, twice/day, 12 weeks</td>
<td>↑ levels of PICP (Tendinosis pat.) - levels of ICTP, PICP (controls) unchanged</td>
<td>(67)</td>
</tr>
<tr>
<td>MD Peritend. space (AT)</td>
<td>Intermittent static plantar flexion, 30 min</td>
<td>↑ levels of PGE2</td>
<td>(66)</td>
</tr>
<tr>
<td>MD Peritend. space (AT)</td>
<td>Running, 36 km (3 h)</td>
<td>↑ levels of IL-6</td>
<td>(68)</td>
</tr>
<tr>
<td>MD Peritend. space (AT)</td>
<td>Training, 2-4 h/day, 4-11 weeks</td>
<td>↑ levels of PICP, ICTP</td>
<td>(69)</td>
</tr>
<tr>
<td>MD Peritend. space (AT)</td>
<td>Running, 36 km (3 h)</td>
<td>↑ levels of PICP, PGE2 - levels of ICTP</td>
<td>(70)</td>
</tr>
<tr>
<td>MD Peritend. space (AT)</td>
<td>Running, 36 km (3 h)</td>
<td>↑ levels of PICP, IGF-1, IGBP-4 - levels of ICTP, IGF-1, IGBP-2, IGBP-3 unchanged</td>
<td>(93)</td>
</tr>
<tr>
<td>Biopsies (PT)</td>
<td>Unloading, 10-23 days</td>
<td>↓ collagen synthesis rate</td>
<td>(30)</td>
</tr>
<tr>
<td>MD Peritend. space (PT)</td>
<td>Running, 36 km (3 h)</td>
<td>↑ levels of PINP - levels of PGE2, unchanged</td>
<td>(23)</td>
</tr>
<tr>
<td>MD Peritend. space and biopsies (PT)</td>
<td>One legged kicking exercise, 1 h (Women)</td>
<td>↑ PINP - collagen synthesis rate unchanged</td>
<td>(86)</td>
</tr>
<tr>
<td>MD Peritend. space and biopsies (PT)</td>
<td>One legged kicking exercise, 1 h (Men)</td>
<td>↑ collagen synthesis rate - levels of PINP - levels of IGF-1, IGBP-3, IGBP-4 unchanged</td>
<td>(87)</td>
</tr>
<tr>
<td>Biopsies (PT)</td>
<td>Knee extension, total 40 repetitions</td>
<td>↓ exp. of Coll 1, Coll 3, MMP-2 - exp. of MMP-9, MMP-3, TIMP-1, proteoglycans unchanged</td>
<td>(111)</td>
</tr>
</tbody>
</table>

AIMS OF THE THESIS

General
The general aim of this thesis was to understand more about the response to mechanical loading during Achilles tendon healing.

Specific

Study I: To find out if short daily loading episodes could improve the healing of otherwise unloaded tendons.

Study II: To find out if four short loading episodes were enough to stimulate tendon healing, and if the response to loading differed between the early inflammatory phase and the later proliferative phase of healing.

Study III: To investigate if unloading influenced the expression of specific genes associated with inflammation, ECM and tendon specificity, but also to study the gene expression pattern in healing and intact tendons.

Study IV: To study how the gene expression of the BMP-signalling system was altered during different phases of tendon healing and if unloading influenced this expression.

Study V: To study the gene expression of myostatin and its receptors in intact and healing tendons, with or without mechanical loading, and to study if myostatin administration during tendon healing could stimulate the repair.

Study VI: To investigate how a single bout of loading influenced gene expression in otherwise unloaded tendons and to find out how long this response lasted.
MATERIALS AND METHODS

A short summary of the materials and methods used in the thesis is presented below. Please see the papers in the end for more details.

Study designs

Short summary of the study designs.

Study 1: The effect of short loading episodes on healing tendons

Study one was divided into three experiments (Figure 4). The right Achilles tendon was transected and the rats were either unloaded by tail suspension or kept in normal cages with free activity during the entire experiment. Tail-suspended rats were unloaded for the entire experiment or released from suspension once or twice daily for treadmill walking. The rats were sacrificed 14 days after surgery for mechanical evaluation.

![Figure 4](image)

Figure 4. Experimental setup for study I. The study consisted of 3 experiments with separate research questions. The box below illustrates the daily loading.
MATERIALS AND METHODS

Study II: Early vs late: when is it important to start loading the tendon?

The right Achilles tendon was transected and the rats were unloaded by tail suspension. Half of the rats were unloaded the entire experiment and the other half were released from the suspension for treadmill walking 30 min/day (day 2-5 or day 8-11, Figure 5). The rats were sacrificed on day 5 for histology and day 8 or 14 for mechanical evaluation. We used ten rats in each group.

Figure 5. Experimental setup for study II, where we compared the effect of loading during early and late healing.

Study III: Unloading and tendon healing: inflammation, ECM and tendon specificity

This study consisted of two parts, mechanical evaluation and gene expression analysis. Half of the rats received Botox into the right calf muscles for unloading, the other half remained loaded (Figure 6). The right Achilles tendon was transected and the healing tendons were analysed after 3, 8, 14 and 21 days of healing with mechanical evaluation or for gene expression levels. Intact tendons were also analysed (loaded and unloaded tendons). Only the right tendon was used for analyses, never the contralateral limb. We used five rats in each group of healing tendons and 10 rats in each group for intact tendons.

Study IV: BMP-signalling in tendons: mechanical loading and healing

The animals for the gene expression analysis in study III were also used for study IV, except that only ten intact tendons were analysed (five loaded and five unloaded).
Study V: The role of myostatin in tendon healing

The animals for the gene expression analysis in study III and IV were also used for study V. The same setup as for the gene expression analysis was used for immunohistochemistry staining (n=3 in each group). Healing tendons were also treated with myostatin and analysed by mechanical evaluation. Cell cultures were used for affinity studies in the alkaline phosphatase (ALP) and luciferase assays.

Figure 6. Experimental setup for the gene expression analyses in study III - V as well as the immunohistochemistry in study V and mechanical testing in study III. Both intact and healing tendons were studied.

Study VI: How long does the response last after one single loading episode?

The last study investigated the effect of one single loading episode by gene expression analyses, microarray and real-time polymerase chain reaction (PCR) and mechanical evaluation. The right Achilles tendon was transected in all rats, followed by unloading by tail suspension. All animals, except the unloaded control groups, were released from the suspension day 5 after tendon transection to walk on a treadmill for 30 minutes before they were unloaded again. The animals for microarray analysis were sacrificed 3, 12, 24 and 48 hours after loading was finished, together with continuously unloaded controls at day 5 and 7 (Figure 7). Animals for real-time PCR were sacrificed 1, 3, and 12 hours after the loading was finished, together with continuously unloaded controls day 5. Finally, animals for mechanical evaluation were sacrificed 3 and 7 days after loading was finished (day 8 and 12 after tendon transection) together with continuously unloaded controls at the same time-points.
MATERIALS AND METHODS

Figure 7. Experimental setup for study VI. Animals were either completely unloaded or unloaded with one exception on day 5 when they were allowed to walk on a treadmill for 30 minutes before they were unloaded again. Animals were killed between day 5 and day 12 for gene expression analyses and mechanical evaluation.
Achilles tendon transection model (all studies)

The rats were anesthetised with isoflurane gas and given preoperative subcutaneous injections of antibiotics and analgesics. The right hind limb was shaved and washed, and a skin incision was made lateral to the Achilles tendon. The plantaris tendon was removed. The Achilles tendon was sharply transected, and a 3 mm segment was removed. The skin was sutured, while the Achilles tendon was left unsutured. In study V, myostatin (10µg/rat) was applied onto a collagen sponge and placed in the tendon defected before skin suturing. Untreated controls and controls with collagen sponges without protein were also used.

Unloading and reloading

Unloading by botulinium toxin injections (study III-V)

Irreversible unloading of the Achilles tendon was achieved by botulinium toxin (Botox®) injections into the calf muscles in study III-V. Botox was injected into the gastrocnemius lateralis, medianus and the soleus muscles under anaesthesia at a dose of 1 U per muscle, 5 days before transection. The botulinium toxin is a specific blocker of acetylcholine release from the presynaptic endings of the motor neurons, and it induces a gradual weakness.

Unloading by tail suspension (study I, II and VI)

To be able to easily reverse the unloading and apply short controlled loading episodes, we used tail suspension in study I, II and VI. Unloading was carried out in special cages with an over-head system, allowing the rats to move in all directions. An adhesive tape was secured to the tail, which was connected to the over-head system. The hind limbs were lifted just above the cage floor, ensuring that no unwanted loading occurred during the experiment.

Reloading (study I, II and VI)

Treadmill walking (9 m/min) was used to apply short controlled loading episodes in study I, II and VI. The walking episodes lasted for 15-60 min/day in the different studies. In study I, loading was also applied as unrestricted cage activity for 15 min/day, where the rats were allowed to move around in the cage on their four limbs. Both types of loading were monitored so that the rats were moving throughout the entire training sessions.
Mechanical testing (study I-III, V and VI)

Mechanical testing was used in all studies except study IV. Following euthanasia by CO$_2$ inhalation, the healing tendon was dissected free from the surrounding soft-tissue and harvested together with the calcaneal bone and parts of the calf muscles. The callus’s sagittal and transverse diameters were measured with a slide calliper as well as the distance between the old tendon stumps (gap-distance). The cross-sectional area was calculated, assuming an elliptical geometry. The muscle was removed and the tendon was fixed between two metal clamps with the bone in 30° dorsiflexion relative to the direction of traction. The distance between the top metal clamp and the bone was measured (length). The clamps were fixed in a materials testing machine (Figure 8) which pulled at constant speed of 0.1 mm/s until failure. Peak force, stiffness and energy uptake were calculated by the software of the testing machine. Peak stress and elastic modulus were calculated afterwards. In study III, intact Achilles tendons were also tested mechanically according to the same procedure, after removal of the plantaris tendon.

Figure 8. Materials testing machine
Gene expression analyses (study III-VI)

Animals for gene expression analyses (microarray and real-time PCR) were anesthetised with isoflurane gas (study III-V) or subcutaneous injections of dexmedetomidine and ketamine (study VI). The subcutaneous injections were used in study VI to sedate the rats while suspended, so that the hind limbs were unloaded throughout the whole harvesting procedure. The tendon callus tissue was then harvested under full anaesthesia. The skin on the right hind limb was shaved and washed, and the callus tissue was dissected free from all extraneous soft tissue. A 5-8mm segment of newly formed callus tissue was harvested, quickly rinsed in saline, snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. For gene expression analyses in study III-V, intact loaded and unloaded tendons were also harvested according to the same procedure. The plantaris tendon was not included in these analyses. The right limb was always used for tendon healing. All rats were killed after harvesting by an overdose of pentobarbital sodium.

RNA extraction (study III-VI)

Total RNA was extracted (in study III-VI) from intact tendons and callus tissues by a combination of the TRIzol method and RNeasy Total RNA Kit. The tendons were pulverised one by one in a liquid nitrogen-cooled vessel using a Retsch Mixer Mill. TRIzol Reagent was added, followed by incubation in room temperature. Chloroform was added to the samples, followed by centrifugation. The top aqueous layer was transferred to ethanol, and RNA was further purified using the RNeasy Total RNA Kit according to the manufacturer’s instructions. Potential DNA contamination was eliminated by DNase, and the RNA was stored at -80°C. RNA yield and integrity was analysed by Nanodrop and Agilent bioanalyzer.

Microarray (study VI)

In study VI, samples were analysed by rat microarrays, Gene 1.0 ST array system (Affymetrix). The microarray analysis was carried out by the Bioinformatics and expression analysis (BEA) core facility, Karolinska institute, Sweden. Genes with less fold change than 1.5 or p≥0.05 were regarded as unchanged by loading.
MATERIALS AND METHODS

Reverse transcription and real-time PCR (study III-VI)

Both intact and healing tendons were analysed in study III-V, and 100 ng of total RNA was converted into cDNA using a high-capacity cDNA reverse transcription kit. In study VI, only healing tendons were analysed, which yielded more RNA. Therefore 500 ng were transcribed into cDNA. Primers for all genes except growth differentiation factor (GDF)-5 were bought from Applied Biosystems. The primers for GDF-5 were designed using PrimerExpress 2.0 software. BLASTn ensured gene specificity of the primers. Amplification was performed in 15-µl reactions using TaqMan Fast PCR MasterMix. Each sample was analysed in duplicate, and samples where the cycle threshold (CT) values differed more than 0.5 were reanalysed. Real-time PCR reactions were conducted using a standard curve method to quantitate the specific gene targets of interest. The standard curves were made with rat spleen RNA or embryonic rat RNA depending on the gene. Each sample in study III-V was normalised to 18S rRNA. In study VI, each gene was normalised to a ratio of three house-keeping genes (18S rRNA, Cyclophilin A and Ubiquitin C). Reactions with no reverse transcription and no template were added as negative controls.
Histology (study II)

Hematoxylin-eosin staining was performed on early callus tissue in study II, to estimate the amount of bleeding after loading. The hind limbs were therefore kept unloaded during euthanasia to ensure that the results only came from treadmill walking. Tendon calluses were fixed in 4% phosphate buffered formaldehyde, imbedded in paraffin and sectioned parallel to the longitudinal axis of the tendon. One slide per specimen, comprising the full length of the tendon callus, was stained with Ehrlich hematoxylin-eosin. The slides were analysed in a light microscope and bleeding was defined as large accumulations of erythrocytes in the tissue.

Immunohistochemistry (Study V)

Immunohistochemistry for follistatin was performed in study V. The tissues were fixed in 4% phosphate buffered formaldehyde, imbedded in paraffin and sectioned transverse to the longitudinal axis of the tendon. One slide from the mid part of the tendon callus, representing the entire cross-sectional area of the sample, was used for immunohistochemistry. Sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked in H$_2$O$_2$ followed by wash in tris-buffered saline with tween 20 (TBST). Non-specific bindings were blocked with bovine serum albumin followed by incubation with primary antibody (goat anti follistatin antibody), in antibody diluent. The slides were rinsed in TBST and incubated with a secondary anti-goat antibody. Staining was visualised by incubation with avidin-biotin-peroxidase complex followed by diaminobenzidine (DAB) incubation. The sections were counterstained with hematoxylin. Normal goat IgG was used as negative control.
Alkaline phosphatase and luciferase reporter gene assays (study V)

The alkaline phosphatase assay was performed on C2C12 cells which stably express the Bmpr1b. The cells were treated with recombinant proteins (GDF-5, OP-1, noggin and follistatin) for 3 days and the induced ALP activity was thereafter determined spectrophotometrically. The amount of p-NP released from the substrate p-NPP was recorded at 405 nm and used to calculate ALP activity. The luciferase reporter gene assay was performed on HepG2 which were co-transfected with a Smad Binding Element luciferase construct and a normalisation vector pRL-Tk using Turbofect. The cells were seeded and stimulated with recombinant proteins (noggin, follistatin and myostatin) 1 day later. Luciferase activity was determined using the Dual-Glo Luciferase Reporter Assay System.

Table 5. Overview of methods used in the different studies

<table>
<thead>
<tr>
<th>Study:</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
</tr>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Botox injections</td>
<td></td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Tail suspension</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Treadmill walking</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanical testing</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Real-time PCR, intact tendons</td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Real-time PCR, healing tendons</td>
<td></td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Microarray</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALP &amp; luciferase reporter gene assays</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Statistics

We have mainly used 10 rats in each group for mechanical testing. This group size allows us to detect a change in peak force by 1.25 standard deviations with a power of 80%. This means that we had sufficient power to detect changes about 25-30%.

Study I

Each experiment in study one was analysed by a one-way ANOVA and Bonferroni-Dunn for post hoc comparisons. A regression analysis including 15, 30, and 60 min of treadmill walking was made to evaluate the influence of the duration of a loading episode.

Study II

The results from the mechanical testing were analysed by a two-way ANOVA with time and loading status as independent variables. Student’s t-test was used at each time-point (early and late) to test if loading had an effect on strength at the different time-points.

Study III

Mechanical data were analysed by a two-way ANOVA, with time and loading status as independent variables. Gene expression data were ln-transformed, and intact and healing tendons were analysed separately. Intact tendons were analysed by Student’s t-test. Healing tendons were analysed by a two-way ANOVA. A significant ANOVA was followed by separate Student’s t-tests and Bonferroni’s correction for multiplicity of time points but not for multiplicity of genes. Gene expression in healing tendons, which showed inhomogeneity in variance by Levene’s test, were analysed by nonparametric statistics with the same principle as the parametric test (Kruskal-Wallis test was followed by separately Mann-Whitney tests). Differences between intact and healing tendons were tested by a two-way ANOVA with loading status and type of tendon (intact or healing) as independent variables.

Study IV

Intact tendons and healing tendons were analysed separately. Intact tendons were analysed by Student’s t-test. Healing tendons were analysed by a two-way ANOVA followed by Bonferroni’s correction for multiple testing.
MATERIALS AND METHODS

Study V
Results of the luciferase and ALP assays were analysed by Student’s t-test. Gene expression in intact tendons and healing tendons were analysed separately. Intact tendons were analysed with Student’s t-test and healing tendons were analysed by a two-way ANOVA. Results from the mechanical testing after myostatin treatment were analysed by a two-way ANOVA with time and treatment as independent variables.

Study VI
Results from the microarray analysis were regarded as descriptive. However, p-values from Student’s t-test were used together with fold-change to identify regulated genes. Results from the PCR study (3 and 12h) were seen as confirmatory and thus, for each gene and time-point, where the microarray suggested regulation, the PCR result was tested with Student’s t-test. PCR data points at 3 and 12h (unregulated in the array) were not analysed. We also analysed the PCR results for 1 hour after loading with Student’s t-test. Results from the mechanical evaluation were analysed by Student’s t-test at each time-point.
RESULTS IN BRIEF

Here follows a short summary of interesting results in the six studies.

Unloading by botulinium toxin reduced the strength of the healing tendon dramatically...

The peak force of the healing tendons was influenced by mechanical loading. Loaded tendon calluses were already stronger after 8 days of healing compared to tendon calluses unloaded by botulinium toxin injections (study III, figure 9). The peak force continued to increase with time in both the loaded and the unloaded group but the difference between them became even more evident with time. The greatest difference was found by day 14 when the loaded calluses were 215% stronger. The stiffness showed a similar pattern as the peak force. Loading mainly influenced the amount of tissue produced (i.e. increased cross-sectional area) and did not improve the material properties (peak stress and elastic modulus). In fact, by day 21 of healing, stress and modulus were even lower in the loaded calluses. There was also a significant difference in lengthening between the loaded tendon calluses and the unloaded, where the loaded calluses had a larger gap distance (table 6). This was evident already after 8 days of healing and sustained until 21 days; however, the length begun to decrease between 14 and 21 days in the loaded samples.

<table>
<thead>
<tr>
<th>Peak force (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
</tr>
<tr>
<td>70</td>
</tr>
<tr>
<td>60</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>40</td>
</tr>
<tr>
<td>30</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Intact 3</th>
<th>8</th>
<th>14</th>
<th>21</th>
<th>Days of healing</th>
</tr>
</thead>
<tbody>
<tr>
<td>unloaded (botox)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>loaded (normal cage)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 9. Peak force of intact and healing tendons with continuous loading or unloaded by Botox injections. The horizontal axis shows intact tendons or time (3, 8, 14, and 21 days) after tendon transection. White dots are unloaded tendons, and grey dots are loaded; n = 5 in each group.
RESULTS IN BRIEF

...but short loading episodes increased the strength of the unloaded tendon calluses

Short daily loading episodes on the treadmill increased the peak force of the healing tendons. This was seen with daily loading episodes (15-60 min) for 11 days (Figure 10A, study I) but also with just four loading episodes of 30 min/day (Figure 10B, study II). Even as little as 30 min of treadmill walking once, increased the peak force by 20% (figure 10C, study VI). The effect of one loading episode was only apparent one week after the loading episode was finished. The peak force was increased by 90% after 11 episodes of daily loading (30 min/day) compared to 50-60% after four episodes of daily loading. The response to four daily loading episodes did not differ between early healing (during the inflammatory phase) and later healing (during the proliferatory phase). An increase in the duration of each loading episodes from 15 to 60 minutes resulted in roughly 25% stronger tendon calluses. There was no additional increase in callus strength by dividing the 30 minutes loading episodes into two episodes of 15 minutes walking with 8 hours apart. Full-time cage activity, resulted however in stronger calluses compared to all the treadmill walking groups. In contrast, free cage activity for 15 minutes each day did not increase the peak force of the healing calluses; only stiffness was increased. Stiffness and energy uptake were also increased by daily treadmill walking. This was seen after 11 and four daily episodes of loading but not after one episode where only energy uptake, not stiffness, was improved.

The cross-sectional area was increased after 11 episodes of daily loading as well as after one loading episode, but not after four episodes. This also reflects on the material properties, peak stress and elastic modulus. These remained unaffected by loading after 11 episodes and after one loading episode i.e. the increased peak force and stiffness were an outcome by the increased cross-sectional area. However, this differed in study II with four daily loading episodes, where peak stress was increased.

The lengthening of the calluses that was seen in the continuously loaded tendons compared to the botulinium toxin unloaded ones did not occur in any of the groups with short daily loading episodes (Table 6).
RESULTS IN BRIEF

Figure 10. Peak force of the healing tendons after short daily loading episodes. White boxes represent completely unloaded tendons, and grey boxes are loaded.
A: Study I, healing tendons with daily loading episodes (15-60 min) for 11 days. All animals were killed 14 days after tendon transection.
B: Study II, healing tendons with four loading episodes (30 min each day) during the early or late phase of healing. Animals were killed 8 (early) or 14 (late) days after transection.
C: Study VI, healing tendons with just one loading episode of 30 minutes, on day 5 after transection. Animals were killed day 8 or day 12.
RESULTS IN BRIEF

Table 6. Gap-distance (mm) of the healing tendons in study I, II, III and VI. The tendons were either unloaded by Botox injections or tail suspension or loaded with short daily loading episodes or continuous loading (free cage activity). Values are expressed as Mean±SD.

<table>
<thead>
<tr>
<th>Study</th>
<th>Days of healing</th>
<th>Unloaded</th>
<th>Short loading episodes (Total N of episodes x Time)</th>
<th>Continuous loading</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>3 days</td>
<td>7.1±0.2 (B)</td>
<td>-</td>
<td>11±1.3</td>
</tr>
<tr>
<td></td>
<td>8 days</td>
<td>7.3±1.6 (B)</td>
<td>-</td>
<td>12±1.9</td>
</tr>
<tr>
<td></td>
<td>14 days</td>
<td>6.2±1.3 (B)</td>
<td>-</td>
<td>11±0.5</td>
</tr>
<tr>
<td></td>
<td>21 days</td>
<td>5.9±2.5 (B)</td>
<td>-</td>
<td>9.3±1.3</td>
</tr>
<tr>
<td>I</td>
<td>14 days</td>
<td>9.7±1.1 (TS)</td>
<td>9.8±0.9 (11 x 15 min c.a.)</td>
<td>13±1.0</td>
</tr>
<tr>
<td></td>
<td>14 days</td>
<td>10±1.2 (TS)</td>
<td>8.7±0.9 (11 x 2 x 15 min) 9.7±1.1 (11 x 30 min)</td>
<td>12±1.4</td>
</tr>
<tr>
<td></td>
<td>14 days</td>
<td>-</td>
<td>8.9±1.5 (11 x 15 min) 7.7±1.2 (11 x 60 min)</td>
<td>11±1.6</td>
</tr>
<tr>
<td>II</td>
<td>8 days</td>
<td>9.6±0.9 (TS)</td>
<td>9.7±1.0 (4 x 30 min)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>14 days</td>
<td>9.4±1.3 (TS)</td>
<td>9.1±1.1 (4 x 30 min)</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>8 days</td>
<td>10±1.0 (TS)</td>
<td>9.9±0.9 (1 x 30 min)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>12 days</td>
<td>9.1±0.7 (TS)</td>
<td>9.5±1.2 (1 x 30 min)</td>
<td>-</td>
</tr>
</tbody>
</table>

B means unloaded by Botox injections, TS means unloaded by tail suspension, 15 min c.a. means 15 minutes of daily free cage activity.

The loading episodes increased the amount of bleeding in the callus tissue

Histology after four daily loading episodes during the early healing in study II showed that signs of bleeding were apparent in both loaded and unloaded calluses. The samples in the loaded group, however, had larger regions with bleeding compared to the completely unloaded group.
Healing tendons with continuous loading had less expression of inflammatory genes and more of ECM and tendon specific genes than unloaded

The mechanical properties of the healing tendons were regulated by loading. It is therefore likely that there is also a change in gene expression pattern with respect to loading. After 3 and 8 days of tendon healing with continuous loading or unloading (by botulinium toxin) 10 genes out of the 24 studied (in study III-V) were differently expressed (Table 7). Inflammation-associated genes, as well as pro-collagens and BMP members like myostatin and follistatin, all had a lower expression in the loaded calluses. Conversely, after 14 and 21 days of healing, most regulated genes had instead a higher expression in the loaded calluses. These genes were mainly related to ECM production and tendon cell specificity. The expression of follistatin and activin receptor-2b (ACTR-2b) was however lower in the loaded samples compared to the unloaded ones at 14 and 21 days of healing. COX-2, iNOS, IL-6, osteogenic protein-1 (OP-1), GDF-6, GDF-7, BMPR-1b, BMPR-2 and ACTR-1b were not differentially expressed in loaded and unloaded tendon calluses at any time-point. The growth factor antagonist noggin was not expressed in any of the tendon samples.

Table 7. Genes differentially expressed in continuously loaded and unloaded tendon calluses. ↓ means lower expression in the loaded calluses compared to unloaded calluses. ↑ means higher expression in the loaded calluses. No arrow means no difference between loaded and unloaded calluses.

<table>
<thead>
<tr>
<th></th>
<th>Loaded Tendons Compared With Unloaded</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
</tr>
<tr>
<td>III</td>
<td></td>
</tr>
<tr>
<td>Inflammation</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>↓</td>
</tr>
<tr>
<td>IL-1</td>
<td>↓</td>
</tr>
<tr>
<td>TGF-β</td>
<td>↓</td>
</tr>
<tr>
<td>ECM</td>
<td></td>
</tr>
<tr>
<td>Procoll. 1</td>
<td>↓</td>
</tr>
<tr>
<td>Procoll. 3</td>
<td>↓</td>
</tr>
<tr>
<td>LOX</td>
<td>↓</td>
</tr>
<tr>
<td>COMP</td>
<td>↑</td>
</tr>
<tr>
<td>Tenascin C</td>
<td>↑</td>
</tr>
<tr>
<td>Tendon specificity</td>
<td></td>
</tr>
<tr>
<td>Tenomodulin</td>
<td>↑</td>
</tr>
<tr>
<td>Scleraxis</td>
<td>↓</td>
</tr>
<tr>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>Growth</td>
<td></td>
</tr>
<tr>
<td>GDF-5</td>
<td>↓</td>
</tr>
<tr>
<td>Follistatin</td>
<td>↓</td>
</tr>
<tr>
<td>V</td>
<td></td>
</tr>
<tr>
<td>Myostatin</td>
<td>↓</td>
</tr>
<tr>
<td>ACTR-2b</td>
<td>↓</td>
</tr>
</tbody>
</table>

53
Similar but different effect on gene expression by one single loading episode

Inflammation-associated genes were regulated by one single loading episode as well as by continuous loading. The most pronounced response of these genes to one loading episode was seen 3 hours after loading. Nitric oxide synthesis as well as PGE$_2$ synthesis both appeared to be up-regulated by one loading episode. This was not seen with continuous loading. The response in these genes after one loading was rather short-lived (some hours). Several genes belonging to the IL-1β family (agonist, antagonist and receptors) were also regulated by one loading episode; both IL-1β and IL-1β receptor antagonist were up-regulated by loading. This contrasts the expression with continuous loading/unloading where IL-1β expression was down-regulated in loaded calluses. Wound healing genes (e.g. CTGF, TGFβ-3, mdk etc.) as well as genes involved in coagulation were also regulated by one loading episode. None of the abundant tendon collagens (I and III) were regulated, however collagen VIII and XI were both down-regulated 3 hours after the loading episode. Also continuous loading down-regulated the collagen expression during the early phase of healing (day 3 and 8). There were also other ECM related molecules which were regulated by one single loading episode, proteoglycans were down-regulated, while aggrecanases (ADAMTS) were up-regulated. The tendon specific markers tenomodulin and scleraxis also appeared to be down-regulated by loading. Down-regulation of scleraxis was also seen during the early phases of tendon healing with continuous loading (day 3 and 8).

The microarray after one single loading episode did also highlight some additional fields which appeared to be regulated by loading. Loading altered the expression of several gene involved in angiogenesis, like angiopoietin, angiopoietin like 1, angiometin and VEGF. The majority of these genes were down-regulated by loading but two of these genes, VEGF and inhibin were up-regulated. Also genes involved in adipocyte differentiation or adipocyte markers were regulated by loading, mostly through down-regulation. However the confirmatory experiment, with real-time PCR did not show any changes in the two selected genes. Finally, several genes associated with reactive oxygen species (ROS) production and oxidative stress were influenced by loading. A few genes were up-regulated, but the majority was down-regulated. This included down-regulation of flavin containing monooxygenases and up-
regulation of heme oxygenase 1, S100a9, and pregnancy-associated plasma protein A. Two heat shock proteins were also up-regulated by loading.

The gene expression is regulated up to 24 hours after one single loading episode

Loading twice each day with 8 hours apart (15 min each time) did not increase the strength of the callus additionally compared to loading ones each day (30 min). When studying the gene expression profile after one single loading episode we noticed that the strongest gene expression response (highest number of regulated genes) was seen 3 hours after the loading episode was finished (Figure 11). There was still a reasonably strong response 12 hours after the loading episode but it was virtually gone by 24 hours. Some genes were regulated at both 3 and 12 hours, but only seven genes were regulated during the first 24 hours.

Figure 11. Number of regulated genes by microarray.
To the left: Number of strongly regulated genes (compared to the pooled control group), at each time-point (3, 12, 24 and 48 hours after loading). Fold change ≥2 and p≤0.05.
To the right: Number of strongly and moderately regulated genes, fold change ≥1.5 and p≤0.01, at each time-point (3, 12 and 24 hours) as well as the number of genes regulated at two or more time-points.
RESULTS IN BRIEF

Unloading by botulinium toxin for 5 days did not have much impact on intact tendons

Unloading by botulinium toxin for 5 days did not have any effect on the mechanical properties of the intact tendons. The effect on the gene expression levels were subtle, only procollagen III, tenascin C and myostatin were down-regulated by unloading. The other 16 genes analysed (inflammation-associated genes were not analysed) remained unaltered.

The difference between the healing and the intact tendons, however, comprised more than just strength.

The lower peak force in healing calluses compared to the intact tendons was expected also at 21 days. Due to the strength of the tendon, intact tendons usually ruptured at the insertion while healing tendons mainly ruptured in the lower callus. The healing tendons had also a larger cross-sectional area and poorer material properties. Energy uptake and displacement at rupture differed depending on loading status. Loaded tendon calluses had an energy uptake at day 14 and 21 similar to intact tendons.

The gene expression in the healing tendons compared with the intact tendons showed higher expression of TGFβ-1, procollagen-1 and -3, LOX, tenascin-C, OP-1, GDF-6, BMPR-1 and -2 and ACTR-1 (Table 8). A majority of these genes had the highest expression during the early phase of healing (day 3 and 8). COMP, GDF-5, GDF-7, follistatin, myostatin and ACTR-2b had an overall higher expression in intact tendons compared to the healing tendons. Tenomodulin expression had a transient down-regulation during early healing and an up-regulation later on.

Table 8. Gene expression characteristic of intact and healing tendons at different time points. The gene name indicates when a gene has the highest expression.

<table>
<thead>
<tr>
<th>Intact tendons</th>
<th>COMP, GDF-5, GDF-7, follistatin, myostatin, ACTR-2b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healing, day 3</td>
<td>TGF-β, OP-1, GDF-6, ACTR-1b</td>
</tr>
<tr>
<td>Healing, day 8</td>
<td>Procollagen 3, LOX, tenascin C, procollagen 1 (UL), scleraxis (UL)</td>
</tr>
<tr>
<td>Healing, day 14</td>
<td>BMPR1b, BMPR2</td>
</tr>
<tr>
<td>Healing, day 21</td>
<td>GDF-5, Tenomodulin, procollagen 1, (L) scleraxis (L)</td>
</tr>
</tbody>
</table>
Myostatin stimulates proliferation

Intact tendons had a higher expression of myostatin than healing calluses. Other members of the same growth factor family (GDF-5, -6 and -7) have been shown to improve tendon healing (38, 39, 41, 126). We therefore wanted to study if treatment with exogenous myostatin also could improve healing. Treatment with myostatin on a collagen carrier increased the cross-sectional area of the healing tendon calluses after both 8 and 14 days of healing (Figure 12). The strength of the calluses were however unaffected as well as the stiffness. Elastic modulus and gap distance were both lower in the myostatin treated tendons.

![Graph showing cross-sectional area (mm²) in tendon calluses, 8 and 14 days after myostatin administration.](image-url)

**Figure 12.** Cross-sectional area (mm²) in tendon calluses, 8 and 14 days after myostatin administration. Myostatin-treated groups are white boxes and control groups are light grey boxes. N = 10 for each group.
DISCUSSION

Unloading during tendon healing is detrimental for the mechanical properties of the tendon callus. Botulinium toxin-induced paralysis of the calf muscles resulted in a significantly weaker tendon callus already after 8 days of healing. The detrimental effect of unloading can be partly reversed by short daily loading episodes.

Rest between loadings might allow the tendon callus to contract

Healing tendons with continuous loading were significantly longer than the unloaded ones. Lengthening during tendon healing is one potential clinical adversity after too much loading early on. However, healing tendons exposed to short loading episodes (in all studies) did not show any lengthening compared to the completely unloaded ones. This suggests that the resting time between the loading episodes might give the callus tissue enough time to contract. In fact, loading on intact tendons have been shown to increase the amount of myofibroblasts in the tendon (112). Myofibroblasts can contract tissues. Short loading episodes might therefore be beneficial for the mechanical properties of healing tendons without risk of persistent lengthening.
Loading generates more matrix but not necessarily of better quality

The main response to loading, irrespective of it was continuous or short episodes, was an increased cross-sectional area. This suggests an increased production of matrix. The material properties (peak stress and elastic modulus) appeared however to be unaltered by loading. This indicates that the matrix produced early on after loading is not of any better quality than the matrix produced in the unloaded tendons. Intact tendons have also been shown to respond and adapt to loading by increased cross-sectional area and not by altered material properties of the tendon (47). An exception to the lack of improved material properties was the increased peak stress after four loading episodes in study II. This was seen after loading during both early and late healing. One discrepancy between this study and the first study, besides the number of episodes, was the time between the final episode and euthanasia. The animals with 11 loading episodes in study I were all killed one day after the last loading episode, while the animals with four loading episodes were unloaded for three more days after the last loading episode before they were killed. We therefore unloaded the animals in study VI, with one loading episode, for 3 days after the loading was finished. We wanted to study if the recovery time after loading was the key for improved material properties. However, these animals only differed in stiffness compared to the unloaded animals. An increased peak force was first seen 7 days after the loading was finished, but the peak stress in these tendons was not different from the peak stress in the unloaded ones. However, because the response after only one loading episode was weaker, it is possible that there was not sufficient power to detect improved tissue qualities.
What is optimal loading?

Even though short loading episodes increased the peak force of the healing tendons, the tendon calluses with continuous loading (i.e. free cage activity) were both stronger and stiffer. A longer duration of each loading episode - 15 minutes compared to 60 minutes each day - only increased the peak force to some extent. Therefore, a longer duration of loading in animals with free cage activity, is presumably not the entire explanation for the discrepancy in peak force. Rats with free cage activity have probably more opportunities to regulate the amount of loading which they apply on the injured limb during the early healing. The treadmill rats had perhaps less of a choice about how to load their tendons and more monotonously loading pattern. Animals with free cage activity could perhaps also apply loading of different frequencies and magnitudes and thereby create deformations or fluid flows for more optimal mechanotransduction.

There is also a possible threshold to get a response, because the animals with only 15 minutes of free cage activity each day did not have stronger tendons compared to the unloaded animals. Studies on intact tendons in humans have shown that there appears to be some kind of threshold for the stress or strain level to induce a response (47).

*Frequency - magnitude - number of cycles*

The increase in collagen synthesis after acute loading appears to be independent of the magnitude of exercise (62). The effect of loading on COX-2 expression and PGE2 production in vitro was shown to be dependent on the stretching frequency but not on the number of cycles (124). However, the expression of scleraxis in bioartificial tendons containing mesenchymal stem cells was dependent on the number of cycles, but it was also dependent on the duration and strain (103). The same model showed that the expression of collagen I was influenced by the strain magnitude, and short rests (10 s) between each cycle increased the expression of both scleraxis and collagen I even more. What is most important for the response to loading: the number of cycles, duration, magnitude or frequency? The different factors most likely create different effects in the tissue regarding fluid flow, strain and shear stress which will affect mechanotransduction.

We have previously attempted to alter the frequency of the loading stimulus by loading healing tendons on a vibration plate (unpublished data). This loading stimulus did not increase the peak force of the healing tendons...
DISCUSSION

compared to loaded tendons without vibrations. However it does not exclude that frequency of the loading is important for the response: other frequencies or magnitudes might be better for mechanotransduction, although the chosen ones are efficacious for bone. We therefore still do not know how the optimal mechanical stimulus looks like.
Inflammation: good or bad?

Inflammation-associated genes, among them IL-1β, were regulated by both continuous loading and by one single loading episode. Continuously loaded tendon calluses had a lower expression of IL-1β than unloaded tendons, while one single loading episode induced a higher expression of IL-1β. Other members of the IL-1β family were also regulated by one loading episode, including its antagonist and receptors. This makes it difficult to determine the net effect of IL-1β expression, and its role in tendon healing. However, our results show that IL-1β appears to be mechanosensitive, which is in line with results from previous in vitro and in vivo experiments (6, 8, 116).

The role of IL-1β in tendon healing and adaption to loading appear to be complex. Too much IL-1β could be detrimental, but a small amount appears to be good. Stretched tendon fibroblasts in vitro can produce a potentially harmful response, by an increased expression of TGF-β, COX-2, MMP-27 and ADAMTS-5 (97). This induced gene expression could be reversed by low doses of exogenous IL-1β (100 pM). However, administration of 10 pM of IL-1β to unstretched tendon fibroblasts also induced an increased expression of COX-2 and MMP-1 and PGE₂ production (130). This IL-1β mediated gene expression could be reversed by a 4% stretching of the cells, but an 8% stretching instead increased the levels additionally. IL-1β and loading therefore appears to regulate each other. We have attempted to inhibit IL-1β during tendon healing but have so far not seen any effects on the mechanical properties of the callus (unpublished). The role of IL-1β during tendon healing therefore needs to be further investigated.

IL-6 is another interleukin which is potentially involved in tendon adaption to loading. IL-6 production has been shown to increase in the peritendinous space after loading (68) and IL-6 infusion can increase the collagen production in tendons (with and without loading) (5). IL-6 might therefore play a role in tendon adaption and perhaps also in tendon healing.

Healing, loading, inflammation and tendinosis are all conditions which appear to regulate or be regulated by the gene expression and protein levels for PGE₂ (COX-2) and NO. This has been shown in vitro and in vivo in both animals and humans. Continuous loading in our model did not significantly regulate the gene expression of iNOS and COX-2. These areas (NO and prostaglandin synthesis) were however regulated after a single loading episode. It is not known whether these proteins are good or bad for the tendon. PGE₂ is increased after a long duration of running, and this response could
both indicate injury by the exercise but it could also indicate that PGE₂ is involved in the adaption to loading. Inhibition of PGE₂ during early tendon healing results in a weaker tendon callus, but inhibition later on in the healing process will improve the material properties (40, 127). Exercise-induced PGE₂ can be inhibited by COX-2-inhibitors, and this also inhibits the increased blood flow seen after exercise (66). Inhibition of COX-1 and COX-2 have also been shown to inhibit the increase in procollagen type I N-terminal propeptide (PINP) levels in the peritendinous space of the Achilles tendon after exercise (23). In vitro studies have shown that inhibition of loading induced PGE₂ results in increased levels of LTB₄ (3). The role of leukotrienes in tendon healing and adaption to loading has been even less investigated, and the connection between prostaglandins and leukotrienes could be important.

Addition of exogenous NO has been shown to improve tendon healing (90, 91, 131). However, NO is also mentioned as a potentially harmful molecule, involved in oxidative stress etc. It appears as a lot of inflammation-associated molecules have dual functions. They are needed in a small amount in the beginning of the healing process, but prolonged exposure or too high levels can be detrimental. This needs to be further investigated.

The general opinion is that exercise should be avoided during the inflammatory phase of healing to minimize disruption of the healing process (122). Our data indicates conversely that the healing process is not disrupted but promoted. Four short loading episodes during the inflammatory phase increased the peak force of the tissue. However, the amount of bleeding was also increased. This could be a result from micro-traumas in the tissue and might explain the up-regulation of wound healing and coagulation genes after one loading episode. An alternative to mechanically induced activation of genes involved in healing or regeneration could therefore be that microinjuries in the healing tissue keep the healing response more active.
All research has limitations

Is a rat model for tendon healing relevant for the situation in humans?

The transferability of research from animals to humans can always be questioned. The knowledge from animals is quite often transferable, but not always, and we don’t know when it is. Animal models are reasonably fast and the studies can usually be more detailed due to substrate availability. Compared to cell cultures, they also include, all the mechanisms, like the proper environment, circulation and immune system etc. We have used rats in these studies, which are quadrupeds while humans are not, this could of course result in discrepancies in the response to loading. Rats are for example able to more easily regulate the amount of loading put on each limb. However, cells in humans and animals most likely experience mechanotransduction by similar or identical mechanisms, and it is likely that the response is at least similar. The size of the Achilles tendons in the rats is small and the distance between the old tendon stumps after transection is a few millimetres. This is roughly the same distance as each fibre bundle has in ruptured human Achilles tendon and we therefore guess that the response to healing with regards to cell migration, capillary growth requirements etc. will be quite similar in rats and humans.

Can we extrapolate the research on the Achilles tendon to other tendons?

We have used the Achilles tendon as a model for tendon healing. This tendon is the largest tendon in the body and it is therefore easy to handle in small animals. It is also prone to injuries. The Achilles tendon heals reasonably well compared to other tendons, partly because there is a rich blood supply in close proximity in the surrounding tissues. Other subcutaneous tendons (like the extensor tendons in the hand) are similar to the Achilles tendon in this respect. It is therefore likely that we can extrapolate the results from the Achilles tendon to these types of tendons. However, tendons and ligaments in synovial fluid are different from the Achilles tendon, because they have poorer nutrition due to limited blood supply and they also have adherence problems during healing. The results from the Achilles tendon will probably not resemble the situation in these tendons. Still, early mobilization is even more important in these tendons to avoid the adherence problems.
Frayed ends or sharp transection

The sharp transection of an otherwise healthy tendon like we do is quite different from the ruptured Achilles tendon in patients. The ruptured tendons almost always have frayed ends and a degenerative background. However, when using an animal model, it is important to have an easily reproducible method. This allows us to measure the effect of an intervention by mechanical testing, without involvement of the surgical technique. We can therefore use reasonably small groups and still have enough power in the analyses.

Short term vs long term effects on healing

We have only studied the healing up to day 21 after transection. This is partly because it is difficult to get true measurements of the peak force in the healing tendons at later time-points. The mechanical testing has some limitations. If the tendon becomes too strong (like intact tendons) the failure will not occur in the mid-substance of the tendon, but at the insertion to the calcaneal bone or by the upper clamp. We have therefore used shorter time-points (mostly 8-14 days of healing) to measure improvements of tendon healing. Furthermore, rats also spontaneously develop cartilage and bone formation in the healing tendon after some weeks. This makes it unsuitable to use this model for a longer follow up. A more long term follow up could perhaps give us more information whether the loaded callus tissue results in more resemble to the original tendon tissue or if loading has created more scar-tissue formation.

The potential sex differences

A limitation in our animal model is that we have only used female rats. Human intact tendons respond to loading differently depending on sex, with regards to collagen synthesis rate and tendon cross-sectional area (82). The mechanical properties also appear to differ between men and women. The fascicles from men are stronger then the fascicles from women. We have previously investigated the role of oestrogen on tendon healing by comparing ovariectomised rats to normal controls, and found no difference in healing properties (unpublished). This however, does not rule out any difference in healing properties between male and female rats. We have chosen female rats because they tend to grow slower compared to male rats, and it might influence the healing process.
**Botox, tail suspension or cast**

There are always advantages and disadvantages with different unloading methods. Unloading by botulinium toxin is a quick and simple procedure which allows us to do fairly large studies easily. The animals are also quite unaffected by this method. Botox unloading has also a clear effect on tendon healing. However, it does not resemble the human situation, where the patient’s foot is immobilized and unloaded in a cast. Also, we can not entirely exclude that any loading occurs. This could on the other hand also occur in patients, where some patients, immobilised by a cast, seem to load their tendons more than others (unpublished).

Tail suspension is an appropriate method when you want to reverse the unloading for just short periods each day. This model is also quite different from the situation in patients, with immobilised joints. However, tail suspension allows us to study the effect of loading *per se*, and not just ankle movements without loading. It is important to discriminate between movements and loading when we want to increase the understanding for treatment strategies (e.g. passive mobilization vs loading with forces). During tail suspension, forces in the Achilles tendon are limited by the antagonistic action of the dorsal flexors. These muscles are weak and have a short lever arm. Therefore the Achilles tendon can only be exposed to weak forces, with possible exception for when the rats scratch themselves.

**Discrepancy between genes and proteins**

We have so far only studied gene expression levels in our experiments (except for the staining for follistatin with immunohistochemistry). Regulations at the gene expression level do not always correspond to changes in protein expression and secretion. The findings in these studies would therefore need to be confirmed at the protein level before any solid conclusion could be drawn. Real-time PCR is a fast way to study responses, but it does not say anything about where in the tissue the genes are expressed (e.g. in the centre or in the periphery) or by which cells. All cells in a tissue do not respond equally to loading/unloading (114). If only a fraction of the cells have a higher expression, this will probably go without finding when using real-time PCR, because it will be clouded by the lower expression the rest of the cells. In situ hybridization or immunohistochemistry can be used to avoid this problem.

When analysing with real-time PCR you need to use reliable house-keeping genes that are stably expressed and this can sometime be hard to find.
DISCUSSION

We used 18S in the first three studies. We found it to be stably expressed among the groups. However in the last study, we used a combination of three different house-keeping genes (which were all stably expressed). By using three genes, the risk of house-keeping gen errors was diminished.

*The number of animals is the limit - RRR*

We have used the same material for the gene expression analysis in study III-V. This could mean that the effects seen in these studies can only be observed in this particular group of animals. However, when using animals in research you are always limited to the number of animals you can use, due to ethical reasons (RRR reduce, refine and replace). This is a limiting factor, and animal experiments are not often repeated. This is also why there are no mechanistic studies done in study I-II.
CONCLUSIONS AND FUTURE RESEARCH

Short daily loading episodes during tendon healing improve the strength of the tendon callus. Loading has an effect already during early tendon healing (during the inflammatory phase) although it is generally believed that loading mainly has an effect first later on, during remodelling. It appears as the loading episodes don’t have to be long, but they need to be applied on a daily basis to sustain a response (at least on the gene expression level). Loading more frequently than once per day, does not seem to improve the healing response additionally. It also appears as short loading episodes do not produce any persisting lengthening of the tendon callus. This observation opens up for new possibilities to use short daily loading episodes as a part of the rehabilitation after tendon rupture.

Continuous loading only altered a few of the chosen genes but not dramatically as for the effect on the peak force and cross-sectional area. The reason for this could be that the response to loading is transient and instead of continuous loading for several days, the loading needs to be better defined. There were several genes which were regulated over time during tendon healing, like ECM components and different members of the BMP-family. The different BMP-members might also have diverse roles during the three phases of tendon healing. A few genes appeared even to be more important in the maintenance of the intact tendon rather than having a role during tendon healing. Genes that are responsible for the improved healing by loading are not easy to point-out. Inflammation definitively appears to be regulated by loading, but the exact role for this during tendon healing needs to be further investigated.
CONCLUSIONS AND FUTURE RESEARCH

What’s next?

We began this project by describing which loading parameters that were important for an improved healing response. We managed to diminish loading until we could use just one episode. This opened up a new possibility to study gene expression: with only one episode. We could see which genes were regulated by loading and mechanotransduction, rather than by the fact that loading had stimulated a better healing response. The effect of one single loading episode was studied 3 hours after loading. This is a fairly long time after loading, which probably results in activation of secondary signalling pathways in addition to the purely mechano-driven pathways. It would therefore be appealing to study the effect of loading at an earlier time-point to find the very first responsive pathways after loading in vivo. It would also be interesting to use this model to study the response to loading in intact tendons. This is probably more difficult, since intact tendons are less responsive to loading.

Inflammation or inflammation-associated proteins like interleukins, NO and eicosanoids are important during tendon healing. They are also involved in the response to loading in both intact and healing tendons. Still, there is much more to know about inflammation and its role in tendons and tendon healing. Inflammation is most likely necessary for tendon healing, but it can also probably do harm. The same factors which are involved in the healing response and adaption to loading are also thought to have a role in tendon overloading and injuries. We need to know how these factors are regulated during tendon healing and also what their role is in intact overloaded and degenerated tendons.

The microarray experiment revealed angiogenesis and production of ROS as two potential mechanisms regulated by loading. A lot of the genes involved in these mechanisms have a variety of functions. It would therefore be interesting to find out more about the role of angiogenesis and ROS production during tendon healing and the potential regulation by loading.

These experiments and a lot of other studies on tendon healing have mainly measured the peak force of the healing tendons shortly after transection. A strong tendon early on probably speeds up the rehabilitation and thereby reduces the health care costs. Early mechanical properties of the tendon have also been shown to correlate with late function (99). However, the question remains whether an initially strong tendon have more resemblance to the original tendon or less. A study on the long term effects of early loading
would be useful for a greater understanding of the benefits and response to loading. Does loading drive the tendon towards scar-formation or regeneration? Tendon markers like scleraxis and tenomodulin could perhaps give some clue whether the healing is directed towards scar or tendon tissue. Before we can really distinguish between scar-tissue, tendon-tissue and callus-tissue, we have to understand more about which markers to use. This could be used to understand if the healing progress is heading in the right direction.

**Take-home message:**

15 minutes of daily loading already at an early stage of tendon healing might improve the rehabilitation after tendon rupture.
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