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Tendon Healing

Mechanical Loading, Microdamage and Gene Expression

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*“The scientist is not a person who gives the right answers,
he is the one who asks the right questions.”*

Claude Lévi-Strauss

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ABSTRACT

Mechanical loading and the inflammatory response during tendon healing might be important for the healing process. Mechanical loading can improve the healing tendon but the mechanism is not fully understood. The aim of this thesis was to further clarify the effect of mechanical loading on tendon healing and how mechanical loading affects the inflammatory response during the healing process.

We used a rat Achilles tendon model to study healing. The rats were exposed to different degrees of loading by unloading methods such as paralysis of the calf muscles with Botox, tail suspension, and an orthosis (a boot). Full loading was achieved by free cage activity or treadmill walking. Microdamage in tendons, unloaded with Botox, was also investigated by needling. The healing tendons were evaluated in a materials testing machine (to analyze the mechanical properties), by gene expression analysis (microarray and PCR), or histology.

Our results show that moderate loading (unloading with Botox) improves the mechanical properties of healing tendons compared to minimal loading (unloading with Botox in combination with tail suspension or a boot), especially the material properties. In accordance to these findings, expression of extracellular matrix genes were also increased by moderate compared to minimal loading.

Full loading improved all mechanical properties and the expression of extracellular matrix genes was further increased compared to moderate loading. However, structural properties, such as the strength and the size of the healing tendon, were more affected by full loading. Full loading also affected the expression of inflammation-related genes during the early healing phase, 3 and 5 days after tendon injury, and increased the number of immune cells in the healing tendon tissue. Also microdamage of the healing tendon (detected by blood leakage) was increased by full loading compared to moderate loading during the early healing phase.

Induced microdamage by repeated needling in the healing tendon tissue increased the structural properties of the healing tendon. The gene expression after needling was similar to the gene expression after full loading.

The improvement of mechanical properties by loading in healing tendons was decreased by an anti-inflammatory drug called parecoxib, which decreases the production of prostaglandins by inhibiting COX-2 activity. The effect of parecoxib was reduced when loading was reduced but we could not confirm that the effect of parecoxib was related to the degree of loading. However, parecoxib abolished the stimulatory effect of microdamage.

In conclusion, these studies show that moderate loading improves the quality of the healing tendon whereas full loading also increases the quantity of the healing tendon tissue. Full loading creates microdamage and increases inflammation during the early healing phase. The strong effect of full loading on the structural properties might be due to microdamage. Indeed, the anti-inflammatory drug parecoxib seems to impair mechanical stimulation of healing tendons by reducing the response to microdamage.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hälsenerupturer läker långsamt och rehabiliteringen tar lång tid. Det kan ta 3 månader innan patienten kan gå normalt och upp till 9 månader innan man kan återvända till sportsliga aktiviteter. Hälsenerupturer har länge behandlats med gips eller en ortos (en stel stövel) för att avlasta den läkande senan från belastning och dragkrafter. Den senaste forskningen har dock visat att belastning kan förbättra senans läkningsprocess och förkorta rehabiliteringen. Den nya rehabiliteringen av hälsenor involverar oftast svaga belastningar under en kort tid varje dag. Hur belastning förbättrar den läkande senan är ännu oklart och mer forskning krävs för att optimera rehabiliteringen.

Syftet med denna avhandling har varit att öka förståelsen för hur belastning påverkar senans läkning. Olika belastningsnivåer har studerats samt effekten av ett anti-inflammatoriskt läkemedel som ofta används av patienter med hälseneskador.

Vi har sett, med hjälp av en djurmodell, att svaga belastningar på hälsenan har stor effekt på senans materialegenskaper. Senan blir starkare och kvalitén på sen-vävnaden blir bättre utan några tecken på skador i den läkande senan. Däremot har starka belastningar visats sig orsaka skador i den läkande senan samt ökat inflammationen. Starka belastningar ger dock en starkare sena, jämfört med svaga belastningar, men det beror mest på att kvantiteten av sen-vävnaden verkar öka mer än kvalitén. Vi har också kunnat visa att skador på den läkande sen-vävnaden, i form av nålstick, kan göra senan större och starkare, så skador i vävnaden kan ha en stimulerande effekt på läkningsprocessen. Dessutom verkar ett anti-inflammatoriskt läkemedel kunna hämma belastningens stimulerande effekt på senans läkning genom att minska effekten av de mikroskopiska skador som uppkommer vid starka belastningar. Huruvida starka belastningar och skador är bra för den läkande senan under en längre period är ännu inte utrett och bör studeras vidare.

Sammanfattningsvis har vi kunnat visa att svaga belastningar kan ha stor effekt på den läkande hälsenan utan att ge några större skador på vävnaden eller öka inflammationen. Detta visar att de nya rehabiliteringsprogrammen för hälseneskador ser lovande ut för kommande patienter.

LIST OF PAPERS

- I. Andersson T, Eliasson P, **Hammerman M**, Sandberg O and Aspenberg P.
Low-level mechanical stimulation is sufficient to improve tendon healing in rats.
Journal of Applied Physiology 2012; 113: 1398-1402.
- II. **Hammerman M**, Dietrich-Zagonel F, Blomgran P, Eliasson P and Aspenberg P.
Different mechanisms activated by moderate versus full loading in rat Achilles tendon healing.
Manuscript.
- III. **Hammerman M**, Blomgran P, Dansac A, Eliasson P and Aspenberg P.
Different gene response to mechanical loading during early and late phases of rat Achilles tendon healing.
Journal of Applied Physiology 2017; 123:800-815.
- IV. **Hammerman M**, Aspenberg P and Eliasson P.
Microtrauma stimulates rat Achilles tendon healing via an early gene expression pattern similar to mechanical loading.
Journal of Applied Physiology 2014; 116: 54-60.
- V. **Hammerman M***, Blomgran P*, Ramstedt S and Aspenberg P.
COX-2 inhibition impairs mechanical stimulation of early tendon healing in rats by reducing the response to microdamage.
Journal of Applied Physiology 2015; 119: 534-540.

*Equal contribution

The original papers in this thesis will be referred to by their Roman numerals.

INTRODUCTION

Tendons are important for body movements as they transmit the forces from muscles to bone (25, 38, 42). They consist of extracellular matrix, such as collagens and proteoglycans, and cells called tenocytes (23, 25, 38). Approximately 90% of the dry weight of tendons consists of collagen 1, which is arranged into fibrils in a parallel manner according to the direction of the force, which gives them a high tensile strength (23, 38, 42). Tendons are elastic and the fiber bundles are arranged in a crimp pattern which is stretched out during loading (23, 38).

Tendons adapt to mechanical loading via tenocytes (25, 38, 53). The cells are connected to the extracellular matrix through different cell receptors and can thereby detect mechanical changes via deformations (11, 25, 53). This initiates an intracellular response, which in the end can affect transcription factors and lead to changes in gene expression and protein synthesis (11, 25, 36-38, 53). For example, to enable the tendon to cope with increased loading tenocytes can increase the production of collagen (11, 12, 23, 53), even though the turnover of tendon tissue seems to be very limited (33). The mechanism by which cells detect mechanical loading is called mechanotransduction (11, 23, 25, 53).

Even though tendons can withstand strong forces, rupture of tendons can occur. Achilles tendon ruptures are common in humans performing sports activities such as football, tennis and squash, especially among untrained middle-aged men (23, 48). Age and inactivity weaken the tendons and increase the risk of rupture (13, 14, 25, 31, 39, 42, 50, 51, 57).

The standard treatment for Achilles tendon rupture, which has been used for a long time in the clinics, includes immobilization in a cast or a brace for more than 6 weeks (35). Normal walking is often possible after 3 months, and it takes around 9 months to return to sports activities (48). The healed tendon might not be as good as it was before the rupture, for example due to tendon elongation (23, 42). It is therefore important to study tendon healing to improve the rehabilitation after tendon injuries.

There are major limitations in how you can study tendon healing in humans, especially if you want to investigate the mechanisms more deeply. Therefore, animal models are used to circumvent this. Animal models provide the ability to produce consistent and reproducible injuries that can be treated in a controlled and quantifiable manner (31). Even though animal models cannot truly replicate the human condition they allow us to understand cellular and tissue-level principles in the context of a living organism (31, 42). In this thesis, rat Achilles tendons have been used to study healing (Figure 1). Tendon injury was created by a full transection of the right Achilles tendon and the tendon was allowed to heal spontaneously, without any sutures.

The healing process of tendons can be divided into three overlapping phases: inflammatory, proliferative and remodeling (25, 38). During the inflammatory phase, immune cells are recruited into the site of injury and orchestrate the healing process (10, 25, 38). Neutrophils are the first cells to arrive, followed by macrophages and T cells (25, 38). Inflammation starts with a pro-inflammatory response, which leads to an increase of immune cells and finally turns itself off with an anti-inflammatory response, which enhances the production and

remodeling of new tissue (10, 42). As the healing process moves further in to the proliferative and remodeling phases, fibroblasts are recruited into the healing tendon (25, 38). The fibroblasts, some of which eventually will become tenocytes, start to proliferate and produce extracellular matrix components such as collagens (25, 38). The duration of each healing phase is difficult to define and different in different species. However, we believe that in rats, the inflammatory phase lasts 1-5 days, the proliferative phase spans days 3-12 after injury, and the remodeling phase starts around day 7-10.

Many researchers have tried to improve Achilles tendon healing by adding different factors into the wound, such as growth factors, platelet-rich plasma (PRP), stem cells etc (6, 23, 26, 42). However, our research group has lately focused on the effect of mechanical loading during Achilles tendon healing, because it seems to improve healing more dramatically (3, 16, 18, 42). Mechanical loading can increase the strength of the healing tendon by approximately 60%, and only 5 minutes of exercise each day is needed to increase the strength of the healing tendon tissue (3, 19).

The positive effect of mechanical loading during tendon healing is now well known. Many clinics have developed routines for early mobilization after Achilles tendon rupture, and report favorable results (23, 25, 35, 52). However, the underlying mechanisms are still unclear and need to be further investigated. This thesis tries to clarify some of the questions that have been unanswered.

Figure 1. Achilles tendon from a rat. A) Intact Achilles tendon in rat. B) Healing Achilles tendon in rat, 1 week after transection without suture.

AIM AND HYPOTHESIS

The aim of this thesis was to further clarify the effect of mechanical loading on tendon healing and how mechanical loading affects the inflammatory response during the healing process. This was accomplished by five papers.

Paper I

Aim: To evaluate mechanical properties in healing tendons exposed to different degree of loading.

Hypothesis: Also mild mechanical loading (moderate loading) would increase the strength of the healing tendon.

Paper II

Aim: To evaluate mechanical properties and gene expression in healing tendons exposed to three different degrees of loading and to verify blood leakage, as a sign of microdamage, in fully loaded healing tendons.

Hypothesis: Increased loading, from minimal to moderate, would increase the structural and material properties of the healing tendon tissue. And further increased in loading, from moderate to full, would create microdamage and increased inflammation leading to a larger tendon size.

Paper III

Aim: To investigate the gene response after mechanical loading in two different healing phases during tendon healing; the inflammatory phase and the early remodeling phase.

Hypothesis: This study was descriptive, however the general underlying hypothesis was that the response to loading would be different during different phases of the healing process, especially regarding inflammation-related genes.

Paper IV

Aim: To evaluate mechanical properties and gene expression in healing tendons exposed to microdamage induced by needling in the absence of full loading (unloading with Botox).

Hypothesis: Needling would make the tendon stronger. The gene expression analysis, a part of the experiment, was descriptive, where we investigated if the gene response to needling was similar to the gene response to loading.

Paper V

Aim: To investigate if the impairment of mechanical properties in healing tendons by a COX-2 inhibitor is related to the degree of loading (mechanotransduction mechanisms) or to microdamage.

Hypothesis: In the first experiment, regarding different degrees of loading, we hypothesized that the negative effects of COX-2 inhibition would only appear when loading is applied. The findings in the first experiment led to a second separate experiment where we hypothesized that COX-2 inhibition would inhibit the response to microdamage.

COMMENTS ON MATERIALS AND METHODS

To study the effect of mechanical loading during tendon healing, many unloading models have been developed. Unloaded tendons, which have not been stimulated by mechanical loading, serve as a control for the mechanically loaded tendons. In this thesis, the rats have been unloaded by tail suspension, a boot, or by paralyzing the calf muscles with Botox, alone or in different combinations.

Improvement of the healing tendon tissue formation can be confirmed by histology or measurements of the mechanical properties of the healing tendon. The last method has mainly been used in this thesis. The healing tendon has been dissected out and tested in a materials testing machine (Figure 2 and 3). The properties of the tendon tissue can be described by a number of mechanical parameters, which can be divided into structural and material properties. The structural properties are the properties that can be measured by a caliper or a materials testing machine, such as transverse area, tendon length, gap length, peak force, stiffness, and energy. The material properties are calculated afterwards to remove the effects of dimensions, such as peak stress and elastic modulus. These properties show the quality of the healing tendon tissue.

To study the effect of mechanical loading in tendon healing more deeply, we used gene expression analysis. A gene is a section in the DNA that is coding for one specific protein. Proteins are essential for the organism and participate in almost every process within cells. When a protein is needed in the cell, a copy of the gene is made. This gene copy is made of RNA and is called messenger RNA (mRNA). Messenger RNA is then used as a code to build the specific protein that is needed. Gene expression analysis measures the number of specific mRNA molecules in the cells, which is an indirect measure of the production rate of the specific protein at this time point.

By comparing the gene expression to a control, for example moderate loading (control) versus full loading, it will show how the expression of a gene changes when more loading is applied, and this is described as fold change (FC). If the fold change has a positive number it means that there is more mRNA of this gene in the full loading group compared to the control group, and the gene expression has increased. This is likely to indicate an increased production of the coded protein. If the fold change has a negative number the gene expression is decreased, meaning that the mRNA of the gene studied is less in the full loading group.

Microarray and polymerase chain reaction (PCR) have been used in this study to measure gene expression. In microarray analysis, all genes that are coding for a protein in the cell are measured. It means that approximately 25 000 genes are studied at the same time. This leads to a high number of false positive results as the probability of finding a significant difference by chance increases when the number of tests is increased. In PCR, only one gene at a time is analyzed which increases the probability of a reliable result. Therefore, genes that show a significant difference in microarray analysis are often confirmed by PCR. However, microarray results can be analyzed by data programs such as Ingenuity pathway analysis, that connect the genes to each other and different pathways and functions, enabling us to detect

patterns and understand multiple gene changes in a broader perspective. It gives us a clue of what these gene changes might affect at a cellular and tissue level.

Figure 2. Evaluating mechanical properties of healing Achilles tendons from rats with a materials testing machine. A) The materials testing machine used in this thesis. B) Healing Achilles tendon measured by a caliper before mounted in the machine. C) Healing Achilles tendon from a rat tested in the machine. D) A typical curve from a healing Achilles tendon. The healing tendon is pulled to failure and the highest point of the curve shows how much force the tendon can withstand before it ruptures (peak force = tendon strength) E) The slope of the curve shows the stiffness of the healing Achilles tendon. Y-axis = Force (N). X-axis = Distance (mm).

Figure 3. Measuring of a healing rat Achilles tendon by the materials testing machine. A) The healing tendon is first mounted in the material testing machine without being stretched. B) The machine starts to pull the tendon with a speed of 0.1 mm/s. C) The tendon is stretched out while the force is increased until it cannot withstand the force and it ruptures. The picture is taken with permission from Franciele Dietrich-Zagonel's thesis "Efeito do plasma rico em plaquetas no reparo do tendão de aquiles em ratos".

STUDY DESIGNS

Full loading - Rats exposed to full loading were allowed free cage activity during the whole experiment.

Moderate loading - Rats exposed to moderate loading were either treated with Botox injections or tail suspended (Figure 4). Moderate loading is also called partial unloading in Paper V.

Botox was injected into the calf muscles 3 or 4 days before tendon transection surgery to paralyze the muscles. Paralysis of the calf muscles prevents the rats from contracting their muscles that normally pull on the tendon, which reduces the stimulation of loading. However, the rats can still walk on the injured leg and the remaining stiffness of the muscle will cause traction forces in the tendon with passive motion of the joints leading to mild mechanical stimulation.

Tail suspension of the rats was done the day after tendon transection surgery. An adhesive tape was attached to the rat's tail and the tape was connected to an overhead system by a fish-line swivel and a fish line. The overhead system allowed the rats to rotate and move in all directions using their fore legs, whereas the hind legs were lifted just above the cage floor. This prevents the rats to walk on their Achilles tendon, thereby reducing the stimulation of loading. However, the rats can still scratch themselves and do isometric contractions leading to mild mechanical stimulation.

Minimal loading - To reduce the loading as much as possible two unloading models were combined: Botox & Tail suspension or Botox & a Boot (Figure 4). The boot was fitted over the ankle joint directly after tendon transection surgery. The customized boot is made of metal in two parts, and held together with 2 screws. Minimal loading is also called unloading in Paper V.

Microdamage by needling - To study the effect of microdamage without the simultaneous effect of loading, the rats were treated with Botox injections to reduce loading and the healing tendon tissue was penetrated with an insulin needle (Figure 4). The needle was forced into the healing tendon tissue 5 times in four different directions, resulting in a total of 20 punctures each day. In studies evaluated by mechanical testing the needling was performed once a day, 2-5 days after surgery. For gene expression analysis, needling was done only the same day as the analysis.

Treadmill walking - Treadmill walking was used to make sure that the rats were mechanically loading their healing Achilles tendon during a specific time (Figure 4). The rats walked on the treadmill for 5 minutes at 9 m/min and were monitored during the whole episode. The rats had also been acclimatized to the treadmill before the experiment started.

Figure 4. Different methods used in this thesis. Our most often used method to reduce loading on the healing Achilles tendon in rats was to inject Botox in the calf muscles to paralyze the muscles. We also used tail suspension or a boot, or a combination of these unloading models. To study microdamage in the healing tendon, needling of the healing tendon tissue was performed to induce microdamage. Treadmill walking was used to make sure that the rats load their healing Achilles tendon during a specific time. Illustrations by Per Aspenberg.

SUMMARY OF THE RESULTS

Mechanical testing

Taken together, all the mechanical testing results show that mechanical loading has a big impact on the mechanical properties with a good reproducibility (Table 1). Moderate loading can increase both structural and material properties without increasing the size of the tendon. Full loading also increased the structural and material properties, but it also increased the size. Additionally, the structural properties were mostly more affected by full loading compared to moderate loading, as the increase in these parameters was much higher in full loaded tendons. For example, the mean increase of peak force by full loading is 190% whereas the mean increase by moderate loading is 68%. Microdamage by needling affects the healing tendon tissue by increasing structural properties.

The results in Table 1 show that each loading condition has a good reproducibility and the different unloading models seems to influence the healing process in a similar way, although Botox in combination with a boot has less effect compared to the other combined models. The properties that have less reproducibility are transverse area, stiffness, peak stress, and elastic modulus. All of these properties are derived from values where the investigator has played an important role, for example measurements from a caliper, which may explain a poorer reproducibility between the experiments. The caliper was used by only one person for each experiment, but measurement in different experiments has been done by different persons. However, we have recently investigated the difference of transverse area measurements between two investigators and found that the error was 2.7 mm², which corresponds to 19% of the mean, and the correlation was 0.935.

Table 1. Difference (%) in mechanical properties of healing rat Achilles tendons between different loading conditions and induced microdamage.

Paper	Moderate vs minimal loading				Full vs moderate loading				Microdamage vs control	
	I	I	II	V	I	I	II	V	IV	V
Unloading models	B&T vs B	B&T vs T	B&Bo vs B	B&T vs B	B	T	B	B	B	B
Structural properties										
Transverse area (mm ²)	2	7	4	-5	105	95	62	54	20	79
Peak force (N)	79	77	33	81	200	204	178	183	45	46
Stiffness (N/mm)	114	105	43	100	53	60	64	76	19	-7
Energy (Nmm)	43	51	14	19	475	446	433	350	126	150
Material properties										
Peak stress (N/mm ²)	58	83	29	83	58	36	71	92	14	-14
Elastic modulus (MPa)	119	155	33	120	6	-9	53	67	-7	-48

The rat Achilles tendons were evaluated either 7 or 8 days after tendon transection. The numbers shows the increase or decrease (-) in percentage (%) between the two groups analyzed. B&T means Botox & Tail suspension. B means Botox. T means Tail suspension. B&Bo means Botox & a Boot.

Gene expression

Our gene expression analysis shows that inflammation-related genes were affected by full loading during the early healing phase, i.e. 3 and 5 days after injury (Table 2). Most of these genes were up-regulated by full loading and involves pro-inflammatory mediators, chemokines and interleukins among others. The regulation of these genes by full loading can be seen both after only one episode of loading, in an otherwise unloaded tendon, and in tendons that have been loaded continuously during the whole experiment. Inflammation-related genes were also up-regulated by microdamage by needling. Moderate loading does not strongly affect inflammation-related genes as only iNOS was up-regulated during this condition.

Table 2. Inflammation-related genes affected by different loading conditions and induced microdamage.

Paper	A & B		III	IV	II	II
Stimulation	Short time full loading immediately after tail suspension			Induced microdamage	Moderate loading (continuous)	Full loading (continuous)
Day after injury	3	5	14	5	5	5
CCL20	↑					
CCL7	↑	↑ [□] *				
IL-4RA	↑					
IL-6	↑	↓ [□] *				↑
NFIL3	↑					
PTX3	↑					
SBNO2	↑					
SOCS1	↑					
TLR2	↑					
IL-1B		↑ [□]		↑		
iNOS		↑ [□]		↑	↑	
PTGES		↑ [□]		↑		↓
IL-1RN		↑ [□]		↑		
TNF						↑
IFN-γ						↑
VCAM-1						↓
IL-10						↓
COX-2						↑

Paper A means Eliasson et al. 2012 (17) and Paper B means Eliasson et al. 2013 (19).
↑ means up-regulation. ↓ means down-regulation. □ means data from Paper A and • means data from Paper B. * means microarray results, not confirmed by PCR.

Our gene expression analysis also shows that loading and microdamage affects other genes related to angiogenesis, blood coagulation, extracellular matrix, and transcription factors etc. (Table 3). Full loading, applied continuously, during the early healing phase increased the expression of extracellular matrix genes (collagen 1 (COL1a1) and 5 (COL5a1)) but this was also seen during moderate loading conditions (collagen 1 (COL1a1) and 3 (COL3a1), and lysyl oxidase (LOX)). The gene expression directly after stimulation by full loading and microdamage by needling, 5 days after injury, affected a lot of genes in a similar pattern. Vascular endothelial growth factor (VEGF), blood coagulation factors (F3 and F5), and extracellular matrix protease (ADAMTS4) were all increased in both conditions. Transcription factors (EGR1, C-FOS and FOSB) were also increased at day 5, and also by full loading 3 days after injury (EGR1 and C-FOS). Scleraxis (SCX), a tendon specific gene, was decreased by both full loading and microdamage at day 5.

Table 3. Other genes affected by different loading conditions and induced microdamage.

Paper	III	A & B	III	IV	II	II
Stimulation	Short time full loading immediately after tail suspension			Induced microdamage	Moderate loading (continuous)	Full loading (continuous)
Day after injury	3	5	14	5	5	5
VEGF		↑ [□]		↑		
ANGPTL1		↓ [□]		↓		
F3		↑ ^{□*}		↑		
F5		↑ ^{□*}		↑		
C3				↑		
COL1a1					↑	↑
COL3a1					↑	
LOX					↑	
COL5a1						↑
ADAMTS4		↑ [□]		↑		
EGR1	↑	↑ [▪]		↑		
C-FOS	↑	↑ [▪]		↑		
FOSB		↑ [▪]		↑		
RGS1		↑ [▪]		↑		
PAPPA		↑ [□]		↑		
SCX		↓ [□]		↓		

Paper A means Eliasson et al. 2012 (17) and Paper B means Eliasson et al. 2013 (19).

↑ means up-regulation. ↓ means down-regulation. [□] means data from Paper A and [▪] means data from Paper B. * means microarray results, not confirmed by PCR.

DISCUSSION

Different loading conditions have different effects on healing tendons

Mechanical loading has been shown to have a big impact on the healing tendon tissue by improving both the structural and material properties (3, 4 (Paper I), 16, 19, 22, 43, Paper II). As little as 5 minutes of daily loading is sufficient to improve the strength of a healing tendon (19). Mechanical loading also has a huge impact on gene expression. Only few minutes of mechanical loading can change the expression of hundreds of genes (17, 29 (Paper III)). However, the gene response to mechanical loading lasts for about 24 hours, which suggests that only a few minutes of daily exercise of the healing tendon should be sufficient to improve the healing process in patients (3, 19). However, these results are based on animal models where the animals are unloaded by tail suspension and the healing tendon is stimulated by full loading on a treadmill, and these conditions might not correspond to the situation in patients.

Patients with Achilles tendon injury are immobilized with a brace for several weeks, which prevents them from loading their healing tendons (35, 46). The clinical rehabilitation programs that have recently been introduced with favorable results may involve weight bearing on the foot, but still only minor loading of the tendon (15, 23, 25, 35, 47, 52). We therefore do not think that full loading in rats corresponds to the human situation. The rats seem to walk normally on the injured leg after tendon injury and may load their healing tendon heavily. Our unloading models with Botox or tail suspension might correspond to the human situation better as these models might still stretch the tendons, although only mildly.

Therefore, to investigate if mild mechanical stimulation can improve healing Achilles tendons in rats we needed to reduce the loading even further. This was achieved by combining two unloading models, either Botox & tail suspension or Botox & a boot (4 (Paper I), Paper II). These two unloading models (minimal loading) were compared to unloading with Botox (moderate loading) which showed that even moderate loading can improve tendon healing dramatically, compared to minimal loading. There were significant differences between minimal and moderate loading for structural properties (peak force and stiffness) and material properties (peak stress and elastic modulus). Gene expression analysis also showed an increase of extracellular matrix genes which corresponds to the improvement of mechanical properties (Paper II). Collagen 1 (COL1a1), collagen 3 (COL3a1), and lysyl oxidase (LOX) were increased by moderate loading compared to minimal loading.

Moderate loading was also compared to full loading which showed a significant difference for all parameters tested (Paper II). Full loading increased the structural properties (peak force, stiffness, transverse area, energy and gap length) by 62-433% and the material properties (peak stress and elastic modulus) by 53-71% (Paper II). The most important effect of full loading was that the size of the healing tendon tissue (transverse area) was increased, which was not seen with moderate loading (Table 1). Also the strength of the healing tendon tissue (peak force) was more affected by full loading compared to moderate loading.

Moderate loading increased peak force by 33-81% compared to minimal loading whereas full loading increased peak force by 178-204% compared to moderate loading (Table 1). These results suggest that moderate loading improves the material properties of the healing tendon tissue, while full loading has a stronger effect on the structural properties. In other words, moderate loading improves the quality of the healing tendon tissue while full loading increases the quantity of the healing tendon tissue.

Gene expression analysis from full loading showed an increase of extracellular matrix genes (collagen 1 (COL1a1) and collagen 5 (COL5a1)) which corresponded to the improvement of mechanical properties, especially regarding the material properties as seen with moderate loading (Paper II). Additionally, full loading also affected a lot of inflammation-related genes, especially pro-inflammatory mediators which are the first genes in an inflammatory response (Table 2). The observation that only tendon size and inflammation genes were affected by full loading suggests that there might be a connection between these two events which will be further discussed below.

Overall, these results suggest that moderate loading increases the expression of extracellular matrix genes, such as collagen 1, and improves the healing tendon tissue, especially the quality of the tendon tissue. As the moderate loading model in rats probably corresponds better to the human situation, it shows that rehabilitation with moderate loading will dramatically improve the healing process of the healing Achilles tendon. In other words, our results might explain why these rehabilitation programs, with moderate loading, have such a favorable result in patients.

Full loading affects inflammation in healing tendons

Mechanical loading can improve tendon healing both when applied during the inflammatory, proliferative and remodeling phase of the healing process (16, 22). Although beneficial in all three phases, the mechanism and the response to mechanical loading might be different as the tissue composition is different in the different phases (25, 41). During the inflammatory phase, the healing tissue contains a high proportion of immune cells and a loose fibrous stroma rich in collagen 3. This tissue gradually matures during the proliferative phase when fibroblasts migrate into the tissue, proliferate, and start to produce collagen 1. Later in the remodeling phase, the healing tendon contains a denser connective tissue dominated by collagen 1 together with fibroblasts or tenocytes and few immune cells. To better understand how mechanical loading improves tendon healing in different healing phases, we studied gene expression after one isolated episode of mechanical loading during the respective phases, in otherwise unloaded healing tendons with tail suspension (17, 29 (Paper III)). This enabled us to study a time sequence, i.e. which genes are the first ones to be regulated after the loading episode.

The results from these studies showed that the immediate gene response, 15 minutes after loading, seems to be similar regardless of the healing phase (19, 29 (Paper III)). The immediate gene response after mechanical loading mainly involves an up-regulation of the transcription factors EGR1 and C-FOS. Both of these genes seem to be important for collagen 1 production, and EGR1 is important for tendon development and healing.

In the late gene response, 3 hours after loading, 90-150 genes were regulated, and as expected, the gene response was quite different between the healing phases (17, 29 (Paper III)). During the inflammatory phase, 3 days after tendon injury, mechanical loading regulated a lot of genes involved in inflammation. The recruitment of leukocytes increased as well as the number of leukocytes in the healing tendon tissue. Chemokines, attracting different leukocytes, were increased by loading such as CCL7 and CCL20. Mechanical loading also regulated the inflammatory response by affecting macrophages and T cells by an up-regulation of IL-6, TLR2, NFIL3, SBNO2, and SOCS1. Interleukin-6 (IL-6) was highly up-regulated and might be important for both regulating the inflammatory response and improving the healing tendon tissue by increasing collagen production.

During the proliferative phase, 5 days after tendon injury, mechanical loading still strongly affected inflammation, as Eliasson et al. has shown (17). Inflammation-related genes, such as IL-1 β , iNOS, PTGES, IL-6, and CCL7, were increased. However, mechanical loading also affected genes involved in scar formation, extracellular matrix production, angiogenesis, oxidative stress, and differentiation of adipocytes and tenocytes. It seems that mechanical loading induces more scar formation, decreases angiogenesis, and inhibits differentiation of adipocytes as well as tenocytes.

During the early remodeling phase, 14 days after tendon injury, mechanical loading seemed not to affect inflammation as strongly as in the previous healing phases (29 (Paper III)). The effect of full loading at this phase is more uncertain but might affect cellular growth, proliferation, and development.

Even though the late gene response in the three different healing phases was quite different, some genes showed the same response both day 3 and 14. These genes might be important for the response to full loading in tendon healing regardless of the healing phase (29 (Paper III)). These genes affect extracellular matrix remodeling, promote migration and proliferation of fibroblasts, increase collagen production, regulate the immune response, and increase the recruitment of immune cells.

In summary, full loading seems to have a big impact on inflammation during the inflammatory phase. The effect on inflammation gradually declines as the healing process progresses to the later healing phases. In the proliferative and remodeling phases, full loading also affects other events such as extracellular matrix, angiogenesis, proliferation, and differentiation. However, there might also be a general response to full loading that affects inflammation, extracellular matrix and fibroblasts regardless of the healing phase.

It does not come as a surprise that full loading has a big impact on inflammation during the inflammatory phase as there are a lot of immune cells in the tendon tissue during this phase. And it is not surprising that full loading affects fibroblasts or the extracellular matrix in the later phases as the tissue during these phases are more mature and contains of more fibroblasts compare to immune cells. Instead, the most surprising result is that full loading affects inflammation during the proliferative phase, as Eliasson et al. has shown (17). However, recent studies have brought an explanation to this effect, namely the creation of microdamage.

Full loading cause microdamage thereby stimulating tendon healing

The first tissue forming after a tendon injury is mechanically weak. After an Achilles tendon rupture, humans protect their injured tendon from mechanical forces by limping. However, the behavior in humans and rats is quite different when it comes to protecting an injured limb, because rats have evolved to avoid predation. Predators prefer weak prey, and rats therefore are forced to avoid showing weakness by limping, and thus are unlikely to protect their injured limbs as humans do. Directly after tendon transection surgery, the rats are up walking and jumping on their injured leg as if it has not been injured. This way of unprotecting their injured tendon from mechanical loading leads to small bleedings in the healing tendon tissue, indicating microdamage (Paper II). This phenomenon was first discovered from histology where extravasated erythrocytes were found interspersed in the healing tendon tissue (16). Further studies have shown that these events are common after full loading during the inflammatory and proliferative phases of tendon healing in rats (Paper II).

Tissue damage release alarmins, also called damage-associated molecular patterns (DAMPs), and bleedings activates platelets (34, 56). Alarmins and activated platelets leads to an inflammatory response, where immune cells are activated and inflammatory mediators are increased (34, 56). Therefore, microdamage in the healing tendon tissue should increase inflammation, which was also shown in the gene expression studies described previously (28 (Paper IV)). Additionally, microdamage seems to affect the mechanical properties of the healing tendon tissue (28 (Paper IV), 30 (Paper V)). Microdamage by itself, created by needling, can increase the structural properties such as peak force, transverse area and energy by 20-150% (28 (Paper IV), 30 (Paper V)). Interestingly, the structural properties are more affected by full loading compared to moderate loading (4 (Paper I), Paper II). This suggests that an additional stimulation has been added by going from moderate loading to full loading, and this additional stimulation is probably due to microdamage.

To confirm this theory even further, gene expression analysis has shown that the gene response seen after microdamage by needling is very similar to the gene response seen after full loading (28 (Paper IV)). The only big difference between these two stimulations was the inflammation-related genes, such as IL-1 β , iNOS, and PTGES. These genes were increased to a much higher level by needling compared to loading. However, these findings strengthen our

theory even more, as these genes are probably more affected by microdamage, and needling is likely to create more damage in the healing tendon tissue compared to full loading.

Mechanotransduction and microdamage stimulates tendon healing differently

Mechanical loading during tendon healing seems to stimulate the tendon tissue both via mechanotransduction and microdamage (4 (Paper I), 28 (Paper IV), 30 (Paper V), Paper II). The role of mechanotransduction and microdamage depends on the degree of loading (4 (Paper I), Paper II).

During mild mechanical stimulation (moderate loading) the healing tendon tissue is not damaged, and only mechanotransduction stimulates the healing tendon tissue (4 (Paper I), Paper II). This stimulation improves both structural and material properties of the healing tendon without increasing the size of the tendon. This suggests that stimulation via mechanotransduction mechanisms improves mostly the quality of the healing tendon tissue.

During strong mechanical stimulation (full loading) the healing tendon tissue is not strong enough to withstand the force that is applied, and this will create microdamage in the healing tissue (Paper II). Damage of the tissue increases inflammation by an up-regulation of inflammation-related genes such as pro-inflammatory mediators (34, 56). This leads to an increased recruitment of immune cells, especially of the pro-inflammatory types, such as M1 macrophages (34, 56). Microdamage by full loading might therefore prolong the pro-inflammatory response and delay the switch of the inflammation to the anti-inflammatory response in the healing process, as Blomgran et al has shown (8). Strong mechanical stimulation of the healing tendon improves almost all mechanical properties but the structural properties are much more affected compared to mild mechanical stimulation (4 (Paper I), Paper II). Most of these improvements are probably due to mechanotransduction mechanisms. However, the additional improvements on the structural properties, such as transverse area and peak force, are probably due to the stimulatory effect from microdamage. Microdamage increases inflammation which can also affect fibroblasts, by increase the recruitment of them, their proliferation and their deposit of extracellular matrix (34). More fibroblasts in the healing tendon tissue and an increase of extracellular matrix deposit, such as collagen, are likely to increase the size of the tendon tissue. A bigger tendon might withstand stronger forces, which lead to an increased strength of the healing tendon.

The additional improvements on the structural properties can be abolished by treatment of a non-steroidal anti-inflammatory drug (NSAID) called parecoxib, which inhibits the enzyme cyclooxygenase-2 (COX-2). This enzyme can be induced during inflammation and is important for the production of prostaglandins (49). These mediators are important during inflammation but they can also have other functions such as during mechanotransduction (12, 49, 53, 54, 56). Prostaglandin E2 and its enzyme prostaglandin E synthase (PTGES), as well

as COX-2, are increased by mechanical loading and seem to play a role during mechanical stimulation in tendons and tenocytes (12, 40, 45, 53, 54). The enzyme, PTGES, is up-regulated by both full loading and microdamage by needling in healing tendons, which suggests that prostaglandin E2 is important during these stimulations (17, 28 (Paper IV)). Inhibition of COX-2 by parecoxib in healing tendons most likely decreases the levels of prostaglandin E2, as shown by Langberg et al. (40). Our findings show that parecoxib completely abolishes the stimulatory effect of microdamage by needling, which suggests that prostaglandin E2 is mainly increased due to microdamage and not due to mechanotransduction (30 (Paper V)). Indeed, parecoxib impairs tendon healing much more during full loading when microdamage occurs and PTGES is increased (4 (Paper I), 17, 30 (Paper V), Paper II). Additionally, there seems to be no interactions between loading and parecoxib, so COX-2 inhibition seems not to interfere with mechanotransduction (30 (Paper V)). Overall, these results strengthen our theory that microdamage does occur during full loading and that it has a stimulatory effect on the structural properties of the healing tendon. It also shows that microdamage increases inflammation as an anti-inflammatory drug can abolish its stimulatory effect on healing tendons.

Comparison with other studies

We have shown that full loading improves Achilles tendon healing and this has also been shown in other studies (3, 9, 16, 18, 19, 22, 38, 42, 43). The improvement by mechanical loading is usually evaluated in the early phases of Achilles tendon healing, 1-3 weeks after injury (3, 9, 16, 18, 22, 27), but later evaluation has also been made (21, 43). Most animal studies evaluating the effect of full loading during the early phase of Achilles tendon healing have shown beneficial effects (3, 9, 16, 18, 19, 22). Apart from the improvement of mechanical properties, an increase in collagen has also been confirmed in other studies (9). The later evaluation studies have also shown beneficial effects by full loading. Palmes et al. showed in a mice model that the full loading group had reached to non-injured levels of mechanical properties after 16 weeks, whereas the immobilized group had not (43). This effect could not be seen in a rat model (21). However, an immobilized group was not evaluated in this study so the full loading tendons might still be better compared to immobilization. Overall, most animal studies suggest that mechanical loading can accelerate the healing process and reduce the rehabilitation time, which has also been shown in clinical studies (15, 35, 47, 52).

The timing of return to mobilization has been tested by other groups and some suggests that immobilization during the early phase of tendon healing is beneficial for the healing process (22, 27). Godbout et al. showed that full loading immediately after injury impaired mechanical properties after 3 weeks (27). However, if the rats were immobilized with a cast the first week and then allowed full loading the mechanical properties improved. Immobilization in a cast during the early phase of healing might not be detrimental as we

have shown that moderate loading, with Botox or tail suspension, still improves tendon healing, especially the quality of the healing tendon tissue (4 (Paper I), Paper II). Although, El-Akkawi et al. have shown that there seems to be no risks of doing early weight bearing rehabilitation after Achilles tendon rupture in patients, starting rehabilitation immediately or 2 weeks after injury compared to late rehabilitation (6 weeks after injury) (15). However, in these rehabilitation programs mild mechanical loading is probably applied which might not be comparable to full loading in animals, probably more related to our moderate loading model, or even less.

We have shown that mild mechanical loading (moderate loading with Botox or tail suspension) can improve tendon healing compared to minimal loading (Botox & tail suspension or Botox & a Boot). Unfortunately, to the best of our knowledge, there seems to be no other studies done comparing minimal and moderate loading that can confirm our results. However, the same experimental setup has been done three times so the reproducibility strengthens our findings (4 (Paper I), 30 (Paper V), Paper II).

We have shown that full loading increases inflammation during the first 5 days after injury by affecting inflammation-related genes and increase leukocytes in the healing tendon tissue (17, 29 (Paper III)). In accordance to these findings, Godbout et al. showed in an Achilles tendon model that full loading immediately after injury increased the amount of neutrophils and macrophages in the healing tendon during the first week after injury compared to immobilization (27). Manning et al. have shown in a flexor tendon model that pro-inflammatory mediators are up-regulated by full loading 1, 3, and 9 days after injury (41). However, the full loading group was compared to intact tendons and not an immobilization group, so these results only shows that pro-inflammatory mediators are increased during the tendon healing process.

Microdamage in healing tendons by full loading has unfortunately not been studied by other groups. Eliasson et al. discovered extravasated erythrocytes in healing tendons in histology samples in 2012 (16). This led to the hypothesis that full loading might create microdamage in the healing tendon tissue which might have a stimulatory effect, which we later could show (28 (Paper IV)). However, Heinemeier et al. have shown that needle penetration in healthy human patellar tendons up-regulates tendon cell activity and gene expression of collagens, which support our findings that microdamage can have a stimulatory effect on tendons (32).

NSAID's effect on tendon healing is not clearly understood. Both impairments and improvements of tendon healing have been shown (49). However, the timing of treatment seems to be important. Virchenko et al. have shown that NSAIDs impairs tendon healing when given during the early phase but improves it when given during the later phase of the healing tendon process (55). Ferry et al. showed that COX-2 inhibition reduced collagen content in the healing tendon, which correlates to our results where transverse area is reduced by COX-2 inhibition in the full loading group and in the microdamage group (20). However, other studies have shown that NSAIDs inhibit proliferation of tendon cells but increases

collagen synthesis (49). Furthermore, mechanical loading can up-regulate prostaglandin E synthase which in turn is regulated by COX activity (12, 45). This suggests that the impairment of tendon healing by COX-2 inhibition might be dependent on the degree of loading, which we could not show.

Neural regulation can actively be involved in healing processes such as tendon healing (2). Neurotransmitters and neuropeptides might affect inflammatory cells, endothelial cells and fibroblasts, thereby affecting release of inflammatory mediators, angiogenesis and fibroblast proliferation (2). For example, neuropeptide receptors for substance P are up-regulated by full loading in healing Achilles tendons, and might therefore be important for tendon healing (9). We use Botox in our rat model, which interferes with nerve actions. The substance blocks the release of acetylcholine in the nerve ends, which inhibits muscle contraction and paralysis the muscle (1, 5). Botox binds specifically to motor nerves and once it is bound to the nerve terminal the neuron takes up the toxin into a vesicle (5). Botox might also interfere with sensory nerves and inhibit the release of substance P, at least when it is injected subcutaneously (5). However, Botox binds strongly and specifically to receptors on the motor nerves (5). Therefore, local injections of Botox, far away from the tendon, should not affect the tendon substantially.

Instead of studying tendon healing in humans, we used a rat model and there is always a difference between rats and humans that may impair the generalization in our findings. One important difference is the size between human and rats. Rats are small, leading to higher metabolic rate. Importantly, diffusion distances are shorter, which might lead to different concentrations of nutrients and signaling substances at a site of the injury. Rats are also quadrupeds so the loading of intact and injured Achilles tendons in rats might be different compared to humans. Rats can for example more easily regulate the amount of loading that is applied to each limb.

Gender and hormone status seems to affect tendons and their healing processes. Tendons from female rats seem to have better material properties compared to males (44). On the other hand, studies on humans has shown that women's response to exercise in Achilles tendons is differently compared to men, and might reduce their ability to adapt to exercise (7). Additionally, female rats seem to improve their mechanical properties faster in healing Achilles tendons compared to males and ovariectomized females (24). We have only studied female rats and that might be a limitation of our findings, as there seems to be a difference between genders.

CONCLUSION

Different degrees of mechanical loading seem to stimulate tendon healing differently. Mild mechanical stimulation (moderate loading) improves the quality of the healing tendon tissue, probably via mechanotransduction mechanisms. It increases the expression of extracellular matrix genes, such as collagen 1, without increasing the size of the tendon. Additionally, inflammation is not affected substantially by moderate loading and it seems that microdamage in the healing tendon tissue rarely occurs.

Strong mechanical loading (full loading) improves the quality of the healing tendon tissue further, compared to moderate loading, but it also increases the quantity of the healing tendon tissue. It increases the expression of extracellular matrix genes, such as collagen 1, together with an increased size of the tendon. Additionally, microdamage occurs and inflammation increases when strong mechanical loading is applied to the early healing tendons. Microdamage, in itself, has a stimulatory effect on healing tendons, as shown under reduced loading stimulation. Therefore, it is most likely that microdamage during strong loading is one of the factors that improve the healing tendon tissue. In accordance, we could show that the anti-inflammatory drug parecoxib could abolish the stimulatory effect of microdamage on healing tendons but could only reduce the effect of strong mechanical loading, not abolish it. This suggests that strong mechanical loading stimulates the healing tendon tissue with different mechanisms, i.e. by both mechanotransduction and microdamage.

Strong mechanical loading gives the strongest healing tendon but might not be the best model, as it might not correspond to the situation in patients. Unloading of the healing tendon in rats, with Botox in combination with tail suspension or a boot, corresponds more likely to the situation in patients with a cast or a brace, as their injured Achilles tendon most likely is not exposed to mechanical stimulation at all. The new rehabilitation programs in clinics, with early mobilization, correspond more likely to mild mechanical loading in rats. Our results might explain why early mobilization improves the healing after Achilles tendon rupture in patients, as mild mechanical loading improved the quality of the healing rat tendon tissue and increased the gene expression of collagen 1 without any increase of inflammation or microdamage.

FUTURE

Gene expression analysis has so far only been studied at three different days during the healing process in our model. It might be interesting to study the gene expression at other days to get a broader perspective of the gene response after mechanical loading. The gene response after minimal, moderate and full loading has only been studied at day 5. As this experiment has some clinical relevance, it would be interesting to see if the positive effect of moderate loading is seen at later time points.

Protein production is only measured indirectly by gene expression analysis and the correlation between gene expression and protein production does not always match. Therefore, protein analysis is needed to really confirm the results of our gene expression analysis.

Our hypothesis about mechanotransduction and microdamage mechanisms during different loading stimulation has not been studied directly. It would be interesting to study these mechanisms more deeply. Maybe by inhibiting mechanoreceptors in order to inhibit the mechanotransduction responses, if this is possible in vivo.

Mechanical data suggests that COX-2 inhibition impairs tendon healing by reducing the response to microdamage. However, the underlying mechanisms are not clear. Therefore, it might be interesting to investigate these results further with gene expression analysis, to see which genes are affected by COX-inhibition when the healing tendon is stimulated by loading or needling.

It is obvious that full loading gives the best effect on tendon healing. However, it also increases the size of the tendon, such as transverse area and tendon length, which is sometimes a disadvantage in the clinic. This effect is seen after only 15 minutes of exercise each day. Our results show that the increase in size is mostly a consequence of the creation of microdamage, and parecoxib can abolish this effect. Therefore, it would be interesting to do a study combining boot, treadmill walking and parecoxib. Maybe we can improve tendon healing further compared to moderate loading by letting the rats with a boot walk a few minutes on a treadmill each day (full loading with boot removed) without increasing the size by injecting parecoxib. These results would be interesting from a clinical point of view. Maybe we can improve the healing tendon in patients and shorten the rehabilitation time by making a tougher rehabilitation program in combination with NSAIDs treatment during later phases of healing.

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*"I don't know anything, but I do know that everything is interesting
if you go into it deeply enough."*

Richard Feynman

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