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Title:

Prolonged Immobilization Compromises Up-regulation of Repair Genes after Tendon Rupture in a Rat Model

Running head:

Immobilization post Injury Impact mRNA Levels

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ABSTRACT

It was hypothesized that mobilization versus immobilization after injury would promote tissue healing by regulating gene expression for molecules associated with repair. Cast immobilization versus free mobilization was studied after rat Achilles tendon rupture. RT-PCR was performed at 8 and 17d post rupture to assess different growth factors (BDNF, bFGF, NGF and IGF-1) and inflammatory mediators (COX 1-2, iNOS and HIF-1α) in the healing region. At 8d post-injury, tendon mRNA levels were comparable in both groups. However, by day 17, the mRNA levels for BDNF, bFGF, COX 1, and HIF-1α in the mobilized group had increased significantly. Corresponding mRNA levels in the immobilized group decreased during the same period. There were no significant differences in the expression of NGF, IGF-1 or COX 2 between the different groups, indicating that injury-associated expression of these molecules is not overtly influenced by loading. This study supports the notion that prolonged immobilization post-rupture hampers the healing process by compromising the up-regulation of repair gene expression in the healing tendon. It might be speculated that a shorter period of immobilization, i.e. 1 week, would not impair the healing process significantly. The findings support the current development of earlier and more active rehabilitation programs after tendon injuries.

Key Words: Achilles tendon, healing, immobilization, growth factors, cyclooxygenase, inducible nitric oxide synthase, hypoxia-inducible factor-1α

INTRODUCTION

Tendons possess a poor healing capacity after injuries compared to many other tissues. The suboptimal healing process frequently leads to prolonged periods of rehabilitation, often with a poor result (Fahlstrom et al. 1998; Don et al. 2007). Immobilization during the early repair process tends to impair the outcome, while early mobilization has been shown to promote tissue repair after injury to tendons and ligaments (Kangas et al. 2007; Bring et al. 2008), although the specific mechanisms for this mechano-biological transduction are still largely unknown.

Stimulating the synthesis and release of growth factors and inflammatory mediators could be one mean by which mobilization could promote healing (Clarke & Feeback 1996; Morrison et al. 2000; Standley et al. 2001; Wang et al. 2003; Petersen et al. 2004). Thus, basic fibroblast growth factor (bFGF) and insulin-like growth factor-1 (IGF-1) have been shown to be involved in angiogenesis, fibroblast proliferation and tendon matrix production following injury (Roesel & Nanney 1995; Okumura et al. 1996; Molloy et al. 2003). Nerve growth factor (NGF) and the brain derived neurotrophic factor (BDNF), are factors mainly known for their regulation of central and peripheral neurons including axonal growth and neurotransmission (Lykissas et al. 2007), which have recently been identified as important features of tendon healing (Steyaert et al. 2006). In addition, NGF is now recognized as a strong promoter of wound healing on structural, biochemical and molecular levels, partly by stimulation of angiogenesis and regulation of fibroblasts and myofibroblasts (Mammoto et al. 2008). The inflammatory mediators cyclooxygenase 1 and 2 (COX-1 and -2), inducible nitric oxide synthase (iNOS) and hypoxia-inducible factor-1α (HIF-1α) are also believed to contribute to tendon repair by enhancing the process from the inflammatory healing phase through to scar remodelling (Elder et al. 2001; Kampfer et al. 2003; Hickey & Simon 2006; Molloy et al. 2006).

In the present study it was hypothesized that mobilization as opposed to immobilization after injury, i.e. mechanical stimulation, promotes tissue repair by regulating the local expression of growth factors and inflammatory mediators in a model of Achilles tendon repair after rupture.

MATERIALS AND METHODS

The study included 40 male Sprague Dawley rats (SD) with an initial weight of 177 – 303g (mean = 204g, SD = 12), housed three or four per cage at 21° C in a 12:12h light:dark cycle with water and food pellets *ad lib*. The rats were allowed one week of undisturbed acclimatization before initiation of the experiments. All experiments were performed in accordance with Karolinska Institutets protocols and approved by the local animal ethics committee.

Thirty two rats were operated upon and subsequently divided into two groups of 16 rats each. One group (*freely moving group,* ***mob***) was allowed free mobilization post surgery, i.e. free cage activity. The other group (*plaster-treated group,* ***immob***) was immobilized with a plaster cast on their operated leg (see below). The remaining 8 rats were used as normal controls (*control group,* ***Ctrl***).

Surgery: The rats were anaesthetized by an injection of a mixture of ¼ Midazolam® (5mg/ml, Pharma Hameln, Germany) and ¼ Hypnorm® (Janssen Pharmaceutica, Belgium) in sterile water (Fresenius Kabi AB, Sweden) (2ml/kg bw, s.c). The surgical procedures were all performed using aseptic technique. A 1-cm longitudinal incision was made in the midline over the Achilles tendon on the right leg. The incision was deepened through the crural fascia, exposing the Achilles and plantaris tendons. The Achilles tendon was ruptured with a blunt instrument, tearing the fibers apart, approximately 0.5 cm from the calcaneal insertion. The Achilles tendon was left unsutured, while the plantaris tendon was left intact, potentially serving as an internal splint. This procedure was chosen in order to mimic the clinical situation as closely as possible, and at the same time attempting to limit the size of the scar tissue facilitating later laboratory analyzes. It is recognized that this method may limit the mechanical stimuli to the healing area to some extent. The skin was closed with three stitches

of 5/0 non-resorbable monofilament suture material (2x2, Ethilon®II, Ethicon, USA). Postoperatively, the animals were returned to their cages. One rat in the immobilization group was lost during surgery.

Immobilization: Before being returned to their cages, the rats in the plaster-treated group had their injured leg immobilized with a padded Plaster of Paris cast (Jarvinen et al. 1992; Bring et al. 2008). The cast was applied from the toes up to approximately 2.5-cm above knee height. The ankle and knee were fixed at 60º and 70-80º of flexion, respectively. The outer layer of the cast was covered with black pepper for protection. Inspections were performed regularly in order to assess the need for reinforcements or replacements to the casts. The immobilization was maintained until sacrifice and dissection.

Reverse transcriptase-polymerase chain reaction (RT -PCR):

Eight rats from both the freely moving- and plaster-treated groups were euthanized at the eighth and 17th post-operative day, respectively. Four rats from the control group were also euthanized at each time point. The rats were anaesthetized by an injection of Pentobarbitalnatrium® (60mg/ml, Apoteket, Sweden) (60mg/kg bw, i.p), subsequently decapitated and exsanguinated before dissection. All surfaces and instruments were thoroughly cleaned and treated with RNAse *AWAY*® (Invitrogen™, life technologies, CA, USA) before and after the dissection of each animal. The right Achilles tendon was dissected. All samples were immediately frozen in liquid nitrogen. The tissues were kept at -70ºC until extraction of total RNA. The protocols for extraction have been described in detail elsewhere (Reno et al. 1997). Briefly, the frozen tissues were powdered in a Braun Micro-dismembrator (B.Braun Biotech International, Allentown, PA, USA) at 2600 rpm for 30 seconds and immediately treated with Trizol™ (Gibco Life Technologies, Carlsbad, CA, USA). The

samples were subsequently extracted with chloroform (EMD Chemicals Inc., Gibbstown, NJ, USA) and 70% EtOH. The aqueous phase was collected and total RNA separated using the column fractionation step of the RNeasy® Total RNA Kit (Qiagen, Chatsworth, CA, USA). The mRNA was eluted using molecular grade water (VWR International), quantified using Sybr Green, and stored at -80ºC until RT-PCR was performed as described previously (Reno et al. 1997).

All samples were reverse transcribed at the same time to minimize variability in the generation of cDNA. Subsequently, PCR was performed for each individual molecule at the same time for all groups. The number of cycles was chosen in order to maintain the PCR amplification within the range of exponential progression. Since the abundance of the studied mRNAs varied, the number of cycles used for the different validated primer pairs varied accordingly. Glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) was used as the housekeeping gene, a gene used in previous studies (Pohjanvirta et al. 2006; Bring et al. 2008), and a gene that did not vary significantly between the different groups (Bring, Reno and Hart, unpublished data). Additional controls included analysis of no-RT samples to ensure genomic DNA contamination was below detection limits, and cDNA from tissues with known high mRNA levels for the genes of interest was used as a positive control. The oligonucleotide primer sets used for the PCR reactions are listed in Table 1. The primers were designed using the software Primer Designer© Version 2.0. All primer sets were evaluated using GenBank data to avoid potential confounding sequence homologies. The amplicons were sequenced to verify primer specificity. Twenty microliters of each PCR reaction was subjected to electrophoresis on a 2% agarose gel as described previously (Marchuk et al. 1998). The gels were stained with ethidium bromide, then destained and photographed. The negatives were analyzed using the MasterScan Interpretive Densitometer (CSPI INC.,

Billerica, MA) and the RFLP Scanalytics software. The densitometric values obtained were corrected for background and normalized against the housekeeping gene (GAPDH).

To verify the results, additional aliquots of some RNA samples were subjected to repeat RT and again analyzed for a subset of selected molecules using PCR. Results from those re-evaluations were not different from the results reported.

Statistics: Standard descriptive statistics were used to summarize the variables, i.e. means and standard deviation. $p \leq 0.05$ was considered to be statistically significant. Due to the limited sample size (n=7-8) and, thus, lower power in the statistical analyses, it is recognized that only pronounced group differences could be statistically determined. The data was controlled and found not to be skewed. The statistical analysis was therefore performed by the ANOVA test followed by the Tukey´s post hoc test.

RESULTS

Growth factor mRNA levels in the Achilles tendon after rupture

The growth factors of interest (BDNF, NGF, IGF-1, bFGF) (Fig. 1-2) were each expressed in all Achilles tendon samples analyzed. Overall, 8 days of plaster-treated immobilization post injury did not lead to any significant differences in growth factor mRNA expression, whereas by 17 days of immobilization post injury, significant differences in expression levels were noted when compared to the freely mobilized group.

At 17 days post rupture, two of the growth factor genes, BDNF (Fig. 1A) and bFGF (Fig. 2B), exhibited an increased expression in the mobilized group as compared to both the immobilized- and the control group. In contrast, BDNF and bFGF mRNA levels were equal to or even below control values in the immobilized specimens at the same time point. However, when examined at 17 days post-rupture, mRNA levels for NGF (Fig. 1B) and IGF-1 (Fig. 2A) were not significantly influenced by immobilization, and IGF-1 mRNA levels were increased in both the mobilized and immobilized groups as compared to controls.

Inflammatory mediator mRNA levels in the Achilles tendon after rupture

In all tendon tissue samples examined, detectable mRNA levels for the pro-inflammatory substances (COX 1, COX 2, iNOS, HIF-1α) (Fig. 3-4) were found. The data showed that 8 days of immobilization post injury was not associated with any significant differences in mRNA levels for inflammatory mediators compared to levels in the mobilization group. However, 17 days of immobilization post injury did lead to significantly decreased levels of mRNA for these inflammatory mediators compared to the mobilization group.

At 8 days post rupture, HIF-1α mRNA levels were increased in both mobilized and immobilized tendons compared to the controls (Fig. 4B). COX 2 mRNA levels in the mobilized group were elevated compared to the control values, although not significantly

higher than the immobilized group (Fig. 3B). The mRNA levels for COX 1 and iNOS did not significantly differ in any of the groups.

At 17 days post rupture, mRNA levels for all inflammatory mediators assessed were higher in the mobilized group than the immobilized (Fig. 3A and Fig. 4A-B), although apparent increases for COX 2 did not reach statistical significance (Fig. 3B). Interestingly, 17 days of immobilization led to inflammatory mediator mRNA levels equal to or even (in the case of COX 1), below the values in control samples.

DISCUSSION

This study on tendon repair supports the concept that prolonged immobilization during healing leads to potentially detrimental effects on connective tissue healing outcomes. Although a short immobilization period during the initial inflammatory phase does not seem overtly harmful, a more long-lasting immobilization tends to selectively compromise the metabolic response to injury.

The data clearly demonstrate that more than two weeks of plaster immobilization of the healing Achilles tendon led to impairment of gene expression for tissue repair stimulating mediators, most pronounced being growth factors (BDNF, bFGF) and pro-inflammatory mediators (COX 1, iNOS, HIF-1α). Thus, 17 days of free mobilization post injury led to significant increases in mRNA levels for BDNF (~9-fold) and bFGF (~2.5-fold) as compared to intact controls. These increases in growth factor mRNA levels were completely abrogated by 17 days of immobilization, suggesting that the effects mediated by BDNF and bFGF, including promotion of nerve regeneration, angiogenesis and fibroblast cell proliferation (bFGF) (Okumura et al. 1996; Grothe & Nikkhah 2001; Vogelin et al. 2006; Kermani & Hempstead 2007) are largely dependent on the increased mechanical stimulation associated with mobilization. In support of this, recent studies in our laboratory revealed that mobilization post-injury induced a higher density of blood vessels, more differentiated fibroblasts and a higher degree of collagen organization by 28 days post-injury, as compared to immobilization (Bring et al. 2008). We were also able to show that some of the enhanced effects on the healing process are proportional to the amount of mechanical stimuli (Bring et al. 2007). Upregulated BDNF and bFGF signaling already at 17 days post-injury may contribute to these observed structural changes. Moreover, more than two weeks of mobilization also resulted in elevated mRNA levels for pro-inflammatory mediators essential to tissue repair, when compared to levels in the immobilization groups.

The increases in mRNA levels for COX 1 (~80%) and COX 2 (~16-fold), although the latter not significant, at 17 days of mobilization post injury presumably represents augmented prostanoid effects on blood vessels, nerve endings and inflammatory cells, since cyclooxygenases convert arachidonic acid into various pro-inflammatory and healing stimulatory molecules (Savla et al. 2001; Braun & Kietzmann 2004). The ~70% up-regulation of inducible nitric oxide synthase (iNOS) mRNA also suggests a promoted effect of nitric oxide, a potent vasodilator and an important signalling molecule in wound healing, from the inflammatory phase through to scar remodelling (Shabani et al. 1996; Corbett et al. 1999). The increase in HIF-1α mRNA levels (~6.5-fold) supports a vital role for this molecule in wound healing as a promoter of angiogenesis (Mace et al. 2007; Liu et al. 2008). Thus, free mobilization during the first 17 days post injury promotes numerous pathways in the wound healing process, most of which appear to be completely obstructed by immobilization.

One interpretation of the increased mRNA of inflammatory mediators in the mobilized group could be that it is a result of repeated trauma to the immature healing tissue and as such not a benefit for the healing process. However, this seems unlikely considering a recent study using the same experimental model, which demonstrated up-regulated mRNA expression of collagen type I and III, versican, decorin and biglycan at day 17 and improved histological healing at day 28 in the mobilized compared to the immobilized group (Bring et al. 2008). Thus, the recent study strengthens the conception that the up-regulated COX 1, iNOS and HIF-1α mRNA expression promotes the healing process.

Interestingly, not all repair genes examined locally in the tendon appeared to be affected by 17 days of immobilisation after injury. Thus, mRNA levels for NGF and IGF-1 exhibited equal levels in the mobilized and immobilized groups both at 8 and 17 days post injury. The NGF and IGF-1 genes, uninfluenced by immobilization, may provide more fundamental functions in the repair process and thus not influenced by mechanical factors. Thus, the up-

regulated IGF-1 mRNA levels observed in the mobilized and immobilized groups suggest enhancement of fibroblast mitogenesis and tendon matrix production. In fact, IGF-1 is a known strong anabolic growth factor for tendon repair (Tsuzaki et al. 2000; Costa et al. 2006). The observation that levels of NGF mRNA were not significantly altered compared to controls is interesting since NGF is a strong promoter of wound repair (Nithya et al. 2003; Mammoto et al. 2008), in addition to its known effects on nerve regeneration (Sun et al. 2007; Mammoto et al. 2008). The unaltered NGF expression at 8 and 17 days post injury suggest that regulation of NGF is not a major pathway for promoting tendon repair neither in the mobilized nor immobilized condition. This however does not preclude that changes in NGF receptor expression may occur, and thus can alter the NGF end-effects. As exogenous NGF enhances ligament repair in rats (Mammoto et al. 2008), functional receptors likely are expressed by cells in other injured rat connective tissues. However, whether receptors for NGF and BDNF are involved in mobilization/immobilization effects on tendon repair or in the regulation of nerve plasticity seen in the healing Achilles tendon (Ackermann et al. 2002; Bring et al. 2007) will require further studies.

Most inflammatory and growth factor genes assessed locally in the Achilles tendon exhibited a time dependent down-regulation following immobilization (BDNF, bFGF, COX 1, iNOS, HIF-1α). Thus, initial increases in mRNA levels at 8 days in the immobilized group were at 17 days post injury equal or even below intact control values demonstrating that the stress shielding effects of immobilisation are time dependent. The time points studied can not be directly translated to the phases of repair in human tendons. However, the unfavourable effects of prolonged immobilization must be seen as principles supporting the concept of early, limited and controlled movement after surgery, and support avoiding excessively long immobilization times.

In the study one animal in the freely moving group at 17d post injury was identified as a more or less consistent low outlier. This could possibly be explained as an individual response to injury. No methodological errors could be found and the data, although diverging, are included in the analyses above.

In summary, the results presented in this report show that prolonged immobilization after Achilles tendon rupture leads to reduced mRNA levels for molecules associated with wound healing and regenerative capabilities in the rat. In contrast, immediate mobilization shows a multitude of wound healing promoting effects, including up regulation of anabolic growth factors. In the future, it may be possible to develop new interventions, using physical and pharmacological approaches to stimulate endogenous growth factor and inflammatory mediator production and thereby prevent the adverse effects of immobilization after injury.

PERSPECTIVES

The treatment of injuries to joint associated connective tissue has during recent years shifted from prolonged periods of immobilization towards a more active and aggressive rehabilitation. This development has been supported by both pre-clinical (Palmes et al. 2002; Bring et al. 2008) and clinical (Kangas et al. 2007) studies showing healing stimulatory effects of mobilization. The mechano-biological mechanisms though, are still not fully understood.

Our current results clearly demonstrate that early mobilization, post injury, significantly increases the expression of several growth factors and pro-inflammatory substances. Prolonged periods of immobilization, however decrease the mRNA expression to levels even below control group values. Interestingly, we also observed isolated growth factor mRNA expressions, which were unaffected by mobilization. Presumably, these growth factors

provide more fundamental functions in the repair process and therefore stand unchanged by external mechano-biological manipulation.

The present findings are in concert with the current clinical development of more aggressive rehabilitation plans after this kind of injuries. Importantly, the data implies that shorter periods of immobilization, i.e. 1 week, does not negatively affect the healing process.

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FIGURE LEGENDS

Figure 1A-B. Normalized expression of mRNA for the nerve growth factors BDNF (A) and NGF (B) at 8 and 17 days post Achilles tendon rupture in the healing tendon of immobilized and mobilized rats, i.e. **Control** (n=8), **8d immob** (n=7), **8d mob** (n=8)**, 17d immob** (n=8) and **17d mob** (n=8) (mean ± SD). (* $P \leq 0.05$; n.s $P > 0.05$). There was a significant increase of the BDNF expression in the mobilized group after 17 days post rupture as compared to immobilization and control. Interestingly the mRNA expression of NGF was not influenced by the different levels of physical activity and did not vary significantly over time.

Figure 2A-B. Normalized expression of mRNA for the growth factors IGF-1 (A) and bFGF (B) at 8 and 17 days post Achilles tendon rupture in the healing tendon of immobilized and mobilized rats, i.e. **Control** (n=8), **8d immob** (n=7), **8d mob** (n=8)**, 17d immob** (n=8) and **17d mob** (n=8) (mean ± SD). (* $P \leq 0.05$; n.s $P > 0.05$). The expression of IGF-1 increased significantly over time in both the mobilized and immobilized group and was notably not influenced by the different levels of physical activity. The expression of bFGF increased over time in the mobilized group, while the expression decreased in the immobilized group.

Figure 3A-B. Normalized expression of mRNA for the pro-inflammatory mediators COX 1 (A) and -2 (B) at 8 and 17 days post Achilles tendon rupture in the healing tendon of immobilized and mobilized rats, i.e. **Control** (n=8), **8d immob** (n=7), **8d mob** (n=8)**, 17d immob** (n=8) and **17d mob** (n=8) (mean ± SD). (* $P \leq 0.05$; n.s $P > 0.05$). The expression of COX 1 increased over time in the mobilized group and was significantly increased 17 days post rupture when compared to control and immobilization. The expression of COX 2 was significantly increased in the mobilized group already at 8 days post rupture, and remained elevated for the duration of the experiment as compared to control. There were no statistically

significant differences between the different levels of physical activity, most likely due to the limited sample size.

Figure 4A-B. Normalized expression of mRNA for iNOS (A) and HIF-1α (B) at 8 and 17 days post Achilles tendon rupture in the healing tendon of immobilized and mobilized rats, i.e. **Control** (n=8), **8d immob** (n=7), **8d mob** (n=8)**, 17d immob** (n=8) and **17d mob** (n=8) (mean ± SD). (* $P \leq 0.05$; n.s $P > 0.05$). The expression of iNOS in the mobilized group was significantly increased 17 days post rupture when compared to the immobilized group. The mRNA expression of HIF-1α was increased 17 days post rupture in the mobilized group when compared to immobilization and control.