Compression Therapy Promotes Proliferative Repair during Rat Achilles Tendon Immobilization

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Received 18 August 2009; accepted 24 October 2009
Published online 7 January 2010 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jor.21066

ABSTRACT: Achilles tendon ruptures are treated with an initial period of immobilization, which obstructs the healing process partly by a reduction of blood circulation. Intermittent pneumatic compression (IPC) has been proposed to enhance tendon repair by stimulation of blood flow. We hypothesized that daily IPC treatment can counteract the deficits caused by 2 weeks of immobilization post tendon rupture. Forty-eight Sprague-Dawley SD) rats, all subjected to blunt Achilles tendon transection, were divided in three equal groups. Group A was allowed free cage activity, whereas groups B–C were immobilized at the operated hindleg. Group C received daily IPC treatment. Two weeks post-rupture the rats were euthanatized and the tendons analyzed with tensile testing and histological assessments of collagen organization and collagen III-LI occurrence. Immobilization significantly reduced maximum force, energy uptake, stiffness, tendon length, transverse area, stress, organized collagen diameter and collagen III-LI occurrence by respectively 80, 75, 77, 22, 47, 65, 49, and 83% compared to free mobilization. IPC treatment improved maximum force 65%, energy 168%, organized collagen diameter 50%, tendon length 25%, and collagen III-LI occurrence 150% compared to immobilization only. The results confirm that immobilization impairs healing after tendon rupture and furthermore demonstrate that IPC-treatment can enhance proliferative tendon repair by counteracting biomechanical and morphological deficits caused by immobilization. © 2010 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 28:852–858, 2010

Keywords: tendon injury; immobilization; intermittent pneumatic compression devices; biomechanics; collagen type III

Achilles tendon ruptures, whether operated or not, are treated with an initial period of immobilization.[1] Accumulating data demonstrate that already 2 weeks of immobilization after tendon rupture impedes the healing process, leading to downregulated production of growth factors, matrix proteins, collagens, and subsequently reduced biomechanical tissue properties.[2–5]

Immobilization additionally inhibits blood circulation and nerve regeneration at the site of injury, which are vital for early proliferative healing.[6] In the rat, the intact tendon proper is practically devoid of nerve fibers and blood vessels, which are located in the paratenon and surrounding connective tissue. Following injury, however, new blood vessels and nerve fibers grow into the tendon proper supplying growth factors and releasing different neuronal mediators vital for repair.[7,8]

One way to counteract the reduction in blood flow and neurovascular supply following immobilization after injury could be the use of external compression therapy. Intermittent pneumatic compression (IPC) treatment aims at passive increase of blood flow by cycling external pressure leading to reduced venous stasis and pressure and enhanced arterial blood flow.[9] IPC is also clinically used to prevent thrombosis in immobilized patients.[10] The circulatory increase after compression treatment has also been hypothesized to benefit tissue healing.[11,12] A recent study demonstrated that daily IPC treatment in a rat model of mobilized Achilles tendon healing enhanced neurovascular ingrowth as well as fibroblast proliferation at 2 weeks postrupture.[13] However, whether IPC treatment could counteract the negative influence on healing due to immobilization has not previously been studied.

Thus, in this study we examined quantitatively the biomechanical properties as well as histological healing with respect to the occurrence and organization of collagen in mobilized, immobilized, as well as immobilized IPC-treated fully ruptured Achilles tendons at 2 weeks postinjury, corresponding to the proliferative phase of healing.[14]

MATERIALS AND METHODS

The study included 48 male Sprague-Dawley rats (B&K Universal, Stockholm, Sweden; 8 weeks old, 180–200 g), housed three or four per cage at 21°, 45–55% humidity, in a 12:12-h light:dark cycle with water and food pellets ad libitum. The rats were allowed 1 week of undisturbed acclimatization before initiation of the experiments. All experiments were approved by the Local Committee for Animal Research and Ethics and conducted in accordance with the Institute's protocols.

All rats were subjected to blunt complete Achilles tendon transection in the right hind leg and subsequently divided into three groups of 16 rats each. Group one (*mobilized*) was allowed free cage activity post surgery. Group two (*immobilized*) was immobilized with a synthetic cast on the operated leg. Group three (*immobilized + IPC treated*) was immobilized in the same way and additionally received intermittent pneumatic compression (IPC) treatment.

Surgery

The rats were anesthetized by an injection of a mixture of one-quarter Midazolam® (5 mg/mL, Pharma Hameln, Germany) and one-quarter Hypnorm® (Janssen Pharmaceutica, Belgium) in sterile water (2 mL/kg body weight, s.c). The operations were performed under sterile conditions. A 1-cm longitudinal incision was made in the midline of the Achilles tendon on the right foot, exposing the Achilles and plantaris

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tendons. Both tendons were fully ruptured with a blunt instrument, tearing the fibers apart, approximately 0.5-cm from the calcaneal insertion. The tendons were left unsutured and the skin was closed with two stitches of 5/0 non resorbable suture material (2×2, Ethilon® II, Ethicon, San Antonio, TX, USA). Postoperatively the animals were returned to their cages.

Immobilization

The rats in the immobilized groups had their operated leg immobilized with a modified padded Plaster of Paris cast,[5,15] using thermosensitive synthetic plastic (Orfit NS), which could be easily removed and readjusted, so that the rats could receive daily IPC treatment. The cast was applied from the toes up to the hip achieving three-point stability (ankle–knee–hip), which ensured a good model of full immobilization of the right hind leg. The ankle was fixed in equinus, at $30 \pm 5^{\circ}$ of plantar flexion and the knee joint was fixed at $20 \pm 5^{\circ}$ flexion (Fig. 1). The proximal part of the plaster ended in a loop with an easy lock–unlock system (Fig. 1) surrounding the waist. For protection of the plaster white pepper covered the outside surface. Inspections were performed regularly to assess the need for reinforcements or replacements of the casts. The immobilization was maintained until the end of the experiment at 14 days postrupture.

IPC Treatment

The treatment started the first postoperative day. All animals were anesthetized 1 h/day using isoflurane (Baxter, Sweden), until day 14 postrupture. The IPC group received treatment during the 1-h anesthesia.IPC cuffs (Aircast Inc., Vista, CA, USA) were tightly attached to the right hind leg of the rat using surgical tape. The device consists of two flat pressure cuffs, covering the calf and thigh on the medial and lateral side of the leg. The cuffs were inflated in cycles of 3 s, pressure of 55 mmHg, followed by 27 s of relaxation. The pressure applied, 55 mmHg, represents around 50% of the rat mean arterial blood pressure, which reflects similar values as used in patients (IPC pressure 45–52 mmHg). The duration and dosage of IPC treatment were chosen according to a protocol used in a previous study.[13]

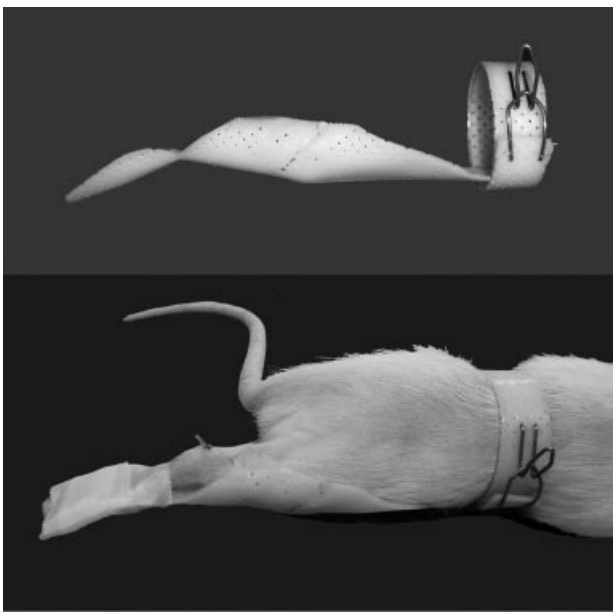

Figure 1. Photographs of the immobilization cast and how it is applied on the rat.

Biomechanical Testing

At 2 weeks 10 rats from each group were anesthetized by an injection of sodium pentobarbitone (60 mg/kg body weight i.p.) and euthanatized. A surgeon blinded to the group identity of the rat dissected the Achilles tendon with the attached calcaneal bone from other tissues. The sagittal and transverse diameters of the callus, the distance between the torn ends as well as the full length of the tendon were measured with a digital slide calliper as previously described.[16] The cross-sectional area of the callus was calculated assuming an elliptical geometry. For clamping, the gastrocnemius muscle was carefully scraped off the proximal tendon by blunt dissection to produce a fan of tendon fibers, which were attached between fine sandpaper and fixed in a metal clamp. Distally, the calcaneus was fixed in a custom-made clamp, in neutral position, that is, 30° dorsal flexion, relative to the direction of traction. The clamps were mounted vertically in a materials testing machine (100R, DDL, Eden Prairie, MN, USA) and tested with stretching in a monotonic phase until failure. The machine pulled at a constant speed of 0.1 mm/s. The biomechanical parameters were maximum force (N), stiffness (N/mm), stress (N/mm^2), and energy uptake (J) until maximum force, which were calculated by the software of the testing machine after that the linear portion of the elastic phase of the curve for stiffness calculation had been marked.

Perfusion

At 2 weeks the remaining six rats from each group were anesthetized by an injection of sodium pentobarbitone (60 mg/kg body weight i.p.) and subsequently euthanatized. Intraarterial perfusion and subsequent tissue fixation followed as described in our previous study.[13] The samples were frozen and sectioned in the coronal plane at 8 μm on a Leitz cryostat and mounted directly on Super-Frost/Plus glass slides from the dorsal to the ventral aspect consecutively.

Quantitative Assessments of Organized Collagen Diameter

Two sections from different levels (i.e., ventral and dorsal parts of the tendon) of each tendon were stained according to the Hematoxylin-Eosin (H&E) method and analyzed on a microscope (Nikon, Eclipse E800, Yokohama, Japan). To assess objectively the progression of healing, the diameter of the tendons was analyzed using pictures taken of the HE-stained sections, as validated previously.[2] For microscopic analysis, a video camera system (DXM 1200, Nikon) was attached to the microscope and connected to a computer. The diameter of organized, parallel collagen at the rupture site as well as the diameter of the whole tendon, were measured three times per section at the rupture site, using the software Easy Image Measurements 2000 (Tekno Optik AB, Skarholmen, Sweden). The mean fraction of *organized collagen diameter*/*whole tendon diameter* from two different sections from the ventral and dorsal portion was calculated for each tendon and group. For the above analysis the mean interobserver coefficient of variation was 10.1% and the intraobserver coefficient of variation was 8.8%.

Semiquantitative Assessments of Sirius Red-Polarized Light

Simultaneous sirius red staining for all sections to be compared was performed,[17] and the sections were examined on the microscope, where the polarizer and analyzer were set according to the Kierman protocol.[18] Three images, micro-

scopic fields (×10 objective), large enough to cover the whole area of the rupture site in each section without overlapping each other, were stored in the computer. Subsequently, the images were analyzed using Easy Image Analysis Software (Technooptik, Skarholmen, Sweden). The software denotes and considers positive staining beyond defined thresholds of color and intensity, which was set to pale green and remained unaltered for all the images assessed. The results were expressed as the fraction area occupied by positive staining in relation to the total area of the picture. The mean fraction of positive/total area in six images from two sections (three images from the ventral and three from the dorsal portion) was calculated for each tendon and group. Previous studies have shown that under polarized light, short thin pale green fibrils denote collagen III-LI (like) structures, that is, short collagens, whereas red fibrils denote collagen I-LI structures.[17,19,20] Thus, the positive staining of short thin pale green fibrils will be denoted as collagen III-LI.

For the above analysis the mean interobserver coefficient of variation was 6.5% and the intraobserver coefficient of variation was 5.4%. Quantification as well as semiquantification was applied by two blinded observers.

Statistical Analysis

All the results were assessed using the softwares Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA) and SPSS 15.0 (for Windows). Nonparametric Kruskal-Wallis tests were used for multiple comparisons between the three groups followed by Mann-Whitney *U*-tests and the level of significance was set to $p = 0.05$. The parameters assessed were: (a) biomechanical data: maximum force, stiffness, stress, and energy uptake at maximum force, as well as tendon length and transverse area of the callus. (b) Histological data: organized collagen III-LI density and organized collagen diameter in relation to the whole tendon diameter.

RESULTS

Weight and Health

No wound swelling or infections were observed during the 14 days postinjury. At the end of the experiment the body weight of the rats varied between 269 and 333 g, and there were no significant differences between any of the groups.

Biomechanical Properties

At 2 weeks post tendon rupture maximum force at failure of the freely mobilized rats, 46.9 ± 3 Newton, had already reached the values of "normal" uninjured rats 43.0 ± 4.2 Newton (Table 1). Compared to mobilization, 2 weeks of immobilization caused significantly lower values ($p = 0.05$) for all parameters assessed quantitatively. Specifically, the immobilized non-IPC-treated group exhibited significant reductions of maximum force 80%, energy uptake at maximum force 75%, stiffness 77%, stress 65%, tendon length 22%, and transverse area of the callus 47%, compared to the mobilized group (Table 1).

At 2 weeks, immobilization combined with IPC treatment, however, demonstrated higher values of maximum force 65%, energy 168%, and tendon length 25% compared to the immobilized untreated group (Figs. 2–4, Table 2). The values for stiffness 31%, stress 34%, and transverse area of the callus 32% were also higher; however, these data were not significant ($p = 0.3$, $p = 0.3$ and 0.1, respectively).

Immobilization with IPC treatment did reach the values of mobilization regarding energy uptake and the length of the tendon, whereas the other parameters remained suboptimal compared to the mobilized group (Figs. 2, 3, and 4).

Collagen III-LI Occurrence

Histological examination showed that the highest density of collagen III-LI fibers at the rupture site was found in the mobilized followed by the IPC-treated immobilized group, whereas the immobilized group exhibited the lowest density. Semiquantitative assessments strengthened this observation by demonstrating that the area fraction of collagen III-LI fibers at the rupture site exhibited 83% lower values in the immobilized untreated group compared to the mobilized group (Table 1 and Fig. 5). However, when immobilization was combined with IPC treatment, a 150% significant, increased density of collagen III-LI fibers was obtained

Table 1. Achilles Tendon Healing at 2 Weeks Postrupture

Group	Intact[a]	Mobilization	Immobilization		Immobilization + IPC treatment	
Parameter	Mean ± SEM	Mean ± SEM	Mean ± SEM	*p*-Level[c]	Mean ± SEM	*p*-Level[c]
Max force (N)	43.0 ± 4.2	46.9 ± 3	9.5 ± 1.4	<0.001	15.7 ± 2.4	<0.001
Energy (J)	82.9 ± 4.2	99 ± 13.5	25 ± 9.7	0.005	67 ± 12.5	0.109
Stiffness (N/mm)	13.1 ± 1.2	16.6 ± 1	3.8 ± 0.4	<0.001	5 ± 0.6	<0.001
Length (mm)	8.9 ± 0.4	14.7 ± 0.9	11.4 ± 1	0.025	14.3 ± 0.5	0.567
Cross area (mm^2)	3.0 ± 0.5	16.2 ± 1.9	8.6 ± 0.5	0.013	11.3 ± 1.6	0.050
Stress (N/mm^2)	17.5 ± 3.9	3.3 ± 0.3	1.1 ± 0.2	0.002	1.5 ± 0.2	0.001
Organized collagen (%)	100	53 ± 3.1	27 ± 3.5	0.004	40 ± 2.5	0.017
Collagen III-LI (%)	NA[b]	5.9 ± 1.9	1.0 ± 0.1	0.004	2.5 ± 0.5	0.052

[a]The intact data were collected from animals of the same branch and age and they are presented in the table above along with the results of the study.
[b]NA: not analyzed.
[b]Compard with the respective value of the mobilized group.

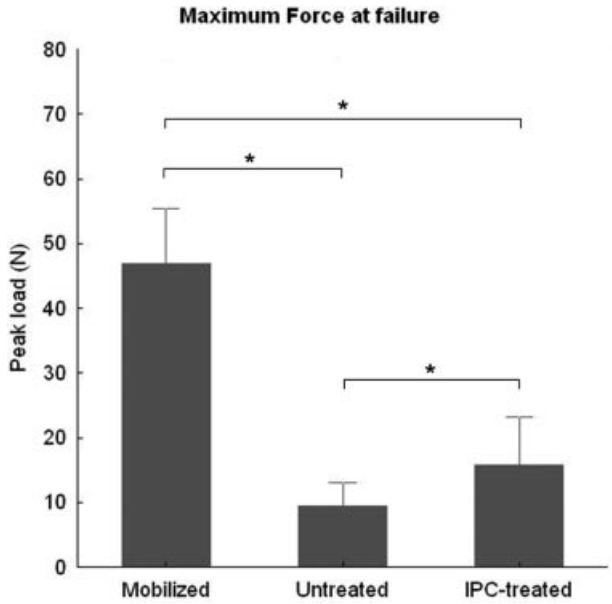

Figure 2. Maximum force expressed in newtons in the mobilized, immobilized-untreated, and immobilized-IPC-treated groups. *Denote $p = 0.05$.

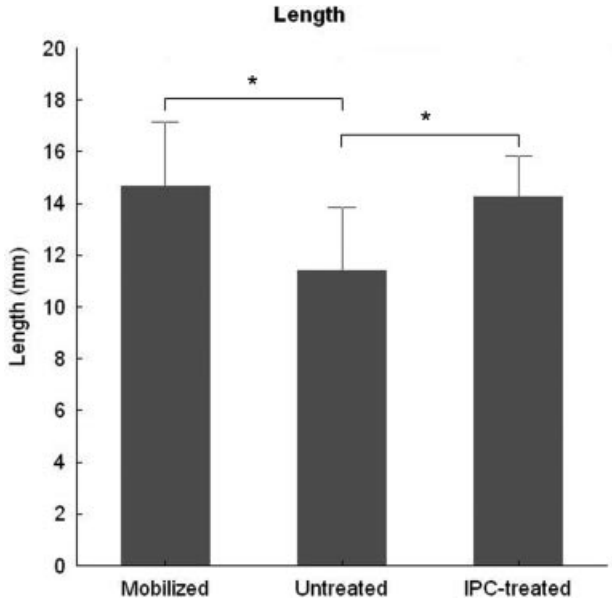

Figure 4. Tendon length (mm) in the mobilized, immobilized-untreated, and immobilized-IPC-treated groups. *Denote $p = 0.05$.

when compared to pure immobilization (Table 2 and Fig. 5).

Organized Collagen Diameter

Histologically, the greatest amount of newly organized parallel collagen fibers in the ruptured tendons was in order found in the mobilized and IPC-treated immobilized groups compared to the immobilized group (Fig. 6). This observation was verified by computerized analysis. Thus, the fraction of the midtendon mediolateral diameter of longitudinally organized, parallel collagen divided by the whole tendon diameter in the immobilized group was 49% smaller compared to the mobilized group (Table 1). When immobilization was combined with IPC treatment, however, the fraction of organized collagen increased by 50% compared to immobilization only (Table 2).

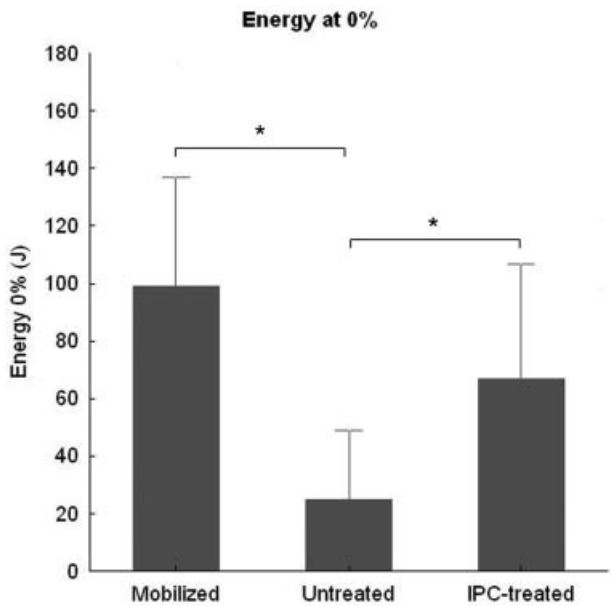

Figure 3. Energy expressed in jouls in the mobilized, immobilized-untreated, and immobilized-IPC-treated groups. *Denote $p = 0.05$.

DISCUSSION

The present study clearly confirms, in concordance with previous knowledge, that immobilization impairs tendon healing during the early proliferative phase.[2–5] Furthermore, this study establishes that external compression treatment can improve and even reverse the values of some of the parameters of tendon repair obstructed by 2 weeks of immobilization.

The combined biomechanical and histological assessments demonstrated that intermittent pneumatic

Table 2. Immobilization Combined with IPC Compared with Pure Immobilization (Set as 0%)

Group	Immob.	Immobilization + IPC treatment	
Parameter	Percentage	Percentage	*p*-Level
Max force	0%	65%	0.050
Energy	0%	168%	0.030
Stiffness	0%	31%	0.278
Length	0%	25%	0.039
Cross area	0%	32%	0.129
Stress	0%	34%	0.278
Organized coll.	0%	50%	0.026
Coll. III-LI	0%	150%	0.002

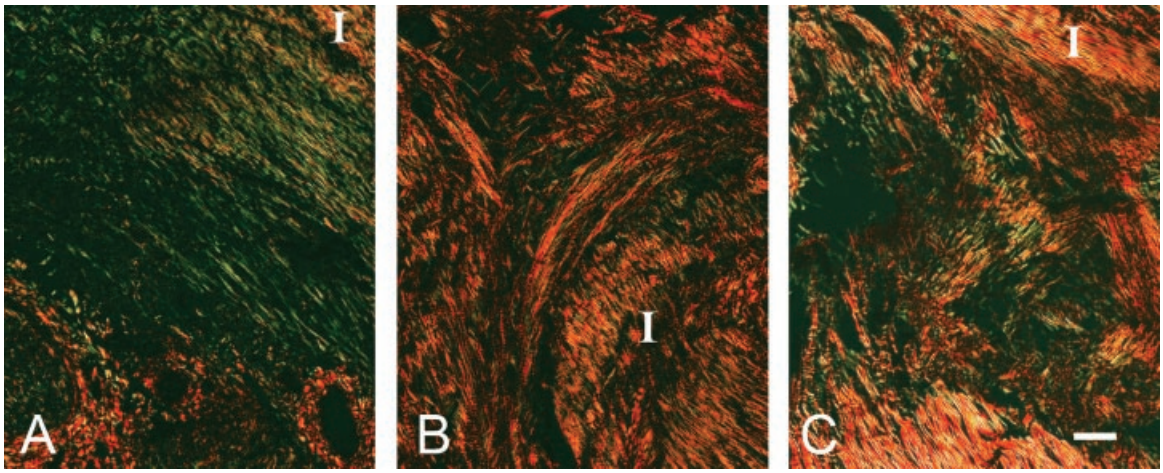

Figure 5. Sirius red-polarized light micrographs of longitudinal sections through the rupture site of the Achilles tendon of (A) mobilized, (B) immobilized untreated, and (C) immobilized IPC-treated rats. Short green fibers denote collagen III-LI (like). "I" denotes intact parts of the tendon (bar = 200 μm).

compression (IPC) promotes the early proliferative healing process during tendon immobilization following rupture. Hence, the increased diameter of organized collagen and collagen III-LI density observed after compression treatment were confirmed by improved biomechanical tissue properties, suggesting that the effects of IPC relate to enhancement of tissue proliferation. Recent studies corroborate a tissue proliferative effect of IPC and indicate that the underlying reparative mechanisms may relate to enhancement of blood flow, neurovascular ingrowth, fibroblast proliferation, and sensory neuropeptide supply to the healing connective tissue.[12,13,21,22]

In this study compression treatment was able to enhance healing with regard to all repair parameters negatively influenced by immobilization, although the values for cross-sectional area, stress, and stiffness did not reach significance. The most significant increases in maximum force (1.5-fold) and energy uptake (2.5-fold) were similar to those for the amount of organized collagen (1.5-fold) and collagen III-LI density (2.5-fold). Further analysis will be needed to detail these potential relationships; one might speculate that newly formed parallel, organized collagen I will be most important for maximum force, whereas collagen III is more involved in regulating energy uptake. In fact, the energy uptake in the IPC treated immobilized tendons increased to reach a level not significantly different from the mobilized tendons.

Interesting to note in this aspect is the remarkable healing of the freely mobilized group. At 2 weeks of mobilization maximum force reached levels equal to uninjured Achilles tendons, suggesting that "free mobilization" after acute Achilles tendon rupture may be the optimal treatment. This indicates that an earlier and more intense mobilization post tendon rupture could be supported.

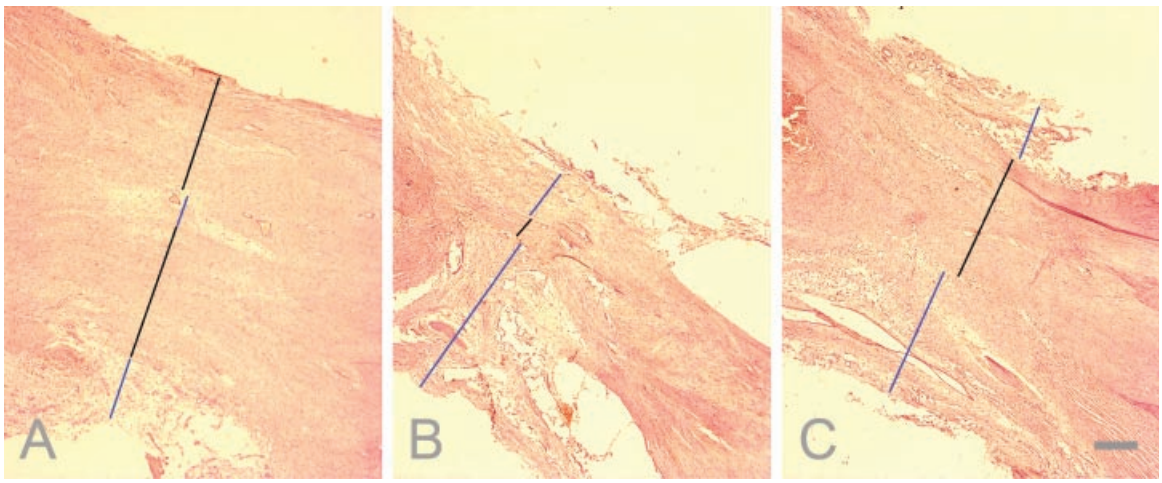

Figure 6. H&E stained micrographs of longitudinal sections through the rupture site of the Achilles tendon of (A) mobilized, (B) immobilized-untreated, and (C) immobilized-IPC-treated rats. The black lines (diameters) denote areas with organized collagen morphology as well as tenocytes orientated with the longitudinal axis of the tendon. The blue lines denote areas of disorganized collagen and granulation tissue (bar = 1 mm).

The conspicuous drop in collagen III-LI density (pale green staining under polarized light), after immobilization, and the subsequent elevation after IPC treatment, likely reflect alterations in type III collagen fiber occurrence. A recent study demonstrated that 2 weeks of immobilization after tendon rupture totally obstructed an increase in collagen III mRNA levels, which were seen at the same time point to be elevated 10-fold after mobilized healing.[23]

Although there was a huge increase in collagen III-LI density after compression treatment compared to immobilization only, the transverse area of the callus did not increase significantly. The most plausible explanation is that IPC treatment reduces inflammatory swelling within the healing area of the callus. In fact, IPC therapy is known to resolve oedema formation.[24] An increase in collagen III and presumed simultaneous reduction in oedema may thus reflect an improvement in tendon material properties.

The nonsignificant increases in stress and stiffness observed would seem to reflect that compression treatment does not have its major effect on tendon material properties. However, the significant elevation in maximum force together with no increase in transverse area might still indicate an improvement in tissue quality secondary to compressive therapy, which may be related to collagen organization, the proliferative capacity to produce collagen III, and to edema reduction. IPC has, in fact, clinically been shown to expedite functional recovery following both fracture and soft tissue injuries.[12]

Whether alterations in the IPC protocol settings may lead to further optimization of healing tendon tissue quality could most likely also be studied by analyzing the mRNA levels of collagen I, and the matrix proteins biglycan, versican, and decorin, which are regulating tendon stiffness. Two weeks of tendon immobilization after rupture clearly inhibited a high increase in the mRNA levels of collagen I, biglycan, versican, and decorin, in fact, observed at 2 weeks of mobilized tendon healing.[23] It may prove that IPC treatment has the capacity to stimulate the production of various matrix molecules. The enhanced production and organization of collagen, that is, tendon remodeling, as observed after IPC treatment, may be initiated by mechanical stimulation of the tendon.[25] When IPC cuffs are inflated, the gastrocnemius muscle is compressed, resulting in longitudinal traction of the tendon. In fact, we observed an increased Achilles tendon length after compression treatment, which may confirm a longitudinal mechanical stimulation of the tendon.

Too much increase in tendon length, however, is not positive, and it could result in decreased contractile capacity, that is, plantar flexion force. Therefore, the time-dependent application of various mechanical forces, such as early mobilization and IPC, has to be further evaluated. One way to allow for earlier mechanical stimuli would be to suture repair the Achilles tendon. However, additional experiments are required to study the outcome of suture repair as well as the dose-dependent effects of compression on biomechanical tissue properties.

The major effects of the current compression treatment protocol are gained by promoting the proliferative healing phase as observed in this study by the 150% increase in collagen III-LI, improvement in collagen organization, and the subsequent 65% increase in maximum force at failure. These proliferative effects are presumably related to enhancement of fibroblast proliferation, blood flow, and regeneration of nerves expressing sensory neuropeptides essential for healing.[13,26–28] In fact, the sensory neuropeptide substance P (SP), which is increased after IPC treatment, has been demonstrated to enhance the production of nitric oxide.[29] SP and nitric oxide likewise upregulated by IPC, are known to stimulate angiogenesis, fibroblast proliferation, and tendon matrix production.[21,30]

In conclusion this study demonstrates that adjuvant external compression treatment can compensate for and even reverse some of the negatively affected parameters of tendon repair caused by immobilization. Given the fact that a period of at least 2 weeks of immobilization is regularly applied clinically when treating ruptured tendons, the results of this study may have a substantial clinical impact for the use of compression therapy during the period of immobilization. Thus, compression treatment may prove to accelerate healing during plaster/splint immobilization of patients, for example, in conservatively managed acute Achilles tendon ruptures. The results warrant long-term follow up studies, also on suture repair, and clinical studies to confirm the observations in this experimental study.

ACKNOWLEDGMENTS

This study was supported by the regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Institutet (project nr. SLL20070317) and the Swedish National Centre for Sports Research. Special thanks to our colleague Aglaia Maleka, M.D. for helping us with the double-blinded analysis.

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