Nitric oxide and tendon healing

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Nitric Oxide and Tendon Healing

George Anthony Calvert Murrell

Doctor of Medicine

The University of New South Wales

2006
Nitric Oxide and Tendon Healing

by

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MBBS (Adel), DPhil (Oxon)

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at

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Dedication

To all my students, who inspire and enlighten.
Declaration

I declare that all the publications enclosed in this report have not been submitted previously by myself for a university degree or other similar award.

George Anthony Calvert Murrell
Publications
Papers Published


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\(^1\) 1991 *American College of Sports Medicine New Investigator Award*

\(^2\) 1992 *American Orthopaedic Association Zimmer Award for Orthopaedic Residents*


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3 1994 American Orthopaedic Association Zimmer Annual Travel Award for Orthopaedic Fellows

4 2001 Australian Conference of Science and Medicine in Sport. Finalist Young Investigator Award


\textsuperscript{5} 2003 \textit{St George Hospital Symposium. Young Investigator Award – Clinical} \\
\textsuperscript{6} 2003 \textit{COMMS Award, American Orthopaedic Society for Sports Medicine Best Paper Annual Meeting}


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7 2003 Runner-up St George Hospital Symposium. Young Investigator Award – Clinical  
8 2004 American Orthopaedic Society for Sports Medicine Herodicus Award  
9 2004 David Garlick Memorial Scholarship, The NSW Sporting Injuries Committee, Sports Safety Award (Best new NSW talent in applied sports medicine research)
Synopsis

This thesis is the summation of a body of work that I performed and supervised over the last 15 years that evaluates the role of nitric oxide in tendon healing. The work began with an interest in sports injuries, oxidative stress and free radicals and culminated in three randomized clinical trials showing the beneficial effect of nitric oxide donation to tendinopathies for the Achilles, supraspinatus and elbow.

My interest in sports injuries started with my own experiences traumatizing my body as a triple jumper and was incubated during my DPhil in Oxford where I began to explore the interactions of oxygen free radicals and soft tissue healing\textsuperscript{15}. My mentors were kind enough to let me establish an animal model when I was completing my orthopaedic residency at Duke University Medical Center. This model and a chance interaction with Dr Csaba Szabo led to the pivotal studies on nitric oxide and tendon healing at the Hospital for Special Surgery New York where I was a research and clinical fellow on the Sports Medicine and Shoulder Service from 1993 to 1995. Since returning to Australia, I have had the great pleasure to establish and direct the St George Hospital Orthopaedic Research Institute and supervise 20 students complete their theses. Some of the work that they have completed forms part of this thesis and has been recognized by a number of awards. These are outlined in the list of publications section at the end of this section.

Tendon injuries

Tendons are the thick fibrous cords of dense connective tissue that attach muscles to bone. They transmit loads generated by muscles to stabilize joints and effect
movement. As such, tendons play a critical role in nearly every musculoskeletal activity - from standing to running to writing and throwing. Tendon injuries result from a single event or multiple episodes of overload, and lead to tendon degeneration (tendinosis) and/or rupture. Tendon injuries are common. In 1999, 6,549,003 Americans visited their doctors for soft tissue injuries. Sprains and strains are the most common workplace injury (64%) in Australia. The injuries are often debilitating - soft tissue strains and sprains were, by far, the leading nature of injury and illness that caused lost time away from the workplace in the USA and in Australia. The disorders are also costly - over 5 billion dollars per annum to the US community. Apart from surgical repair in certain cases and the avoidance of excessive immobilization, there are no methods available to the clinician to enhance the rate and strength of tendon healing.

**Tendon healing**

The process of tendon healing is complex and the roles of cellular, biochemical and biomechanical mediators continue to be elucidated. The key processes include clot formation, inflammation, fibroblast proliferation, matrix synthesis and reorganization. The stimuli for these processes are multifactorial, and are likely to include structural proteins and cytokines. Besides these factors, evidence is emerging for an important role of small diffusible molecules in wound repair. One of these is nitric oxide (NO).
Nitric oxide

Nitric oxide (NO) is a free radical – it has an unpaired electron and as such is highly reactive. It is also very small – one of the 10 smallest molecules. Its size and its high reactivity allow it to travel across nearly all biological structures and to readily react with other atoms or molecules to effect a change. NO is synthesized by a family of enzymes, the nitric oxide synthases (NOS), by catalyzing L-arginine to L-citrulline (Figure 1). Nitric oxide synthases are regulated by a number of co-factors. eNOS (originally found in endothelial cells) and bNOS (originally identified in brain and neuronal tissue) are constitutive, low output isoforms important in blood pressure regulation and memory. iNOS is a high output isoform that can be induced by bacterial cell wall products and pro-inflammatory cytokines (e.g. IL-1 and TNF), and is important in host defence. The isoforms of NOS have been cloned and sequenced from chromosomes 7 (eNOS), 12 (bNOS) and 17 (iNOS).

Rat Achilles tendon healing model

We developed a rat Achilles tendon division model to evaluate the healing process in tendons. The model involves removal of the plantaris muscle (which is rarely present in humans) and sharp transection of the right Achilles tendon in rats. Following this injury the rats have an altered gait consisting of a shortened stride and a longer, narrower footprint on the affected side. These alterations can be monitored from measurements made on their footprints. The defect in the tendon is initially filled with blood clot, then a loose connective tissue matrix with numerous inflammatory cells.
These cells are gradually replaced with fibroblasts and a dense collagenous matrix, with a ten-fold increase in the cross-sectional area of the healing tendon at day 10\(^{20}\). With time this matrix becomes denser, thinner and less cellular. Tendon load to failure properties recover at the same rate as the functional recovery (AFI), over 15 – 20 days\(^{5}\).

**Nitric oxide synthase activity following tendon injury**

We found no nitric oxide synthase (NOS) activity or immuno-activity in normal, uninjured rat Achilles tendon (Figure 2). Following division, however, there was a significant (approximately 5-fold) increase in the conversion of \(^{3}\)H-L-arginine to \(^{3}\)H-L-citrulline within the healing tendon at day 7. The activity was inhibitable by a NOS inhibitor (\(N^{G}\)-monomethyl-L-arginine; L-NMA), and returned to near baseline levels at day 14\(^{20}\). We used semi quantitative PCR and immunoblotting to determine which NOS isoforms were expressed in the healing tendon. All three NOS isoforms were expressed following rat Achilles tendon division. Four days after injury, there were increases in the steady-state levels of mRNA and protein for all three NOS isoforms, with peaks for the inducible isoform (iNOS) (23-fold increase) at day 4 and 7, the endothelial isoform (eNOS) (24-fold increase) at day 7, and the neuronal isoform (bNOS) (seven-fold increase) at day 21\(^{13,14}\).
**Rat rotator cuff tendon healing**

We also examined NOS expression in an acute rotator cuff tendon model in the rat by creating a defect in the supraspinatus tendon with a 3 mm diameter biopsy punch. Care was taken to leave at least 1 mm tacked tendon strip on both sides of the defect to prevent retraction of the tendon. The size of the defect was approximately 50% of the width of the supraspinatus tendon. All three NOS isoforms were expressed following injury in this acute rotator cuff injury model. As determined by competitive RT-PCR nNOS expression was maximum on day 4, eNOS on day 7 and iNOS on day 728.

**Rat rotator cuff tendon overuse model**

Soslowsky and coworkers developed an exercise overuse model of tendon degeneration in the rat\(^8,9\) and assisted us in establishing this model in our laboratory. In this model the rats were subjected to overuse exercise which consisted of treadmill running at 17 meters per minute (1 km/hour), on a 10 degree decline, for one hour per day, five days per week, for 4 weeks. This regime is similar in terms of the number of shoulder movements as that of elite swimmers. Animals subjected to normal cage activity were used as controls. We found that iNOS, eNOS and bNOS mRNAs were over-expressed in the supraspinatus tendon of rats subjected to treadmill running at 14 days\(^28\). These results suggest that nitric oxide synthase activity is induced as a result of tendon injury in this model, and/or that expression of NOS is a part of supraspinatus tendinopathy.
Human rotator cuff tendon injury

During surgical repair of the rotator cuff in humans the edges of the torn tendon are excised and discarded. We have evaluated these samples and found that nitric oxide synthase enzyme activity was detectable in 7 out of 10 human rotator cuff tendon samples. mRNA expression was demonstrated for iNOS and eNOS isoforms in each sample examined, while bNOS mRNA was detectable in 3 out of 8. These results indicate that a similar phenomenon of NOS up-regulation occurs following injury in humans as in rats.

Where does NO come from?

In the rat Achilles tendon model the first isoform to appear was iNOS, followed by eNOS and then bNOS. As one would expect, iNOS was found to be expressed in macrophage-like cells and eNOS was found in endothelial cells. Interestingly, all three isoforms were found to be expressed in fibroblast-like cells, again in temporal fashion with iNOS being expressed first (days 4-7), followed by eNOS (days 4-14) and bNOS (days 14-21).

Is nitric oxide synthase expression important to tendon healing?

We fed rats a competitive nitric oxide synthase inhibitor - $N^\text{6}$-nitro-L-arginine methyl ester (L-NAME) and found that rats taking this inhibitor had significantly reduced healing of their Achilles tendons compared with rats drinking the inactive enantiomer of it - D-NAME. There was a 50% reduction in cross-sectional area of the Achilles tendon at day 7, and a corresponding 24% reduction in the failure load of the rat Achilles tendon.
Nitric oxide synthase inhibition (L-NAME) resulted in a significant reduction in cross-sectional area (30% at day 7, p < 0.01; 50% at day 15, p < 0.001) and failure load (24% at day 7, p < 0.01) of the healing Achilles tendon constructs in rats.

Figure 3

Which nitric oxide synthase gene is important for tendon healing?

We performed an experiment in mice to determine whether the inducible nitric oxide synthase gene was crucial for tendon healing. Mice with the gene for iNOS deleted were compared with wild type mice. iNOS and wild type mice were fed a nitric oxide synthase inhibitor amino guanidine. Their right Achilles tendons were divided and, on day 15, tendon healing was measured by its cross-sectional area and mechanical properties. While inhibition of nitric oxide synthase by aminoguanidine inhibited cross-sectional area by 20%, knocking out the iNOS gene alone did not have any significant effect on tendon healing. These data imply that iNOS alone is not responsible for the beneficial effects of nitric oxide on tendon healing.
What roles does nitric oxide play in tendon healing?

The experiments in animals using nitric oxide synthase inhibitors show that NO is important for the volume of tissue synthesised during tendon healing. NO is likely to be important in a number of processes, including local blood flow and host defence. Work in our laboratory has identified NO to be important in collagen synthesis. Cultured human rotator cuff tendon cells, when exposed to exogenous nitric oxide in the form of S-nitro-N-acetyl penicillamine (SNAP) and when transfected with the iNOS gene via an adenovirus vector, incorporated more collagenase sensitive $^3$H-proline in their matrix (Figure 4). This increased collagen synthesis was inhibited by a nitric oxide synthase inhibitor.

**Figure 4.** The effects of SNAP and iNOS gene transfection on collagen synthesis in human tendon cells measured by [$^3$H] proline incorporation into collagenase sensitive fraction of the cells.  
(a) Control: untreated human tendon cells. SNAP: the human tendon cells treated with different doses of SNAP, 10, 50, 100, 400 and 800 µM. *: P < 0.05, **: P < 0.01, ***: P <0.001 compared with control group (Mean ± SD, n = 6).  
(b) Ad-Empty: human tendon cells transfected with adenovirus 100 pfu/cell without gene insert. Ad-iNOS: human tendon cells transfected with adenovirus at 10, 50 or 100 pfu/cell concentration with iNOS gene insert. *: P < 0.05, **: P < 0.01 compared to Ad-Empty group (Mean ± SD, n = 6).
In our rat model, NO was delivered systemically via flubiprofen, a non-specific cyclo-oxygenase inhibitor. Flubiprofen reduced body weight gain by 25% and this reduction was prevented by the addition of NO (ie NO-flubiprofen). Flubiprofen and NO-flubiprofen decreased healing tendon cross-sectional area by 30% and 20%. This reduction in tendon cross-sectional area was accompanied by a decreased failure load of the healing tendons in the flubiprofen group, but not the NO-flubiprofen group. The additional NO via NO-flubiprofen had a protective or beneficial effect on tendon healing failure load and stress (load/area) (Table 1).

Table 1: Assessment of the drug effects (Mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>Body weight gain (g)</th>
<th>Cross-sectional area (mm²)</th>
<th>Failure of load (N)</th>
<th>Stress (N/mm²)</th>
<th>Hydroxyproline content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO-flubiprofen</td>
<td>41±4 (n=12)</td>
<td>5.3±0.1 (n=5)***</td>
<td>36±1.1 (n=5)≡</td>
<td>6.9±0.3 (n=5)*</td>
<td>51±1.6 (n=12)</td>
</tr>
<tr>
<td>flubiprofen</td>
<td>31±3 (n=10)*</td>
<td>4.7±0.2 (n=5)***</td>
<td>30±2.6 (n=5)*</td>
<td>6.4±0.59 (n=5)</td>
<td>54±1.0 (n=9)</td>
</tr>
<tr>
<td>vehicle</td>
<td>41±3(n=12)</td>
<td>6.9±0.2 (n=5)</td>
<td>39±1.9 (n=5)</td>
<td>5.6±0.2 (n=5)</td>
<td>50±1.5 (n=11)</td>
</tr>
</tbody>
</table>

* Significantly different from vehicle at the 0.05 level; *** significantly different from vehicle at the 0.001 level; = significantly different from flurbiprofen at the 0.05 level.

The beneficial effects of NO on healing tendon were more clear in a similar experiment where we used NO-paracetamol as the vehicle for delivering NO. In this experiment, there were no significant differences between the vehicle and paracetamol groups. In the NO-paracetamol group, the tendon had less cross-sectional area but better material and mechanical properties and, perhaps most interestingly, much more collagen.
per unit dry weight\(^{30}\) (Figure 5). Furthermore, the histological analysis showed the collagen fibres to be better organised than the paracetamol and vehicle alone groups. These data imply that delivering nitric oxide via NO-paracetamol enhances the quality of the healing tendon, perhaps by increasing the amount of collagen and/or decreasing the amount of non-collagenous protein and improving the organisation of healing tendon. These rat results are consistent with cell culture findings for human tendon cells where NO enhanced collagen synthesis, and with the results from the clinical trials described below.

![Graphs showing hydroxyproline content, cross-sectional area, stiffness and stress of healing Achilles tendons in rats treated with vehicle, paracetamol, NO-paracetamol at day 10 post-operation. Values are mean ± SEM, n=11, 11 and 12 for vehicle, paracetamol and NO-paracetamol. ***= p < 0.001, * p<0.05 using un-paired two-tailed Student’s t-test and ANOVA.]

**Figure 5.** Total collagen content, cross-sectional area, stiffness and stress of healing Achilles tendons in rats treated with vehicle, paracetamol, NO-paracetamol at day 10 post-operation. Values are mean ± SEM, n=11, 11 and 12 for vehicle, paracetamol and NO-paracetamol. ***= p < 0.001, * p<0.05 using un-paired two-tailed Student’s t-test and ANOVA.
Clinical trials

To determine whether additional nitric oxide might enhance tendon healing in humans we conducted three randomised double-blind clinical trials in humans. These trials involved the application of a commercially available nitric oxide delivery system (glyceryl trinitrate patches) and a placebo version of the same patch. These patches were applied over the area of tenderness for three conditions: tennis elbow, Achilles tendinosis and supraspinatus tendinosis.

Tennis elbow

Eighty six patients with chronic (symptoms of greater than three months duration) were randomized to one of two groups. The active group received continuous topical nitric oxide donation (1.25 mg / 24 hour glyceryl trinitrate) and the placebo group received the identical patch without GTN. Both the patients and the clinical examiner were blinded as to which group the patients were

Figure 6.
in. The site of application was demonstrated as immediately distal to the lateral epicondyle of the humerus, and patients were instructed to rotate the patch application site around this point with each new patch application. Both groups also received a counterforce brace and a stretching and strengthening program. In other words, NO was applied in addition to “best practice”. The nitric oxide group had reduced elbow pain with activity at 2 weeks (p = 0.01), reduced epicondylar tenderness at 6 and 12 weeks (p = 0.02), and an increase in wrist extensor mean peak force and total work at 24 weeks (p = 0.03). 81% of patients on GTN patches were asymptomatic in activities of daily living at six months compared to 60% of patients with tendon rehabilitation alone (p = 0.005 with Chi square analysis)(Figure 6)

**Achilles tendinitis**

A prospective randomized double blinded, placebo controlled clinical trial was performed with 65 patients (84 Achilles tendons) with chronic (symptoms greater than three months) Achilles tendinitis using the same study design, with the exception that patches were applied to the Achilles tendon rather than the elbow. The nitric oxide (GTN) group showed reduced Achilles activity pain at week 12 (p = 0.02) and 24 (p=0.03), reduced night pain at week 12 (p=0.04), reduced tenderness at week 12
(p = 0.02), decreased pain scores with the hop test at week 24 (p=0.005) (Figure 7), and an increase in ankle plantar flexor mean total work\textsuperscript{24} at 24 weeks (p = 0.04). 78\% of patients on GTN patches were asymptomatic with activities of daily living at six months compared to 49\% of patients with tendon rehabilitation alone (p = 0.001 with Chi square analysis). Mean effect size for all outcome measures was 14\%\textsuperscript{23}.

**Supraspinatus tendinopathy**

53 patients (57 shoulders) with chronic supraspinatus tendinopathy/impingement were recruited and we completed a prospective randomized, double-blinded, placebo controlled clinical trial of continuous topical nitric oxide donation (1.25 mg/24 hour glyceryl trinitrate, GTN). The nitric oxide group had significantly reduced shoulder pain

Figure 8.
with activity, at night, and at rest at week 24 (p = 0.03), reduced internal rotation impingement at week 24 (p = 0.02) (Figure 8), increased range of motion in abduction and internal rotation at week 24 (p=0.04)\textsuperscript{11}, and an increase in force at weeks 12 and 24 with supraspinatus testing (p = 0.001) (Figure 9), external rotation (p=0.04), internal rotation (p=0.01), adduction (p=0.04), and subscapularis push-off (p=0.01)\textsuperscript{10}. 46 % of patients on GTN patches were asymptomatic with activities of daily living at six months compared to 24 % of patients with tendon rehabilitation alone (p = 0.007). Mean effect size of GTN treatment for all outcome measures was 26 %\textsuperscript{25}.

**Summary of clinical trials**

In each clinical trial, there was a significant beneficial effect of nitric oxide on patient-determined pain, function and loss of symptoms. The treatment effects of GTN in
each of these situations are greater than any other reported treatment modality for those tendinopathic conditions. In terms of side effects, the only significant increase was in headaches, which was more prevalent in the active group, particularly in the GTN clinical trial.

Discussion

The work presented in this thesis shows that following injury to a tendon, three isoforms of nitric oxide synthase are expressed by fibroblasts as well as other cells usually associated with the relevant isoforms of nitric oxide synthase. Expression in the healing rat Achilles tendon is in a temporal fashion, with the inducible nitric oxide synthase coming first, followed by endothelial nitric oxide synthase and then a neuronal nitric oxide synthase. Nitric oxide generated by these isoforms is important for tendon healing. The data included in this thesis supports the hypothesis that NO enhances collagen synthesis and results in injured tendons having better material and mechanical properties, ie the healing tendons are stronger on a per unit area basis than those not exposed to additional nitric oxide. The clinical trials support this concept and show that delivering nitric oxide via a patch enhances the healing of tendinopathy. The enhancement manifests itself in a reduction in pain, increase in range of motion and an increase in strength.

There are however many unanswered questions. The exact mechanism whereby nitric oxide enhances collagen synthesis, and the nature of and contribution of NO to other mechanisms to improving wound healing need to be identified. The optimal dose and mode of delivery, timing and duration of delivery of nitric oxide for tendinopathies
and in torn tendons in humans needs to be explored further. Hopefully, however, the work presented in this thesis will provide a foundation and a framework of understanding for this interesting molecule in tendon healing.

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The Achilles Functional Index

George A. C. Murrell, Edward G. Lilly, Helen Davies, Thomas M. Best, Richard D. Goldner, and Anthony V. Seaber

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Summary: The literature regarding the management of spontaneous rupture of the Achilles tendon is controversial and confusing. The relative infrequency of the condition in any one center prohibits the completion of well-designed clinical studies. Many of the disputes could be addressed and innovations tested if an appropriate animal model were available. We present a method for evaluating Achilles tendon function from measurements of the prints, preserved in bromphenol-blue-impregnated photocopying paper, of the hindfeet of walking rats. The stimulus for this study was derived from de Medinaceli’s method for assessing the functional condition of rat sciatic nerves (de Medinaceli L, Freed WJ, Wyatt RJ: An index of the functional condition of rat sciatic nerve based on measurements made from walking tracks. Exp Neurol 77:634-643, 1982). Four variables were measured from these walking tracks, and comparisons between the damaged (experimental) and intact (normal) side were converted to proportional deficits. The relative contribution of each parameter to the overall deficit was determined by multiple linear regression analysis, and the variables were weighted accordingly to obtain an "Achilles Functional Index" (AFI). A sham operation produced no functional deficit, whereas animals subjected to a 0.5-cm mid substance Achilles tendon defect demonstrated a markedly impaired AFI. Animals with repaired transected Achilles tendons also demonstrated a significant, but less severely impaired AFI. The functional deficit in this repair group returned to control values by postoperative day 15, whereas animals with a defect remained impaired at day 15. Furthermore, an excellent correlation was found between the functional recovery and biomechanical properties (ultimate failure load) of the healing tendon \( r = 0.94; p < 0.001 \). In summary, this method provides a relatively inexpensive, sensitive, and reproducible means of assessing the functional performance of the Achilles tendon after injury in the rat. Key Words: Achilles tendon—Tendon injuries—Functional model.

Although an Achilles tendon injury can be studied biochemically and biomechanically, the most important clinical criterion of recovery is return in function. To our knowledge, there is no quantitative method for evaluating the functional recovery of Achilles tendon injuries in animals. The present study describes an index based on the measurements of the hindpaw prints of walking rats, which provides a reliable and quantifiable method for evaluating the functional condition of the Achilles tendon.

MATERIALS AND METHODS

Eighty-five outbred male Sprague–Dawley rats (Dominion Laboratories, Dublin, VA, U.S.A.)
weight 250-300 g (just before skeletal maturity) were used in the following studies. All animals were housed in 6-chip-lined plastic cages, three animals per cage, with a 12-h light, 12-h dark cycle in a central animal care facility. Animals were fed rat chow and water ad libitum. Animals were assigned to the experimental groups in a random fashion. Anesthesia was achieved with 50 mg/kg intraperitoneal sodium phenobarbital (Anthony Products Co., Acadia, CA, U.S.A.) and the surgical procedures were performed under sterile conditions with the assistance of an operating microscope (Zeiss Mottorstativ, Zeiss Instruments, Germany).

**Surgical Procedures**

**Sham Operation**

The right hindlimb was prepared with povidone-iodine (Betadine). A 3-cm midline incision was made over the Achilles tendon, and the Achilles tendon and plantaris were dissected free from the surrounding fascia. The skin was then sutured in a subcuticular fashion with 5-0 absorbable polyglactin-910 (Vicryl) suture on a PS-6 cutting needle (Ethicon Inc., Somerville, NJ, U.S.A.). No cast or dressings were applied and the animals were unrestricted and fed a normal diet during the healing phase.

**Achilles Tendon Repair**

The same procedure was followed as above. In addition, the Achilles tendon was transected with a scalpel in an axial fashion 0.5 cm from its calcaneal insertion. In rats, the plantaris muscle is well developed, being approximately one-fifth the size of the Achilles tendon and lying on the medial side of the Achilles tendon. If left intact, it may act as an "internal splint." For this reason the tendinous portion of the plantaris was removed to prevent any possible action as an internal splint. The Achilles tendon was then repaired with 5-0 ehtilon monofilament nylon on a PC-1 cutting needle (Ethicon) using a modified Kessler-type suture (Fig. 1).

**Achilles Tendon Defect**

A 0.5-cm segment was removed from the central portion of the Achilles tendon to create a significant defect in the Achilles tendon. The tendinous portion of the plantaris was removed. The Achilles was not repaired.

**Sciatic Nerve Contusion**

The right gluteal region of the animal was shaved and prepped with povidone-iodine. Using a gluteal-splitting approach, the sciatic nerve was isolated and the orifice of a strain gauge (Lebow model 3168, Measurement Products, Troy, MI, U.S.A.) paired with a micrometer (Mitutoya, Tokyo, Japan) and strain indicator (Model P-3500, Measurements Group, Raleigh, NC, U.S.A.) were introduced across the nerve (Fig. 2). The strain gauge was then tightened to apply a 0.98 N force (100 g mass) across the sciatic nerve for 10 min. The skin was closed with a running subcutaneous 5-0 polyglactin-910 suture on a PS-6 cutting needle.

**Sciatic Nerve Division**

The same procedure was performed as described as for sciatic nerve contusion, with the exception that the sciatic nerve was divided sharply with a scalpel, rather than being subjected to contusion.

**Functional Assessment**

Animals were tested in a confined walkway 8.7 cm wide x 43 cm long with a dark shelter at the end.
as described by Hruska et al. (5). An 8 × 42-cm piece of photocopying paper impregnated with a 0.5% solution of the anhydrous form of bromphenol blue (Sigma, St. Louis, MO, U.S.A.) in absolute acetone (6), was placed on the floor of the rat walking corridor. The rats were held by their chest, their hindpaws were dipped in a dish containing a water-soaked sponge, and they were allowed to walk down the corridor, leaving blue footprints on the yellow paper. The paper was coded by animal number and stored for later measurements in a blinded fashion.

Distance to opposite foot (TOF), print length (PL), distance between first and fifth toes or toe spreading (TS), and distance between the second and fourth toes or intermediary toes (IT) was measured as described by de Medinaceli et al. (4) (Fig. 3). de Medinaceli recorded the longest measurements for each parameter on each side. We recorded the measurements of all footprints on each strip of paper (range four to eight prints per paper) and calculated the mean value for each parameter. The coefficient of variance for the Achilles Functional Index (AFI) from measurements using the mean value was less than that using the longest measurements (21 c.f. 25; n = 18). A factor was generated for each of the following measurements.

Distance to opposite foot factor (TOFF) =

\[
(\text{ETO}F - \text{NTOF})/\text{NTOF}
\]

de Medinaceli’s print length factor (PLF) (4) =

\[
(NPL - \text{EPL})/\text{EPL}
\]

Toe-spread factor (TSF) =

\[
(\text{ETS} - \text{NTS})/\text{NTS}
\]

Intermediary toe-spread factor (ITF) =

\[
(\text{EIT} - \text{NIT})/\text{NIT}
\]

where TS is total spreading, PL is print length, IT is distance between intermediate toes, TOF is distance to opposite foot, N is normal, uninjured side, and E is experimental side (Fig. 3).

Multiple linear regression analysis was performed using each of the preceding factors and using analysis of variance to determine which factors contributed to the regression (7). The multiple linear regression used the defined functional deficit as the dependent variable (defined as −100 in animals with transected Achilles tendons, 0 in sham-operated or unoperated animals; n = 54 for each group). Each of the preceding factors was used as the independent variable in the regression. To determine whether the factor contributed to the regression, analysis of variance was performed, and any factors that did not significantly contribute were excluded from the final predictive equation for Achilles function. An α value of 0.05 was taken to determine significance.
Statistical Analysis

Statistical analysis between the experimental groups was performed using two-way Student's *t* tests. The confidence limit was predetermined at an α value of 0.05.

RESULTS

Transsection of the Achilles tendon produced an obvious gait disturbance in walking rats, a disturbance that was not apparent in sham-operated animals. The hindpaw prints of animals with injured Achilles tendons were considerably longer and narrower on the injured side during the immediate postoperative period. These alterations in paw prints gradually resolved during a 15-day period as the animals recovered (Fig. 4). The functional deficit resulting from an Achilles tendon transection (and repair) manifested itself in a statistically significant (p < 0.001) 20-fold, 30-fold, and 6-fold greater deficit in footprint length (PLF), and toe spreading (TSF and ITF), respectively (Fig. 5). There was a twofold greater deficit in the ability to push off the injured leg (TOFF), but it did not reach statistical significance (Fig. 5A). Further compromise in the integrity of the Achilles tendon by an artificially created 0.5-cm defect resulted in additional deficits in PLF, TSF, and TOFF, whereas ITF was unchanged (Fig. 5).

The immediate deficits resulting from Achilles tendon transection and partial removal were, for the most part, comparable to the deficits resulting from sciatic nerve contusion and division (Fig. 6). The group with sciatic nerve contusion had less impairment in TOFF than did the group with a complete sciatic nerve lesion (Fig. 5A). There was no evidence of foot dragging or autophagia in any group. Animals subject to a 0.98-N sciatic nerve contusion recovered during a 9-day period (Fig. 7). Animals with a sciatic nerve division remained impaired (AFI < -100) during the 15-day study period (Fig. 7).

AFI Index

Measurements from 108 tracks were used to generate a formula to represent the functional deficit of injured Achilles tendons. Each factor (TOFF, PLF, TSF, ITF) demonstrated a normal Gaussian distribution (not shown). Multiple linear regression analysis of variance of these factors as independent variables against the dependent variable of the defined Achilles tendon injury indicated that three of the variables contributed to the dependent variable: PLF (standard regression coefficient β = 0.26; p < 0.0001), TSF (β = 0.57; p < 0.0001), ITF (β = 0.17; p < 0.0007), whereas TOFF did not significantly contribute (β = 0.05; p = 0.33). Thus, TOFF was discarded, the multiple linear regression analysis repeated, and an AFI generated:

$$AFI = 74 \text{(PLF)} + 161 \text{(TSF)} + 48 \text{(ITF)} - 5$$

The *F* value for this equation was 279, significant at the p < 0.00001 level. The same analysis was performed using Bain’s PLF (1), and was found to contribute less to the analysis than de Medinaceli’s PLF (β = 0.22, c.f. β = 0.26).

Sensitivity of the AFI

AFIs were calculated for three groups of animals (sham operation, Achilles transection and repair, and Achilles defect) during a 15-day period. The apparently normal gait of sham-operated animals was confirmed by AFI scores, which all remained ~0 (Fig. 8). Animals subject to a 0.5-cm Achilles tendon defect showed a significant reduction in AFI (−128 ± 5, mean ± SEM, p < 0.001), which gradually improved during a 15-day period to −60 ± 16, while remaining significantly more impaired than the sham-operated group. Animals subject to Achilles tendon transection and repair showed a statistically significant impairment in AFI, an impairment that was not so great as that in the Achilles defect group (Fig. 8). The AFI worsened in the Achilles repair group on postoperative days 2 and 3, and then gradually improved during a 15-day period, such that at day 15, the AFI was not statistically significantly different from that of the sham-

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**FIG. 4.** Illustration of the initial changes and rate of recovery of pawprints in rats with divided and repaired Achilles tendons.
DISCUSSION

To our knowledge, this is the first time that a functional assessment of healing Achilles tendons has been made in an animal model. Hruska et al. (5) were the first to show that the locomotor pattern in normal rats was constant for a range of speeds, sizes, and ages of rats. de Medinaceli et al. (4) went on to use measurements from the hindpaw prints of rats to calculate a formula that reflected the functional state of damaged sciatic nerves. His method of recording prints has since been improved by Lowdon et al. (6), and minor modifications to the...
formulas have been made by Carlton and Goldberg (3) and Bain et al. (1). We adapted these methods to objectively assess the function of walking rats with Achilles tendon injuries. Sham-operated animals (in which the skin was incised and the Achilles and plantaris tendons were mobilized, but not injured) exhibited no functional deficit, whereas all animals subject to Achilles tendon transection and repair showed a significant, immediate reduction in function that gradually improved during a 15-day period. Three variables (PLF, TSF, ITF) provided a sensitive and reliable indicator of Achilles tendon function. The TOFF (the hindlimb stride length) measures the capacity to push off the experimental limb. It was the factor showing the most variation and was eliminated from the final equation. Bain et al. (1) and Carlton and Goldberg (3) had also found it to be the most variable of the factors in sciatic nerve injuries and eliminated it from their formulas. The PLF (length of footprint) is short in normal animals that walk only on their toes. Animals with a damaged Achilles tendon placed the whole foot much more flatly, leaving a longer pawprint. As expected, animals subject to Achilles tendon transection had an immediate impairment in PLF, which gradually returned to normal. TSF (toe-spread factor) and ITF (intermediate toe factor) are measures of the ability of the rat to spread its middle three toes (ITF) and all five toes (TSF). The ability to spread toes was markedly impaired in both Achilles tendon and sciatic nerve injuries. The measures were, in fact, the most sensitive indices. Although it is easy to rationalize impairment of the intrinsic foot muscles as being secondary to denervation in nerve
injuries, similar observations in Achilles tendon injuries (where care was taken to avoid nerve damage) suggest that ITF/TSF are more likely to represent the inability of the animal to support weight and to "push off" the affected hind limb. The return of the factors to normal by day 15 in the Achilles tendon transection and repair group also indicates that denervation may not be the only cause for an impairment of ITF and TSF.

The functional deficit resulting from an Achilles transection was found to be very similar to that of a sciatic nerve contusion or division. One difference was that animals with chronic sciatic nerve injuries often drag their feet, making measurements difficult (1,3,4). This behavior was not observed in animals subject to Achilles tendon injury. A second difference was that animals subject to sciatic nerve division often autophagia the toes of the affected hind limb, again making measurements difficult. There was no evidence of autophagia in any group in this study. The index generated from the walking track measurements of animals with transected Achilles tendons \( \text{AFI} = 74 \text{(PLF)} + 161 \text{(TSF)} + 48 \text{(ITF)} - 5 \) was similar to that generated by Bain et al. (1) for sciatic nerve injuries \([\text{Sciatic Functional Index (SFI)} = -38.3 \text{(PLF)} + 109.5 \text{(TSF)} + 13.3 \text{(ITF)} - 8.8]\). The PLF value expressed here is negative because the PLF used by de Medinaceli and in this article \((\text{NPL} - \text{EPL}/\text{EPL})\) is the inverse of that used by Bain \((\text{EPL} - \text{NPL}/\text{NPL})\).

Although not particularly specific, the AFI was sensitive enough to reflect the additional initial impairment and reduction in rate of recovery in animals with a 0.5-cm Achilles tendon defect. Concurrent studies on the biomechanical properties of healing Achilles tendons suggest that the rate of functional recovery is very similar to the mechanical recovery of the Achilles tendon \((\text{AFI} \text{ versus experimental failure load}; r = 0.94; p < 0.001)\) (2).

The outlined method for quantifying Achilles tendon function has the advantage that it is reproducible, noninvasive, objective, inexpensive, and easy to perform. It must be stressed, however, that there are limitations to any model. The rat is a small quadruped with a more developed plantaris muscle than the human has. In this model, the plantaris tendon was removed so that the anatomy would be closer to that of the human. No form of external splint was provided to any of the animals during the postoperative period. The animals were, however, able to "protect" the injured limb with the other three limbs, a situation most akin to touch-down or partial weight bearing in a biped.

The rat has a more rapid rate of metabolism with an associated increase in rate of repair and shorter life span than humans have. Care was taken to follow functional recovery at narrow intervals to minimize these effects. The model of clean transection of the midsubstance of the Achilles tendon is not a direct mimic of Achilles tendon rupture, because in the clinical setting the tendon does not rupture cleanly, but pulls apart with frayed ends. Methods for creating more ragged ends were not pursued, because the resulting lesions are less likely to be reproducible. Another consideration is age. Achilles tendon rupture classically occurs in middle-aged individuals, in whom the vascular impairment and degenerative changes are usually more prominent. The animals included in this study were approaching skeletal maturity and were chosen because they were relatively easy to handle and demonstrated highly reproducible walking tracks.

Taken together, the results of this study indicate that the AFI provides a precise and reliable, noninvasive measure of the functional condition of the Achilles tendon in the rat. It is hoped this method may provide a valuable, alternative means to assess tendon healing in an animal model.

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Achilles Tendon Healing: A Correlation Between Functional and Mechanical Performance in the Rat

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Summary: The pathogenesis and treatment of rupture of the Achilles tendon remain a source of controversy. This study presents the results of a biomechanical, functional, and morphological evaluation of a group of rats that had division and repair of the Achilles tendon. A total of 46 rats were used: 18 for biomechanical testing, 18 for functional evaluation, and 10 for histology. Morphological examination revealed an early inflammatory response with loose connective tissue formation that was replaced gradually by fibroblasts and a collagenous matrix. The functional evaluation (Achilles functional index [AFI]) was made from measurements of the hind pawprints of walking rats. Division and repair of the Achilles tendon produced a significant functional impairment (mean [±SEM] AFI = -87 ± 8; p < 0.001), which gradually improved with healing time. The load to failure for the repaired tendons consistently improved with healing time, in a manner similar to the functional recovery. The average deformation (repair/control) varied considerably and was not related to healing time. The stiffness of the repaired tendons increased with healing time and was 60% of the corresponding control side by day 15. The major finding of this study was a strong correlation between the AFI and the failure load of the healing tendon-bone constructs (250-300 g group, r = 0.97, p < 0.001; 325-375 g group, r = 0.96, p < 0.001).

Rupture of the Achilles tendon has been documented in the literature for several centuries (11). The controversy regarding the pathophysiology and treatment of this injury continues, as many authors advocate primary operative repair (1,3,4,9,19,22,32) whereas other authors favor a nonoperative approach (7,8,11,16,29,39).

In contrast to experimental studies on the mechanical and histological properties of healing anterior cruciate and medial collateral ligaments (41) and digital flexor tendons (15,23,25,33), there have been comparatively few laboratory investigations on the healing of Achilles tendons (2,5,20,31,34,36,38). This may be due in part to problems in methodology for biomechanical testing, including adequate fixation of the specimen (2,38) and the attainment of consistent mechanisms of failure (2). One study investigated healing of the Achilles tendon and the evaluation of its biomechanical properties at physiological rates of deformation (36). The major findings were that the return of stiffness in a healing tendon correlated with the presence of fibroplasia and that the return of other tensile properties was a function of the amount and degree of organization of the fibroplasia.

Although the biomechanical properties of healing tendon are important, from a clinical perspective the
functional performance of the injured individual is crucial to the success or failure of any given management strategy. Clinical studies in which function was evaluated after injury to the Achilles tendon have included objective testing by static lifts (21) and assessment by dynamometry (19,35). To our knowledge, no quantitative methods are available for the evaluation of functional recovery after injury of the Achilles tendon in animals. The current study presents the results of a biomechanical, functional, and morphological evaluation of a group of rats that had division and repair of the Achilles tendon. We hope that the development of an animal model that includes a functional and biomechanical assessment to study the progress of recovery after injury to the Achilles tendon will advance our knowledge and understanding of the treatment of this common and often debilitating problem.

MATERIALS AND METHODS

Animal Model

Forty-six outbred male Sprague-Dawley rats (Dominion Laboratories, Dublin, VA, U.S.A.) weighing 250-375 g were used in this study: 18 for biomechanical testing, 18 for functional evaluation, and 10 for histology. All animals were housed in cages lined with Beta Chips (Northeastern Products, Warrensburg, NY, U.S.A.), three animals per cage, and a standard 12 h light and 12 h dark cycle was used. Animals were fed rat chow and water ad libitum. Anesthesia was achieved by intraperitoneal injection of Nembutal (pentobarbital) (50 mg/kg) (Anthony Products, Arcadia, CA, U.S.A.). The operative procedures were performed under sterile conditions with the aid of a 15 mm/s operating microscope (Op. Mi 15; Zeiss Instruments, Oberkochen, Germany).

Operative Procedures

Sham operation: With aseptic technique, a 3 cm midline incision was made in the skin overlying the Achilles tendon of the right hindlimb. Blunt dissection was used to free the plantaris and Achilles tendon from the surrounding fascia. The skin was closed in a subcuticular fashion with a 5-0 absorbable Vicryl (polygactin; Ethicon) suture on a PC-I cutting needle (Ethicon). Achilles tendon repair: The same incision was made in the left leg, and the Achilles tendon was transected transversely 0.5 cm proximal to its calcaneal insertion with a No. 10 scalpel blade. The tendinous portion of the plantaris was removed to prevent it from acting as an internal splint. A 5-0 Ethilon (Ethicon) monofilament nylon suture on a PC-I cutting needle (Ethicon) was used to repair the divided tendon with a modified Kessler-type suture.

Biomechanical Testing

Eighteen animals were assigned prospectively to one of two groups based on the initial body weight. Group 1 consisted of nine animals weighing 250-325 g, and group 2 consisted of nine animals weighing 325-375 g. In each animal, repair of the Achilles tendon was done on the left hindlimb. One animal from each group was killed on postoperative days 1, 2, 3, 5, 7, 9, 11, 13, and 15 for subsequent biomechanical testing of both the control (sham operation) and experimental limbs. After death, the hindlimbs were transected at the knee joint, snap frozen in liquid nitrogen, wrapped in gauze soaked with BSS (balanced salt solution) Plus (Alcon Laboratories, Fort Worth, TX, U.S.A.), covered with plastic wrap (Saran Wrap; Dow Chemical, Indianapolis, IN, U.S.A.), and stored at −20°C.

Two hours before testing, specimens were thawed at room temperature in BSS Plus solution. All soft and hard tissues were dissected from the calcaneus-Achilles tendon complex. The gastrocnemius-soleus muscle fibers were removed with the blunt end of a scalpel handle, leaving the formerly intramuscular tendinous fibers. A portion of the Achilles tendon (mean ± SD, 1.02 ± 0.12 cm) was measured proximally from the distal attachment of the tendon to the calcaneus with precision calipers (±0.0016 cm). The remaining proximal portion of tissue was secured between two pieces of laboratory tape with Thick Gel Super Glue (Super Glue, New York, NY, U.S.A.). The plantar aspect of the calcaneus was mounted with Thick Gel Super Glue at 45° to the surface on a triangular piece of Epon (Pelco, Tustin, CA, U.S.A.) embedding bloc (measuring 1.0 x 1.0 x 0.5 cm) (Fig. 2). The Epon bloc-calcaneal complex was embedded in a 13 mm diameter aluminum pot with self-curing plastic cement (Teledyne Getz, IL, U.S.A.). Throughout the procedure, care was taken to ensure that neither the Super Glue nor the self-curing cement contaminated the tendon itself. The tendinous portion of the construct was wrapped in BSS Plus-soaked gauze during the fixation period.
to prevent dehydration of the specimen.

All mechanical testing was carried out with a servohydraulic materials testing machine (model 1321; Instron, Canton, MA, U.S.A.). The room temperature was 25°C, and the specimens were kept moist with BSS Plus solution. Before testing, the previously measured length of the tendon was reestablished by adjustment of the actuator. The specimens were not preconditioned or cyclically stretched prior to tensile testing. All tendons underwent a test of constant velocity ramp to failure at 1 cm/s. Force-time and displacement-time curves were digitally recorded on an oscilloscope (Nicolet; Instron) and transferred to a computer (model 80; IBM, Research Triangle Park, NC, U.S.A.) for subsequent post-processing and data analysis. Three structural properties for the tendon-bone construct were measured: load to failure, total deformation, and stiffness (slope of the load-deformation curve over the linear range). At the completion of testing, each specimen was inspected for mode of failure (substance tear or avulsion) and any evidence of suture pullout.

**Functional Evaluation**

Function was evaluated in 12 animals that were grouped according to weight (250-325 and 325-375 g) and six animals that had only a sham operation. A quantitative method for the evaluation of functional performance after injury to the Achilles tendon in animals was developed from the modification by Lowdon et al. of the sciatic functional index of de Medinacleri et al. (10,24,28). The hindpaws of the 18 rats were moistened, and the rats were allowed to walk down a confined runway that had been covered with bromphenol blue-impregnated photocopy paper, which changes color on contact with water.
Measurements were made from each walking track as illustrated in Fig. 1. The distance to the opposite foot (TOF), print length (PL), and distance between the second and fourth or intermediary toes (IT) were measured as previously described (10). A factor was generated for each of the following measurements.

distance to opposite foot factor (TOFF) = (ETO - NTOF)/NTOF
de Medinacelli's print length factor (PLF) = (NPL - EPL)/EPL
toe-spread factor (TSF) = (ETS - NTS)/NTS
intermediary toe-spread factor (ITF) = (EIT - NIT)/NIT

where N is the normal uninjured side, E is the experimental side, and TS is total spreading (Fig. 1).

A multiple linear regression analysis indicated that the TOFF did not significantly contribute to the regression. An Achilles functional index (AFI) thus was calculated as

\[ AFI = 74(PLF) + 161(TSF) + 48(ITF) - 5 \]

**Histology**

One repaired and one uninjured (control) Achilles tendon were harvested on days 0, 3, 9, 13, and 15 (n = 10). The tendons were fixed immediately in buffered formalin (pH 7.4) for 24 h, dehydrated, and embedded in paraffin wax. Coronal sections of the midsubstance of the tendon were cut, stained with hematoxylin and eosin, and examined in a blinded fashion.

**Statistical Analysis**

A two-way Student t test was used to assess the biomechanical differences between control (uninjured) and experimental (repaired) tendons. The confidence limit was predetermined at an alpha level of 0.05.

**RESULTS**

**Biomechanical Testing**

All load-deflection curves were nonlinear and showed a stiffening response (increasing load with increasing deformation) (Fig. 3). There was no slippage of the tendon from the clamp and no suture failure or retraction of the tendon ends in any of the specimens.

After the second postoperative day, load to failure was greater for the group of heavier animals at each time point (Fig. 4). For both groups, the failure load of the repaired tendon was equal to that of its control by the thirteenth postoperative day, and the specimen failed at the tendon-bone interface. Prior to day 13, all repaired tendons failed at the tenotomy site. All control (uninjured) specimens failed at the tendon-bone insertion site.

The average deformation for the 18 tendons, expressed as a percentage of the control, varied consid-
Neither deformation nor stiffness correlated with erably and showed no relationship with healing time (mean ± SEM, 2.31 ± 0.35 cm). Stiffness improved with healing time in the repaired tendons and was 60% of the corresponding control value by day 15. Neither deformation nor stiffness correlated with the AFI.

### Functional Evaluation

Six animals that had only a sham operation showed no evidence of protective weight-bearing and had no objective functional deficit, as determined by the AFI (Fig. 5). The animals that had division and repair of the Achilles tendon demonstrated a significant functional impairment with longer, narrower pawprints and a shorter stride length (Table 1) (mean ± SEM AFI = –86 ± 8 for the six animals in group 1, p < 0.001; and –88 ± 14 for the six in group 2, p < 0.001). The animals initially protected the injured hindlimb and were reluctant to place the injured limb on the ground. The exact weight-bearing status was difficult to assess, but pawprints were recorded immediately postoperatively on day 1. The pawprint parameters and the AFI gradually improved with healing time; by day 15, all rats had a nearly normal gait (Fig. 5 and Table 1).

### AFI Compared with Load to Failure

The time course of recovery of functional performance was almost identical to that of the failure load for the repaired tendons. When the AFI was compared with the ultimate failure load in rats of equivalent body weight, the correlation coefficient (r) was 0.97 (p < 0.001) in group 1 (250-325 g) and 0.96 (p < 0.001) in group 2 (325-375 g).

### Histology

Macroscopically, the control tendons appeared as glistening, pearl-white longitudinally arranged structures, 1 cm long and 2 mm² in cross section. In contrast, by day 15, all of the tendons that had been repaired were thickened, salmon-pink structures, 1 cm long and an average of 5 mm in diameter. All tendons were examined at harvest for disruption at the suture site. No evidence of suture failure or pull-out was noted.

At the microscopic level, the control tendons consisted of dense, parallel bundles of homogeneous eosinophilic material aligned with the long axis of the tendon. Interspersed within this matrix were a few longitudinally aligned spindle and oval shaped fibroblasts (1,400 mm²⁻²) with prominent nuclei and sparse cytoplasm (Fig. 6A).

In the divided Achilles tendons, the area between the cut ends of the tendon initially was filled with a loose connective tissue matrix containing numerous inflammatory cells (neutrophils, basophils, macrophages, and lymphocytes) (3,500 mm²⁻²) and erythrocytes (2,300 mm²⁻²) (Fig. 6B). By day 9, this loose connective tissue matrix had been replaced by a more

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**TABLE 1. Change (mean ± SD) in length and width of pawprint after division and repair of Achilles tendon in the group of six rats weighing 325-375 g**

<table>
<thead>
<tr>
<th>Plantar length (mm)</th>
<th>Toe spread (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Experimental</td>
</tr>
<tr>
<td>Day 0</td>
<td>Day 1</td>
</tr>
<tr>
<td>25 ± 2</td>
<td>38 ± 3</td>
</tr>
<tr>
<td>20 ± 1</td>
<td>14 ± 3</td>
</tr>
</tbody>
</table>

*p < 0.001 when compared with sham control (two-way Student t test).

*p < 0.01 when compared with sham control (two-way Student t test).

*p < 0.05 when compared with sham control (two-way Student t test).

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**FIG. 5.** The mean (± SEM) Achilles functional index for each group (n = 6) versus time. □ = group of rats that had a sham operation. ● = group of rats that had repair of the Achilles tendon (weight, 250-325 g) and ▲ = group of rats that had repair of the Achilles tendon (weight, 325-375 g).
FIG. 6. Light microscopy of rat Achilles tendon. A comparison of coronal sections of normal (uninjured) rat Achilles tendon (A) and the reparative process after division on postoperative days 3 (B), 9 (C), and 15 (D). The large arrows indicate the site of division. Note the original well organized collagenous tissue (M) and the new loose connective tissue matrix, infiltrated with inflammatory cells. The new matrix soon was populated by more fibroblasts and a denser, somewhat disorganized collagenous matrix. On day 9, the junction of old and new matrix was characterized by a dense band of fibroblasts (large arrow); the band was almost indistinguishable by day 15 (hematoxylin and eosin, x170).
homogeneous, denser eosinophilic matrix. Plump fibroblasts with large nuclei (2,700 mm\(^2\)) were the predominant cell type, and generally they were aligned in the longitudinal axis of the tendon. The junction between the old tendon and the new matrix was characterized by a dense (0.1 mm) band of oval shaped fibroblasts (8,000 mm\(^2\)) with little organization (Fig. 6C). With time, the tendon-new matrix
interface and the band of disorganized fibroblasts became less distinct. The collagenous tissue also became more organized, with fewer fibroblasts (2,200 mm\(^2\) on day 15) and more matrix (Fig. 6D).

**DISCUSSION**

This study presents the results of new methods of biomechanical testing and functional evaluation after injury of the Achilles tendon in a rat model. Both techniques were reliable, repeatable, and sensitive to healing time. Division and operative repair of the Achilles tendon resulted in an initial functional impairment that improved consistently throughout the study period. An excellent correlation was found between the functional performance of the rat, as determined from measurement of walking tracks, and the failure load of the repaired tendon-bone constructs during the 15 day period.

In contrast to other soft tissues, there have been relatively few reports on the healing of Achilles tendon injuries in animal models. Male Sprague-Dawley rats were chosen for this study because they are well characterized experimental animals with a reproducible gait pattern and a relatively rapid rate of healing. Because of this rapid healing response, the return of function and strength were followed closely during the postoperative period. Tissues were snap frozen in liquid nitrogen to facilitate subsequent removal of the muscular tissues from the tendon and to promote fixation of the proximal portion of the specimen. Immediate freezing has been shown to preserve tissue integrity (14) without compromising the mechanical properties of the specimen (6,14,37).

Most of the previous investigations on healing of the Achilles tendon have been complicated by difficulties with either failure at the tendon-clamp interface (27,38) or slippage from the clamp (2,38). Failure of the muscle belly frequently has been observed in control muscle-tendon complexes (2,36). In this study, the muscle fibers of the gastrocnemius-soleus complex were removed by freeze-thawing and blunt dissection, thereby permitting the tendon fibers to be secured between tape with cyanoacrylate glue. With these techniques, constructs consistently failed at the suture site (repaired tendons prior to day 13) or at the tendon-calcaneus interface (controls at all time intervals and from day 13 in repaired tendons).

The mechanical response of biological tissues is dependent on the rate of testing (6,40). The present results were achieved with use of a stretch rate of 100%/s, suggested by others as an appropriate rate for the evaluation of physiological conditions (6). To date, only one study has reproduced this rate of testing without problems of specimen slippage or failure at the clamp (36). Data on failure load and stiffness have been reported elsewhere for Achilles tendons in animal models, but different methods and testing rates preclude any meaningful comparison of our results with other studies. In the only previous study carried out at comparable rates, Steiner (36) did not report a failure load for normal tendon because all of the control specimens failed by rupture of the muscle. Using test preparations similar to those of Steiner, Barfred reported an occasional failure load of 54.0 N for control specimens (2). When the specimens were matched for body weight, the mean failure load of the controls in the present study was 64.8 \(\pm 5.4\) N. The methods presented here were sensitive to healing time. Both failure load and stiffness of the repaired tendons improved with healing time. A second group of heavier animals (group 2) were tested under identical conditions and the same response to healing time was found, but failure loads were consistently greater. These results are in agreement with those in a study by Jackson et al. showing increased breaking strength of the Achilles tendon with increased body weight (20). Although it may be argued that the method of clean transection of the midportion of the tendon does not directly mimic a clinical injury, methods for creating more ragged ends were not pursued, as it is less likely that these lesions would be consistent and reproducible. In support of our methods is a recent study of rabbit medial collateral ligaments that demonstrated that both types of injury appear to heal at the same rate and with the same mechanical properties (13).

The functional test also was shown to be sensitive to healing time, with improvement in test scores throughout the healing period for both groups of animals after the second postoperative day. In comparison, the animals that underwent a sham operation demonstrated no functional impairment throughout the healing period. A previous study demonstrated that this test is not specific to injuries of the Achilles tendon, as the functional deficit resulting from transection and repair of the Achilles tendon is similar to that resulting from contusion or division of the sciatic nerve (28).

There was a strong correlation between the ani-
mal's functional performance, as measured by the AFI, and the failure load of the repaired tendon (group 1: \( r = 0.97 \) and \( p < 0.001 \); group 2: \( r = 0.96 \) and \( p < 0.001 \)). By the thirteenth day postoperatively, the failure load of the repaired tendon-bone complex was the same as its control in both groups and the corresponding AFI was close to full recovery. A similar correlation was not found between the AFI and stiffness or deformation of the tendon. Previous reports have documented a return of stiffness before load to failure in healing collagenous tissues (30,36,42). Our results are not in agreement with this finding, as failure load of the repaired tendon normalized with respect to the control side by the thirteenth postoperative day, while stiffness was 60% of the control value.

The morphological results presented here correlate well with the healing process described elsewhere (24,26). It is important to remember that the present results do not include a consideration of the cross-sectional area of the healing tendon, and therefore the structural properties of the tendon-bone complex, rather than the material properties of the healing tendon itself, are reported. The gross hypertrophic response seen in the repaired tendons suggests that, in mature Achilles tendons, scar tissue has inferior material properties compared with those of normal Achilles tendons. The hypertrophy of immature scar tissue with inferior material properties may be important in the early reestablishment of biomechanical failure properties and functional recovery of healing Achilles tendons in the rat.

As with any animal model, application to the human condition must be reviewed carefully. In this study, we attempted to address the topic of healing of the Achilles tendon by developing an animal model that would permit evaluation of the biomechanical, functional, and morphological properties of healing tendon. We did not observe the phenomenon of early softening seen in other studies on ligament and tendon healing. Our hypothesis is that immobilization may have contributed to this observation in previous studies and that early softening did not occur in our model because the animal's hindlimb was not formally immobilized. Observation of the animals in the postoperative period indicated that the animals were able to "protect" the injured limb with the other three limbs, a situation most similar to touch-down or partial weight-bearing in a biped. This protective behavior did not extend to complete nonweight-bearing or complete immobilization and gradually changed with the AFI over 15 days. By day 15, the animals had a normal gait, as measured by the AFI, and objectively were fully weight-bearing.

In summary, this study presents experimental techniques that can be used to study the healing of Achilles tendon injuries in the rat. These techniques include an assessment of both the biomechanical characteristics and the functional performance of the healing tendon. Both of these methods are reliable, repeatable, and sensitive to healing time. Our major finding was an excellent correlation between the properties of load to failure and the functional performance of the healing tendon.

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¹ 1991 *American College of Sports Medicine New Investigator Award*
Achilles Tendon Injuries: A Comparison of Surgical Repair Versus No Repair in a Rat Model∗

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ABSTRACT
Controversy exists regarding the treatment of Achilles tendon ruptures. The aim of this study was to determine whether surgical repair of the rat Achilles tendon offered any biomechanical, functional, or morphological advantage over no repair. Thirty-two male Sprague-Dawley rats were randomly allocated into four groups: (1) sham operated (skin incision only), (2) no repair (complete division of the Achilles tendon and plantaris tendon without repair), (3) internal splint (plantaris left intact), and (4) Achilles repair (with a modified Kessler-type suture). Functional performance was determined from the measurements of hindpaw prints utilizing the Achilles Functional Index. On day 15, the animals were killed, and biomechanical and histological evaluations were performed on both the injured and uninjured Achilles tendon constructs.

All groups subjected to Achilles tendon division had a significant initial functional impairment that gradually improved so that by day 15 there were no functional or failure load impairments in any group. The injured tendons in all three groups subjected to Achilles tendon division had a 13-fold increase in the cross-sectional area and were less stiff and more deformable than uninjured and sham-operated tendons on day 15 (P < .001). The magnitude of the biomechanical and morphological changes at postoperative day 15 and the initial impairment and rate of functional recovery were similar for no repair, internal splint, and Achilles repair groups. In summary, this study demonstrates that surgical repair of the Achilles tendon in the rat does not offer any advantage over nonoperative management.

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INTRODUCTION
We have recently developed an animal model for evaluating the functional recovery of rats subjected to Achilles tendon injuries. The method involves dipping the hindpaws of rats in water and allowing them to walk down a confined runway covered with bromophenol blue-impregnated photocopy paper. Measurements can be made, and an Achilles Functional Index (AFI) can be calculated from these footprints. The index is sensitive to the severity of the Achilles tendon injury and the time after injury and has a good correlation with the ultimate failure load of the Achilles tendon construct. The index is, however, not specific to Achilles tendon injuries; injuries to the sciatic nerve produce similar changes. We were interested in using the AFI and our biomechanical model to address clinical problems. One such problem is the management of ruptured Achilles tendons. Controversy exists regarding the treatment of this injury. A number of authors propose surgical repair using a variety of techniques, and a corresponding number advocate nonoperative management. The aim of this study, therefore, was to determine whether surgical repair of the Achilles tendon offered any functional, mechanical, or morphological advantage over no repair in a rat model.

MATERIALS AND METHODS
Animal Model
Outbred male Sprague-Dawley rats (Dominion Laboratories, Dublin, VA), weight 250–300 g, were housed in beta chip-lined plastic cages, three animals per cage, with a 12-hr light: dark cycle in a central animal care facility. They were fed rat chow and water ad libitum. Thirty-two animals were randomly divided into four groups (Fig. 1). Anesthesia was achieved by intraperitoneal Nembutal (50 mg/kg). All surgical procedures were performed under sterile conditions with the aid of...
an operating microscope (OpMi 1S, Zeiss, Germany). A 3-cm midline incision was made over the right Achilles tendon, and the Achilles tendon and plantaris were isolated from the surrounding fascia. The Achilles tendon and plantaris were left intact in the sham-operated group. In the other groups, the Achilles tendon was divided cleanly in its midsubstance with a scalpel, 0.5 cm from its calcaneal insertion. Care was taken not to disrupt the surrounding tissues. The tendinous portion of the plantaris was removed in the no repair and Achilles repair groups and left intact in the internal splint group. In the Achilles repair group, the Achilles tendon was repaired with 5-0 ethilon monofilament nylon on a PC-1 cutting needle (Ethicon Inc., NJ) using a modified Kessler-type suture technique (Fig. 1). The skin was then sutured in a subcuticular fashion with 5-0 absorbable polyglactin-910 (Vicryl) suture on a PS-6 cutting needle (Ethicon). No operation was performed on the left, uninjured, hindlimb. No cast or dressings were applied, and the animals were unrestricted during the healing phase.

Functional assessments (see below) were made for all animals before surgery and postoperatively on days 1, 2, 3, 5, 7, 9, 11, 13 and 15. On day 15, the animals were killed with intraperitoneal Nembutal (500 mg/kg), and biomechanical evaluations were performed on both lower limbs of six of the eight animals in each group. Morphological examinations were performed on the remaining two animals in each group.

**Functional Assessment**

The functional assessment is outlined in detail elsewhere. In summary, animals were tested in a confined walkway with a dark shelter at the end as described by Hruska et al. Photocopy paper impregnated with 0.5% solution of the anhydrous form of bromophenol blue (Sigma St. Louis,M) in absolute acetone was then laid on the floor of the rat-walking corridor. The anhydrous form of Bromophenol blue is yellow but readily changes to blue on contact with water. The rats were held by their chest, their hindpaws were dipped in a small dish containing a water-soaked sponge, and they were allowed to walk down the corridor leaving blue paw prints on the yellow paper. The paper was coded by animal number and stored for later measurements in a blinded fashion.

Distance to opposite foot (TOF), print length (PL), distance between first and fifth toes or toe spreading (TS), and distance between the second and fourth toes or intermediary toes (IT) were measured as described by de Medinaceli (Fig. 2). A factor (F) was generated for each of these measurements (where E = experimental, N = normal, uninjured side): distance to opposite foot factor (TOFF) = (ETOF-NTOF)/NTOF, de Medinaceli's print length factor (PLF) = (NPL-EPL)/EPL, toe-spread factor (TSF) = (ETS-NTS)/NTS, intermediary, and distance between intermediate toes; TOF = distance to opposite foot. From reference 15 with permission.
ary toe-spread factor (ITF) = (EEIT-NIT)/NIT. The AFI was then calculated: AFI = 74 (PLF) + 161 (TSF) + 48 (ITF) - 5.

Biomechanical Testing

Harvested lower limbs were immersed in liquid nitrogen, wrapped in a BSS Plus® (Alcon Labs, TX)-soaked cotton gauze and placed in 30-ml plastic containers. The specimens were stored at -20°C for an average of 7 days before testing. On the day of testing, the specimens were thawed in BSS Plus solution for 2 hr. All extraneous soft and hard tissues were removed by blunt and sharp dissection from the calcaneus-Achilles tendon complex. Area measurements of the uninjured and sham-operated tendons were made with an area micrometer (Mitutoya, Tokyo, Japan) at 0.25, 0.5, and 0.75 cm from the calcaneal insertion. Area measurements in the remaining specimens were calculated from the circumference of the tendon (using 0-silk suture material) at 0.25, 0.5, and 0.75 cm from the calcaneal insertion. The formerly intramuscular tendinous fibers were then secured between two strips of white labeling tape with Thick Gel Super Glue® (Super Glue Corp., NY) so that the distal edge of the tape was 1.0 cm from the calcaneal insertion. The calcaneus was fixed to a plastic block at 45°, and the block-calcaneal complex was embedded in a 13-mm diameter pot with self-curing plastic (Teledyne Getz, IL) (Fig. 3). Throughout the procedure, care was taken to ensure that neither the Super Glue nor the self-curing plastic contaminated the tendon. Specimens were kept moist by application of BSS Plus solution. Mechanical testing was carried out using an Instron model 1321 Servohydraulic materials testing machine (Instron Corp., MA). The in situ resting length of the tendon was re-established at 1 cm, and each specimen underwent a constant velocity ramp-to-failure test at 1 cm/sec (100%/sec). Voltage-time and displacement-time histories for each test were captured on a Nicolet oscilloscope (Instron) and transferred to an IBM model 80 computer (IBM, Research Triangle Park, NC) for subsequent data analysis. At the completion of testing, each specimen was inspected for type of failure, i.e., substance tear versus avulsion.

Morphology

Experimental and uninjured Achilles tendons were immediately fixed in buffered Formalin saline (pH 7.4) for 24 hr, dehydrated, and embedded in paraffin wax. Coronal sections of the midsubstance of the Achilles tendons were cut at 5 μm, stained with hematoxylin and eosin, and examined in a blinded fashion.

Statistical Analysis

Statistical analysis among the sham-operated group and the experimental groups was performed using two-way Student's t-tests. Differences between uninjured and operated tendon complexes were analyzed with a two-way paired Student's t-test. The confidence limit was predetermined at an alpha level of 0.05.

RESULTS

Morphology

The macroscopic appearance of uninjured and sham-operated tendons on the 15th postoperative day was glistening, pearly white, longitudinally arranged structures 1 cm long and 2 mm² in cross section. In contrast, all tendons previously subjected to surgical division were salmon pink structures 1 cm long and 26-27 mm² in cross-sectional area (Fig. 4). The morphological appearance and cross-sectional area were similar in the injured tendons in the no repair, internal splint, and Achilles repair groups. No evidence of rerupture was noted in any of these experimental groups.

At the microscopic level, uninjured and sham-operated tendons consisted of dense, parallel bundles of homogeneous eosinophilic substance aligned with the long axis of the tendon. Interspersed within this matrix were a few longitudinally arranged spindle- and oval-shaped fibroblasts with prominent nuclei and sparse cytoplasm (Fig. 5A).

At day 15, the area of transection of all experimentally divided tendons was filled with a dense eosinophilic matrix populated in a rather disorganized fashion with
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Fig. 4. Cross-sectional area of Achilles tendons at day 15. Mean (SEM) experimental (injured) tendon/control (uninjured) tendon. N = 6 for each group. Mean cross-sectional area of sham operated groups = 2.37 mm². ** = P < .001 when compared with sham-operated tendons using two-way Student's t-tests.

numerous fibroblasts (Fig. 5 B-D). No differences in the microscopic appearance of the tendons were observed between the no repair, internal splint, or Achilles repair groups.

Function

Sham-operated animals exhibited no functional deficits throughout the experimental period (Fig. 6). Animals subjected to Achilles tendon division demonstrated an initial obvious functional deficit, with the hindpaw prints considerably longer and narrower on the injured side. These alterations in paw prints gradually resolved over a 15-day period so that at day 15 the AFI of the no repair, internal splint, and Achilles repair groups were not significantly different from those of the sham-operated animals (Fig. 6).

There were no statistically significant differences between the experimental groups (no repair, Achilles repair, internal splint) with respect to the initial impairment or the rate of functional recovery (Fig. 6).

Biomechanics

Firm proximal fixation was obtained by removing the muscle fibers of the gastrocnemius and soleus (by freeze-thawing) and sandwiching the remaining, formerly intermuscular tendinous fibers between labeling tape with Super Glue. Distal fixation was achieved by potting the cleaned calcaneus in plastic cement with the plantar surface mounted at 45°. Under these conditions, the failure site of all sham-operated and uninjured tendons was at the distal tendon-calcaneus interface. In the previously divided tendons, failure occurred at both the midsubstance and the tendon-calcaneus interface. The relative distribution between these two failure sites was similar (3.3, 3.3, 4.2; midsubstance:tendon-calcaneal interface) for the no repair, internal splint, and Achilles repair groups respectively.

The biomechanical properties of Achilles tendon-calcaneal complexes in the sham-operated and uninjured (internal control) limbs of all groups were similar at day 15 (Fig. 7). There were no statistically significant differences in failure load, ultimate deformation, or mean stiffness between any of the experimental groups at day 15; i.e., all tendons subjected to division alone, internal splintage, or surgical repair had similar failure load, ultimate deformation, and mean stiffness properties. Furthermore, by day 15 all three experimental groups regained an ultimate failure load equivalent to uninjured and sham-operated tendons (Fig. 7A). The previously divided calcaneal-Achilles tendon complexes in the repair, no repair, and internal splintage groups were, however, approximately twice as deformable and half as stiff as uninjured and sham-operated Achilles tendon-calcaneal complexes on day 15 (Fig. 7, B and C). The tendons initially subjected to division in the repair, no repair, and internal splintage groups had a 13-fold greater cross-sectional area on postoperative day 15 than the uninjured or sham operated tendons, suggesting that the tensile strength (failure load/cross-sectional area) of the previously divided tendon complexes was an order of magnitude less than the tensile strength of uninjured and sham-operated tendons.

DISCUSSION

To our knowledge, this is the first time that both biomechanical and functional assessments have been used to objectively determine the effectiveness of surgical repair of the Achilles tendon in an animal model. Sham-operated animals exhibited no functional deficit, and all animals subjected to Achilles tendon division (with or without repair) showed a significant, immediate reduction in function that gradually improved over a 15-day period so that at day 15, the AFI was not statistically significantly different from that of sham-operated animals (Fig. 6). The magnitude of the initial impairment and the rate of recovery were almost indistinguishable in the experimental groups subjected to repair, no repair, or internal splintage.

The rat is a small quadruped with a more developed plantaris muscle than that in humans. In this model, the plantaris tendon was removed so that the anatomy would be closer to human plantaris muscle. In the internal splint group, the plantaris tendon was left intact to act as an internal splint. No form of external splint was provided to any group during the postoperative period. The animals were, however, able to protect the injured limb with the other three limbs, a situation most akin to touch down or partial weightbearing in a biped.
Fig. 5. Light microscopy of rat Achilles tendon. A comparison of coronal sections of A, normal sham-operated rat Achilles tendon; B, the reparative process in divided Achilles tendon; C, plantaris tendon left intact to act as an internal splint; and D, repaired tendon, on postoperative day 15. Note the well organized collagenous tissue in A, compared to the relatively poorly organized collagenous matrix in B, C, and D, (hematoxylin and eosin, x170).

Fig. 6. Time course of the AFI in the sham-operated and experimental groups. Mean (SEM); N = 8 for each group.

The rat has a more rapid rate of metabolism with an associated increase in rate of repair and a shorter life span than humans. The model of clean transection of the midsubstance of the Achilles tendon is not a direct imitation of Achilles tendon rupture; in the clinical setting, the tendon does not rupture cleanly but pulls apart with frayed ends. Methods for creating more ragged ends were not pursued because the resulting lesions are less likely to be reproducible. Another difference between individuals with Achilles tendon ruptures and the rats used in this experiment is age. Achilles tendon rupture classically occurs in middle-aged individuals in whom the vascular impairment and degenerative changes of Achilles tendons are usually more prominent. The animals included in this study were approaching skeletal maturity and were chosen because they
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Fig. 7. Biomechanical properties of Achilles tendon-calcaneal complexes in the four groups of rats at day 15. Mean (SEM) experimental (injured) construct/control (uninjured) construct. N = 6 for each group. A, mean failure load for sham-operated group = 73 N; B, mean stiffness for sham-operated group = 33.867 N/m; C, mean ultimate deformation for sham-operated group = 2.0 mm. ** = P < .01, *** = P < .001 when compared with sham-operated constructs using two-way Student’s t-tests.

were relatively easy to handle and demonstrated highly reproducible walking tracks.

Our previous studies have indicated that the AFI is not specific to Achilles tendon injuries; rats subjected to sciatic nerve contusion or division demonstrated similar changes in print length and width. The AFI was, however, sensitive to the magnitude of the initial injury to the Achilles tendon. Rats with an artificially created 0.5-cm Achilles tendon defect had a greater initial deficit and recovered more slowly than rats with a repaired Achilles tendon. The rate of functional recovery, as measured by the AFI, also followed a time course similar to the recovery of the ultimate failure load of Achilles tendon-calcaneal complexes (r = 0.94, P < .001). This association between functional and mechanical performance was confirmed in the present study: both the functional indices and the failure loads returned to control values on day 15 (Fig. 6 and 7A).

Previous mechanical studies on Achilles tendon healing have been complicated by difficulty with tissue fixation. In Barfred’s study, more than 90% of the specimens failed at the bone-metal pin fixation site. In this study, a construct was designed to minimize clamp damage and slippage. Freezing muscle in liquid nitrogen proved a simple method to help remove unwanted muscle fibers. Super Glue and tape provided a reliable means for fixing the remaining proximal tendinous fibers. Distal fixation was achieved by potting calcanei at 45° in plastic cement. Using this system, all failures occurred within the tendon midsubstance or at the tendon-bone interface suggesting that the stress concentrations at the proximal clamp-tendon interface had been reduced to acceptable levels. The results obtained using this construct under standardized conditions (including maturity of animals, method of harvesting, freezing conditions, temperature, and hydration) demonstrated less variation than previous evaluations of healing rat and rabbit Achilles tendons.

Constructs from animals that had undergone a sham operation (i.e., where neither the Achilles nor the plantaris tendon were violated) exhibited biomechanical properties similar to the uninjured (control) constructs of all the experimental groups. On the 15th postoperative day, constructs from the no repair, Achilles repair, and internal splintage groups had regained failure loads equivalent to sham-operated and uninjured constructs (Fig. 7A). The restoration of failure loads to control values on postoperative day 15 is in contrast to the results for rabbit Achilles tendon in which failure loads after 10 weeks of immobilization and 10 weeks immobilization/remobilization were 30% and 70% of the uninjured tendon. The detrimental effects of immobilization on failure load in the rabbit experiments may account partly for these discrepancies.

It is important to note that whereas all three experimental groups (no repair, internal splint, Achilles repair) had regained the failure load properties of uninjured tendon constructs on day 15, the experimental constructs were approximately twice as deformable and half as stiff as uninjured and sham-operated constructs.
Furthermore, 50% of the Achilles tendon-calcaneal complexes in which the tendon had previously been surgically divided failed in the midsubstance (compared with 0% of the sham operated and uninjured tendons). This relative immaturity in the biomechanical properties of the healing tendons was supported by the disorganized microscopic appearance and the 13-fold increase in cross-sectional area on day 15 and suggests that the hypertrophic response of the healing tendon may compensate for the healing tendon’s inferior tensile strength properties.

Taken together, the results of this study demonstrate that neither surgical re-aposition with a nonabsorbable Kessler suture nor the presence of the plantaris tendon to act as an internal splint offered any mechanical, functional, or morphological advantage to the recovery of divided Achilles tendons in rats. These findings are consistent with the clinical literature supporting nonsurgical treatment of Achilles tendon ruptures.\(^7\)\(^,\)\(^11\)\(^,\)\(^21\) The findings are also consistent with the only prospective randomized trial\(^17\) assessing methods of treating Achilles tendon rupture. In this study, 105 consecutive patients with rupture of the Achilles tendon were randomized to surgical and nonoperative treatment groups. At follow-up (average 2.5 year), there were no statistically significant differences in plantarflexion strength or major complications, including rerupture, between the surgically and nonsurgically treated groups.

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REFERENCES


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Effects of Immobilization on Achilles Tendon Healing in a Rat Model

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Summary: The aim of this study was to evaluate the effects of immobilization and mobilization on the functional and biomechanical recovery of injured Achilles tendons. Male Sprague-Dawley rats were allocated randomly into four groups: (a) sham operation, (b) division only (surgical transection of the Achilles tendon without immobilization), (c) "dummy" external fixation (division of the Achilles tendon and application of Kirschner wires), and (d) rigid external fixation (division of the Achilles tendon and immobilization with Kirschner wires connected by two triangular frames). All procedures were performed on the right lower limb; the left, uninjured, lower limb served as an internal control. Kirschner wires and external fixators were removed on day 12. Functional performance was determined from measurements of hind pawprints of rats walking preoperatively and on postoperative days 1, 3, 5, 7, 9, 11, 13, and 15. On day 15, the animals were killed and biomechanical evaluations were performed on both the injured and the uninjured Achilles tendon constructs. No functional or mechanical deficits were observed in the sham-operation group. Animals subjected to division of the Achilles tendon had an initial functional deficit that returned to near normal by day 15. The application of Kirschner wires was associated with an impairment of the functional performance of the rat as well as of the mechanical properties of the tendon-bone constructs. Immobilization by connection of the Kirschner wires to an external frame had an additional, highly significant (p < 0.001) detrimental effect on the functional and mechanical recovery of Achilles tendon-calcaneal complexes.

The beneficial effects of early motion on the recovery of injured soft tissues other than the Achilles tendon (3,8,15) have stimulated the design and preliminary testing of functional braces that allow patients with rupture of the Achilles tendon early protected motion of the ankle (11,27) rather than immobilization in long and short leg casts (1,4,7,9,12,18,24,26,28,29). Saleh et al. (27), in a prospective randomized study, showed that patients treated with a splint that permitted early mobilization of the ankle regained range of motion of the ankle and were able to return to normal activities sooner than the more rigidly immobilized group. In spite of these clinical investigations and much data concerning the potential benefits of motion on soft-tissue injuries, there is little information regarding the effects of immobilization on the functional and mechanical performance of injured Achilles tendons. We recently developed methods to assess such parameters in a rat model (5,6,22). The aim of this study, therefore, was to determine if early motion of the ankle offered any functional or mechanical advantage compared with immobilization in the rehabilitation of injured Achilles tendons in a rat model.
MATERIALS AND METHODS

The study included 32 outbred male Sprague-Dawley rats (Dominion Laboratories, Dublin, VA, U.S.A.), each weighing 250-300 g (7-8 weeks old in a life span of 2.5-3 years), that were allocated into four groups: (a) sham operation, (b) division only, (c) "dummy" external fixation, and (d) rigid external fixation (Fig. 1). All procedures were performed on the right lower limb, and the left, uninjured, lower limb served as an internal control. All animals were housed in plastic cages lined with Beta Chips (Northeastern Products, Warrensburg, NY, U.S.A.), three animals per cage, with a 12-hour light, 12-hour dark cycle, in a central animal care facility. The animals were fed rat chow and water ad libitum. Anesthesia was achieved with 50 mg/kg intraperitoneal sodium phenobarbital (Anthony Products, Arcadia, CA, U.S.A.), and the surgical procedures were performed under sterile conditions with the assistance of an operating microscope (Motorstativ; Zeiss Instruments, Oberkochen, Germany).

Surgical Procedures

Sham operation: The right hindlimb was prepared with Betadine (povidone-iodine). A 3 cm midline incision was made over the Achilles tendon, and the Achilles tendon and plantaris were dissected free from the surrounding fascia. The dorsal surface of the Achilles tendon pseudosheath was split in a longitudinal fashion. The ventral surface of the pseudosheath was preserved. The skin then was sutured in a subcuticular fashion with 5-0 absorbable polyglactin-910 suture (Vicryl; Ethicon, Sommerville, NJ, U.S.A.) on a cutting needle (PS-6; Ethicon). No cast or dressing was applied, and the animals were unrestricted and were fed a normal diet during the healing phase.

Division only: The same procedure as already described was performed. The dorsal surface of the Achilles tendon pseudosheath was split in a longitudinal fashion, and the ventral surface of the pseudosheath was preserved. In addition, the Achilles tendon was axially transected with a scalpel, 0.5 cm from its calcaneal insertion. The cut ends of the tendon were dissected free from the pseudosheath. The distance between the cut ends of the tendon at the

FIG. 1. Schematic illustration of the experimental groups used in this study. No procedure was performed on the uninjured left lower limb. Ex-fix = external fixation.

FIG. 2. Schematic illustration of the external fixator applied in the group that had rigid external fixation.
time of skin closure was 3-4 mm. In rats, the plantaris muscle is well developed, with its tendon being approximately one-fifth the size of the Achilles tendon and lying medial to it. If left intact, it may act as an "internal splint," and for this reason the tendinous portion of the plantaris was removed.

**Dummy external fixation:** The same procedure was performed. In addition, 0.045-inch (1.2-mm) Kirschner wires were placed through the proximal femur (just distal to the greater trochanter), proximal tibia (at the level of the tibial tuberosity), calcaneus, and metatarsals (at the level of the metatarsal necks). Self-curing plastic (Teledyne Getz, IL, U.S.A.) was placed on the exposed tips of the wires to prevent injury to the animal and to secure the pins during the experimental period.

**Rigid external fixation:** The same procedure was performed. In addition, the Kirschner wires were connected by 0.062-inch (1.6-mm) Kirschner wires in triangular formations (Fig. 2). Self-curing plastic cement was used to secure the joints of the frame and to prevent injury to the animal. The construct was fashioned so that the knee was held in 60° of flexion and the ankle, in 30° of equinus.

Functional testing was performed preoperatively for all animals and on postoperative days 1, 3, 5, 7, 9, 11, 13, and 15 in the sham-operation and the division-only groups. On day 12, the animals in the dummy external fixation and the rigid external fixation groups were anesthetized with 50 mg/kg intraperitoneal sodium phenobarbital and the Kirschner wires were removed. Functional testing was performed on these animals on days 13 and 15. On day 15, all animals were killed and biomechanical testing was carried out on both the injured and uninjured Achilles tendon constructs.

**Functional Assessment**

The animals were tested in a confined walkway, 8.7 cm wide by 43 cm long, with a dark shelter at the end, as described by Hruska et al. (16). An 8 × 42 cm piece of photocopying paper impregnated with a 0.5% solution of the anhydrous form of bromphenol blue (Sigma, St. Louis, MO, U.S.A.) in absolute acetone (19) was placed on the floor of the corridor where the rats walked. The anhydrous form of bromphenol blue is yellow in color but readily changes to blue on contact with water. The rats were held by the chest and the hindpaws were dipped in a dish containing a water-soaked sponge; they then were allowed to walk down the corridor, leaving blue pawprints on the yellow paper. The paper was coded by animal number and was stored in a blinded fashion for later measurements. Measurements were made from all pawprints on each strip of paper (range, four to eight prints per paper) with the method illustrated in Fig. 3. These measurements included print length, distance between first and fifth toes (total spreading), and distance between the second and fourth (intermediary) toes (22).

Biomechanical Assessment

The harvested lower limbs were immersed in liquid nitrogen, wrapped in cotton gauze soaked with Balanced Salt Solution (BSS Plus; Alcon Labs, Fort Worth, TX, U.S.A.), and placed in 30 ml plastic containers. The specimens were stored at -20°C for an average of 7 days prior to testing. On the day of testing, the specimens were thawed in BSS Plus solution for 2 hours. All extraneous soft and hard tissues were removed from the calcaneus-Achilles tendon complex by blunt and sharp dissection. The formerly intramuscular tendinous fibers then were secured between two strips of white labeling tape with Thick Gel Super Glue (Super Glue, New York, NY, U.S.A.), such that the distal edge of the tape was 1.0 cm from the calcaneal insertion. The calcaneus was fixed to a plastic block at 45°, and the block-calcaneal complex was embedded in a 13 mm diameter pot with self-curing plastic (Fig. 4). Throughout the procedure, care was taken to ensure that neither the Super Glue nor the self-curing plastic contaminated the tendon itself. The specimens were kept hydrated by application of BSS Plus solution. Mechanical testing was carried out with a materials testing machine (model 1321; Instron, Canton, MA, U.S.A.). The specimens were preconditioned for 10 cycles with a 1 Hz sinusoidal displacement of 1 mm. Each specimen underwent a constant velocity ramp to failure test at 1.0 cm/sec (approximately 100%/sec). Force-time and displacement-time histories for each test were captured on a Nicolet oscilloscope (Instron) and were transferred to an IBM Model 80 computer for subsequent data analysis. At the completion of testing, each of the specimens was inspected for...
TABLE 1. Functional assessment on day 15

<table>
<thead>
<tr>
<th></th>
<th>Sham operation (n = 6)</th>
<th>Division only (n = 6)</th>
<th>Dummy external fixation (n = 9)</th>
<th>Rigid external fixation (n = 10)</th>
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<td><strong>Toe spread</strong></td>
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<tr>
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<td>5 ± 1.0***</td>
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<td>0.8 ± 0.02</td>
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</tr>
<tr>
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<td>11 ± 0.4</td>
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<tr>
<td>Experimental</td>
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<td>7 ± 0.3**</td>
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<tr>
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<td>22 ± 0.6</td>
<td>24 ± 0.9</td>
<td>24 ± 0.6</td>
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<tr>
<td>Experimental</td>
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<td>1.0 ± 0.01</td>
<td>1.32 ± 0.07</td>
<td>0.8 ± 0.04***</td>
<td>0.2 ± 0.09***</td>
</tr>
</tbody>
</table>

Measurements are given in millimeters and are the mean ± SEM.

* p < 0.01 compared with the division-only group (two-way Student t test with Bonferroni's adjustments for multiple comparisons).

** p < 0.05 compared with the dummy fixation group (two-way Student t test with Bonferroni's adjustments for multiple comparisons).

*** p < 0.001 compared with the division-only group (two-way Student t test with Bonferroni's adjustments for multiple comparisons).

++++ p < 0.001 compared with the dummy fixation group (two-way Student t test with Bonferroni's adjustments for multiple comparisons).

Statistical Analysis

Statistical analysis was performed with use of two-way Student t tests between the experimental groups and, when appropriate, the confidence limits were adjusted downward according to Bonferroni's recommendations for multiple comparisons.

RESULTS

Preliminary investigations showed that other forms of immobilization (synthetic or plaster casts or splints) were either harmful to the animals or ineffective (due to loosening and ingestion of cast material by the rats). With the Kirschner wire method described in this paper, secure fixation could be obtained in the tibia and calcaneus. The size of the wires (0.045 inch; 1.2 mm) is relatively large, however, compared with rat metatarsals, and it is likely that not all metatarsals were penetrated by the wires and that some of the metatarsals may have fractured. There was no evidence of ulceration or autophagia in any group. All wires and external fixators remained intact throughout the study period.

Function

No functional deficits were observed in the sham-operation group. Animals subjected to division of the Achilles tendon protected the injured hindlimbs by flexion of the hip and knee and equinus of the ankle. The hind pawprints of the injured lower limb were longer and narrower than those from the uninjured side. These alterations in gait gradually improved over 15 days (Fig. 5).

Animals in the dummy external fixation group were more reluctant to place the injured hindpaw to the ground. Their hind pawprints on days 13 and 15 were considerably shorter and narrower than those of the sham-operation and division-only groups (Table 1). The animals in which the Kirschner wires were connected by an external frame to provide rigid fixation had even narrower and shorter hind pawprints (Table 1 and Fig. 6).

Biomechanics

To obtain firm proximal fixation for in vitro testing, the muscle fibers of the gastrocnemius and the soleus were removed (by freeze-thawing) and the remaining (formerly intramuscular) tendinous fibers were sandwiched between labeling tape with Super Glue. Firm distal fixation was achieved by potting of the cleaned calcaneus in plastic cement, with the plantar surface mounted at 45°. Under these conditions, the failure site of all of the sham-operation and uninjured tendons was at the distal tendon-calcaneal interface. In the previously divided tendons (division-only group), failure occurred at both the midsubstance and the tendon-calcaneal interface (three of six at the midsubstance and three of six at the tendon-calcaneal interface). All experimental Achilles tendons of the dummy and the rigid...
FIG. 6. The effects of immobilization on function. (A) Toe spreading and (B) distance between intermediary toes. Kirschner wires in the dummy external fixation ("Dummy" Ex-fix) and rigid external fixation (rigid Ex-fix) groups were removed on day 12. Functional measurements (expressed as mean ± SEM experimental/control hindlimbs) were made on day 15. N = 6 for the sham-operation and division-only groups, n = 9 for the dummy external fixation group, and n = 10 for the rigid external fixation group. For the sham-operation group, the mean toe spread was 18 mm and the distance between the intermediary toes was 9 mm. ***p < 0.001 compared with the sham-operation group, ++p < 0.01, +++p < 0.001 compared with the division-only group, ++p < 0.01, and +++p < 0.001 compared with the dummy external fixation group, two-way Student t tests with Bonferroni's adjustments for multiple comparisons.

external fixation groups failed in the midsubstance.

The failure load and stiffness properties of the Achilles tendon-calcaneal complexes in the four groups on postoperative day 15 are illustrated in Fig. 7. The failure loads of the Achilles tendon-calcaneal complex in the sham-operation and uninjured (internal control) limbs of all groups were similar on day 15. On day 15, the control uninjured Achilles tendon-calcaneal complexes of the rigid fixation group were stiffer than those of the control uninjured complexes of the dummy fixation group (59,495 ± 5,130 N compared with 43,258 ± 2,924 N/m [mean ± SEM]) (p = 0.02). The experimental Achilles tendon-calcaneal complexes of the division-only group had regained similar ultimate failure load properties as the uninjured side and the sham-operation adolescent complexes on day 15, but they were twice as deformable and half as stiff as the sham-operation Achilles
FIG. 7. The effects of immobilization on (A) failure load and (B) stiffness. Kirschner wires in the dummy external fixation ("Dummy" Ex-fix) and rigid external fixation (rigid Ex-fix) groups were removed on day 12. Biomechanical assessments (expressed as mean ± SEM experimental/control Achilles tendon-calcaneal complexes) were made on day 15. N = 6 for sham-operation and division-only groups, n = 9 for the dummy external fixation group, and n = 10 for the rigid external fixation group. For the sham-operation group, the mean load was 73 N and the mean stiffness was 33,867 N/m. **p < 0.01, ***p < 0.001 compared with the sham-operation group, +++p < 0.001 compared with the division-only group, and +++p < 0.001 compared with the dummy external fixation group, two-way Student t tests.

tendon-calcaneal constructs (p < 0.001) (Fig. 7). It should be emphasized that failure in the sham-operation group and the control complexes occurred at the bone-tendon interface and therefore may not represent "full force" for a tendinous failure. The Achilles tendon-calcaneal constructs of the dummy fixation group failed at half the load (p < 0.001) and were slightly less stiff than the division-only group, but not significantly so (Fig. 7). Application of an external frame to achieve rigid external fixation had an additional and highly significant detrimental effect on failure load and stiffness. The experimental tendons of the rigid external fixation group failed at half the load and were one-third as stiff as those
of the dummy fixation group (p < 0.001) (Fig. 7).

DISCUSSION

To our knowledge, this is the first report documenting the influence of immobilization and mobilization on the functional and mechanical recovery of injured Achilles tendons. The findings indicate that the insertion of Kirschner wires through the femur, tibia, calcaneus, and forefoot had a significant deleterious effect on the mechanical properties of Achilles tendon-calcaneal complexes and the functional performance of rats subjected to division of the Achilles tendon. Rigid immobilization by connection of the wires with an external frame had an additional and highly significant detrimental influence on the functional and biomechanical performance of the rats.

The rat has a more developed plantaris muscle than is found in humans. In the current studies, the plantaris tendon was removed so that the anatomy would be closer to that of the human. No form of external splint was provided to the division-only group during the postoperative period, but the animals were able to "protect" the injured limb with the other three limbs, a situation most akin to touch-down or partial weight-bearing in a biped. The rat has a more rapid rate of metabolism, with an associated increase in rate of repair and shorter life span, than the human. Large differences between the experimental groups (Figs. 6 and 7, Table 1) were found in spite of the rat's rapid rate of metabolism and propensity to heal. The model of clean transection of the midsubstance of the Achilles tendon is not a direct imitation of rupture of the Achilles tendon since, in the clinical setting, the tendon does not rupture cleanly but pulls apart, with frayed ends. In this model, the pseudosheath was essentially intact and the distance of tendon separation was approximately 3 mm (the length of the tendon is 10 mm). For these reasons, the model may be somewhat analogous to an injury of the extensor tendon. Methods for creation of more ragged tendon ends were not pursued, since the resulting lesions are less likely to be reproducible. Another difference between individuals with rupture of the Achilles tendon and the rats used in this experiment is age. Rupture of the Achilles tendon classically occurs in middle-aged individuals (14), when the vascular impairment (10) and degenerative changes of the Achilles tendon usually are more prominent. The animals used in this study were relatively immature and were chosen because they were comparatively easy to handle and demonstrated highly reproducible walking tracks.

Hruska et al. (16) showed that the locomotor pattern in normal rats is constant for a range of speeds, sizes, and ages. de Medinaceli et al. (13) went on to use measurements from the hind pawprints of rats to develop a formula that reflected the functional state of damaged sciatic nerves. Their method of recording prints was improved on by Lowdon et al. (19). We adapted these methods to objectively assess the function of rats walking with injuries to the Achilles tendon (22). Animals that had a sham operation exhibited no functional deficit, whereas all animals subjected to transection and repair of the Achilles tendon showed a significant, immediate reduction in paw width and a gradually improving increase in the length of hind pawprints over a 15-day period (22). Three variables (plantar length, toe spreading, and distance between intermediary toes) provided sensitive and reliable indicators of Achilles tendon function and were utilized to develop an Achilles Functional Index (22). In that study, indices of paw width followed a pattern similar to that found in our previous investigations (5,6,20-23). Application of Kirschner wires through the calcaneus inhibited the animals' heel-strike ability and rendered the measurement of plantar length inappropriate for this study. Hence, the Achilles Functional Index (22) was not calculated, and total spreading and intermediary toe measurements were used as indices of function. Multiple linear regression analysis of variance of these factors as independent variables against a dependent variable of the defined Achilles tendon injury indicated that they contributed strongly to the dependent variable: for toe-spreading factor, the standard regression coefficient β was 0.57 and p < 0.0001 and for intermediary toe factor, β was 0.17 and p < 0.0007 (22). It is likely that placement of Kirschner wires through the forefoot inhibits the animal's ability to spread the toes, although it is interesting that toe spreading was inhibited to a greater extent when the Kirschner wires were connected to a rigid external frame.

Application of only the Kirschner wire (dummy fixation group) resulted in a reduction in failure load of Achilles tendon-calcaneal constructs. Failure occurred within the tendon midsubstance, well away from the calcaneus. Trauma to bone and soft tissue resulting from application of the Kirschner wires or the presence of Kirschner wires, or both, may have limited motion (and caused a relative immobilization) of the injured lower limb in the recovery period.
The addition of an external frame to the Kirschner wires to permit rigid immobilization (rigid fixation group) had an additional and highly significant detrimental effect on the indices of function and on the failure load and stiffness of the Achilles tendon-calcaneal complexes. The positive correlation between the mechanical and functional performance of injured Achilles tendons noted in this study is consistent with similar associations found in our previous studies (5,6,20,23).

Similar effects of immobilization on soft-tissue healing have been documented in other animal models. Immobilization decreased stiffness and failure load of lateral collateral ligaments in uninjured rabbits (2), anterior cruciate ligaments in uninjured primates (25), and in medial collateral ligaments (8) and canine flexor tendons (15) in injured rats. Immobilization was associated with decreased collagen mass and increased collagen degradation in the medial collateral ligament of the rabbit (3) and with decreased capillary density of the gastrocnemius-soleus muscular tissue of rats subjected to division of the Achilles tendon (17).

In summary, immobilization with a rigid external fixation device had a highly significant detrimental effect on the functional and mechanical recovery of divided Achilles tendon-calcaneal complexes in a rat model. Care must be taken in extrapolation of this data to a “high energy” rupture in the “pathological” Achilles tendon of the middle-aged human. This model and these effects of immobilization may have relevance to injuries in general and to extrasynergial tendons in particular. The mechanism whereby immobilization produces these biochemical and biomechanical changes remains undetermined and is clearly important for our understanding of soft-tissue injury and repair.

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Effects of Exercise on Achilles Tendon Healing in a Rat Model

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ABSTRACT

The effects of motion, or lack of it, on Achilles tendon healing are not well defined. We have recently shown that immobilization has a detrimental effect on tendon healing in a rat model. The aim of this experiment was to determine whether enforced exercise had an additional beneficial effect on the mechanical and functional recovery of divided Achilles tendons in rats. Male Sprague-Dawley rats were randomly allocated into a nonexercise and an exercise group (N = 10 for each group). In both groups the right Achilles tendon was surgically transected. The left, uninjured lower limb served as an internal control. Both groups of animals were housed under identical conditions with the exception that the exercise group swam for 15 minutes per day. Functional performance was determined from the measurement of hindpaw prints of walking rats preoperatively and on alternate postoperative days. On day 15, the animals were killed and weighed, and biomechanical evaluations were performed on both the injured and uninjured Achilles tendon constructs.

There were no differences in weight at time of death. All animals had an initial functional deficit that returned to near-normal by day 15. There were significant differences in the morphological and the mechanical properties of the healing Achilles tendon constructs at day 15 when comparing the injured with the uninjured Achilles tendon constructs. Supplemental exercise, however, had no effect on the functional or mechanical recovery of injured or uninjured Achilles tendons in the rat model.

INTRODUCTION

The beneficial effects of early motion on the recovery of injured soft tissues have stimulated the design and preliminary testing of functional braces that allow early protected ankle motion in patients with Achilles tendon ruptures, as an alternative to immobilization in long and short leg casts. Despite these clinical investigations and much data concerning the potential benefits of motion on soft tissue injuries, little information existed regarding the effects of motion on the functional and mechanical performance of injured Achilles tendons. For this reason, we developed methods to assess such parameters in a rat model and performed a study to determine whether early ankle motion offered any functional or mechanical advantage over immobilization in the rehabilitation of injured Achilles tendons. The results indicated that immobilization with a rigid external fixator had a significant detrimental effect on the functional and mechanical recovery of injured rat Achilles tendons. The aim of the present study, therefore, was to determine whether additional exercise in the form of daily swimming had any effect on the functional and mechanical recovery of injured Achilles tendons in the rat model.

METHODS

Twenty male Sprague-Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) (weight, 280–320 g; 8–10 weeks old; life span, 2.5–3 years) were allocated into two groups: nonexercise and exercise. The rats were housed in beta-chip-lined plastic cages (two animals per cage) with a 12-hour light/12-hour dark cycle in our animal care facility. They were fed rat chow and water ad libitum in accordance with United States Public Health Service Policy on human care and use of laboratory animals, and the National Institutes of Health guide for the care and use of laboratory animals guidelines, and with Institutional Animal Care and Utilization Committee approval.
Anesthesia was administered by intraperitoneal injection of 80 mg/kg ketamine and 5 mg/kg xylazine. Surgical division of the right Achilles tendon was performed as previously outlined.4 A 3-cm midline incision was made over the Achilles tendon and the Achilles tendon and plantaris were dissected free from the surrounding fascia. The Achilles tendon was transected with a scalpel in an axial fashion 0.5 cm from its calcaneal insertion. The tendinous portion of the plantaris was removed to prevent any possible action as an internal splint. The skin was then sutured with two simple nylon sutures of 5-0 Ethilon monofilament nylon on a PC-1 cutting needle (Ethicon, Sommerville, NJ). No operation was performed on the left uninjured hindlimb. No cast or dressings were applied, and the animals were unrestricted during the healing phase.

Exercise consisted of 15 minutes of enforced swimming per day. The animals in the exercise group were placed in a large circular plastic container (100 cm high x 50 cm wide) filled with water (20 °C) to within 20 cm of the top of the container. In this system the animals swam vigorously without the ability to rest on the bottom or jump out of the container. Functional testing (see below) was performed preoperatively in all animals and on postoperative days 1, 3, 5, 7, 9, 11, 13, and 15. On day 15, all the animals were killed by CO2 inhalation and weighed. Biomechanical testing was conducted on both the injured and uninjured Achilles tendon constructs as described below.

Functional Assessment

The functional assessment is outlined in detail elsewhere.16 Animals were tested in a confined walkway 8.7 cm wide x 43 cm long with a dark shelter at the end, as described by Hruska et al.13 An 8 x 42 cm piece of photocopying paper impregnated with a 0.5% solution of the anhydrous form of bromphenol blue (Sigma, St. Louis, MO) in absolute acetone14 was placed on the floor of the rat-walking corridor. The anhydrous form of bromphenol blue is yellow in color but readily changes to a blue color on contact with water. The rats were held by the chest, and their hindpaws were dipped in a dish containing a water-soaked sponge; they were then allowed to walk down the corridor leaving blue foot prints on the yellow paper. The paper was coded by animal number and stored for later measurements in a blinded fashion. Measurements were taken from all foot prints on each strip of paper (range, 4-8 prints per paper) as described previously.15,16 Print length (PL), distance between first and fifth toes or toe spreading (TS), and distance between the second and fourth toes or intermediary test (IT) were measured, as illustrated in Figure 1. A factor was generated for each of these measurements (where E = experimental, N = normal uninjured side): de Medinacelli's print length factor (PLF) = (NPL - EPL)/EPL; toe spread factor (TSF) = (ETS - NTS)/NTS; and intermediary toe spread factor (ITF) = (EIT - NIT)/NIT. The Achilles Functional Index (AFI) was then calculated: AFI = 74(PLF) + 161(TSF) + 48(ITF) - 5.16
Biomechanical Assessment

Harvested lower limbs were placed in phosphate-buffered saline, frozen, and stored at −70°C for 7 days before testing. On the day before testing, the specimens were thawed. All extraneous soft and hard tissue were removed by blunt and sharp dissection from the Achilles tendon, the formerly intramuscular tendinous fibers, and the hindfoot. Area measurements of the uninjured and sham-operated tendons were made with an area micrometer (Mitutoyo, Tokyo, Japan) 0.5 cm from the calcaneal insertion (for intact tendons) and in the midportion of the healing tissue (for the tendons that had been surgically divided previously). Length measurements were made with a 0.02-mm calibrated caliper (Draper, Tokyo, Japan). The formerly intramuscular tendinous fibers were then secured between two strips of white tape with Thick Gel Super® (Super Glue Corporation, New York, NY) in which the distal edge of the tape was 1.0 cm from the calcaneal insertion. The calcaneus was fixed at 45° in an adjustable rubber-gripped clamp (Panavise, Sparks, NV). Throughout the procedure, care was taken to ensure that the Super Glue® did not contaminate the tendon proper, and the specimens were kept moist by application of phosphate-buffered saline. Mechanical testing was carried out using an MTS 810 material test system (MTS Systems Corporation, MN). Each specimen underwent a constant velocity ramp to failure-test at 1 cm/sec (100%/sec). Voltage-time and displacement-time histories for each test were transferred to a 486/33C computer (Gateway 2000, North Sioux City, SD) for subsequent data analysis. At the completion of testing, each specimen was inspected for the location of failure, and the proximal tape was inspected to determine whether any of the tendinous fibers had pulled out from the tape (i.e., evidence of slippage).

Statistical Analyses

Exercise and nonexercise Achilles functional indices were compared by repeated measures analysis of variance (ANOVA). Morphological and biomechanical characteristics were evaluated using independent t-tests. Data from left (uninjured) and right (injured) constructs were evaluated by paired t-tests and by multivariate repeated measures ANOVA. Statistical significance was predetermined at P < 0.05.

RESULTS

Swimming Program

All the rats swam vigorously each day for 15 minutes. At the end of the period of swimming, all rats had a high respiratory rate (>50/min) and were relatively sedentary for an additional 20 minutes. The exercise program did not result in any weight loss (Fig. 2).

Function

Animals subjected to Achilles tendon division protected their injured hindlimbs by flexion of the hip and knee and equinus of the ankle. The hindpaw prints of the injured lower limb were longer and narrower than those on the uninjured side. These changes gradually improved during the 15-day experimental period in linear fashion. There were no differences between the exercise and nonexercise group during the 15-day experimental period (power = 0.9, α = 0.05) (Fig. 3).

Morphology

The macroscopic appearance of uninjured tendons was that of glistening, pearly white, longitudinally arranged structures 11 mm long and 2 mm² in cross section. In contrast, all tendons previously subjected to surgical division were opaque structures 10–17 mm long and 6–10 mm² cross-sectional area. The healing tissue in the midsubstance was relatively clear, with the original cut ends of the tendon visible within the healing tissue. Although the “gap” between the cut ends was 6–7 mm, the overall length of the healing tendons was only 2 mm longer than the uninjured tendons (Table 1).

Biomechanics

Three of the tendon constructs pulled out from the tape in the proximal clamps: one from the injured
nonexercised group and two from the uninjured nonexercised group. The data generated from these specimens were not used. The site of failure for the remaining constructs was distributed between the proximal, distal, and middle portions of the Achilles tendon as follows: nonexercise uninjured group (4, 0, 4), nonexercise injured group (2, 7, 0), exercise uninjured group (2, 1, 7), exercise injured group (5, 4, 1). There were no significant differences in terms of site of failure between the exercise and nonexercise groups.

All load-deflection curves exhibited a typical response for soft tissue structures. There was a small nonlinear response in the toe region (i.e., increasing stiffness or slope of the load-displacement curve), which was followed by a linear response until failure (Fig. 4). The healing Achilles tendon constructs in the injured groups had a greater toe displacement, maximum displacement, less stiffness, and a lower ultimate failure load than did uninjured tendon constructs (Table 1). Exercise did not affect toe displacement, maximum displacement, stiffness, energy, modulus, cross-sectional area, or tendon length.

### TABLE 1

<table>
<thead>
<tr>
<th>Morphological and Biomechanical Properties of Rat Achilles Tendon Constructs*</th>
<th>Nonexercise Uninjured (L)</th>
<th>Nonexercise Injured (R)</th>
<th>Exercise Uninjured (L)</th>
<th>Exercise Injured (R)</th>
<th>Statistical analysis</th>
<th>Uninjured vs. healing</th>
<th>Nonexercise vs. exercise</th>
<th>Paired t-test</th>
<th>Multivariate repeated measures ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-sectional area (mm²)</td>
<td>2 (0.1)</td>
<td>7 (0.9)</td>
<td>2 (0.1)</td>
<td>8 (0.5)</td>
<td>NS</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
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</tr>
<tr>
<td>Tendon length (mm)</td>
<td>11 (0.3)</td>
<td>13 (0.6)</td>
<td>11 (0.4)</td>
<td>13 (0.8)</td>
<td>NS</td>
<td>0.0003</td>
<td>0.0002</td>
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<tr>
<td>Gap between tendon ends (mm)</td>
<td>N/A</td>
<td>7 (0.6)</td>
<td>N/A</td>
<td>8 (0.6)</td>
<td>NS</td>
<td>N/A</td>
<td>N/A</td>
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</tr>
<tr>
<td>Toe displacement (mm)</td>
<td>0.4 (0.1)</td>
<td>2 (0.3)</td>
<td>0.5 (0.2)</td>
<td>2 (0.2)</td>
<td>NS</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>Maximum displacement (mm)</td>
<td>4 (0.4)</td>
<td>6 (0.7)</td>
<td>4 (0.3)</td>
<td>7 (0.2)</td>
<td>NS</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
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<tr>
<td>Failure load (N)</td>
<td>98 (3)</td>
<td>78 (4)</td>
<td>97 (2)</td>
<td>81 (3)</td>
<td>NS</td>
<td>0.0001</td>
<td>0.0001</td>
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</tr>
<tr>
<td>Stiffness (N/m)</td>
<td>34,300 (4,500)</td>
<td>15,700 (1,300)</td>
<td>30,000 (4,700)</td>
<td>16,500 (1,500)</td>
<td>NS</td>
<td>0.0003</td>
<td>0.0001</td>
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</tr>
<tr>
<td>Energy (Nm)</td>
<td>0.25 (0.03)</td>
<td>0.45 (0.08)</td>
<td>0.31 (0.050)</td>
<td>0.40 (0.04)</td>
<td>NS</td>
<td>0.007</td>
<td>0.02</td>
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<td></td>
</tr>
<tr>
<td>Modulus (MPa)</td>
<td>287 (94)</td>
<td>39 (6)</td>
<td>291 (41)</td>
<td>44 (8)</td>
<td>NS</td>
<td>0.0001</td>
<td>0.0004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum stress (MPa)</td>
<td>32 (2.5)</td>
<td>8 (0.9)</td>
<td>31 (2.6)</td>
<td>9 (2.4)</td>
<td>NS</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* in the uninjured left Achilles tendon and the injured right Achilles tendon 15 days after surgical division of the right Achilles tendon. Rats were exercised for 15 minutes a day by swimming.

ANOVA, analysis of variance. Toe displacement is displacement prior to the linear position of the curve. Failure load and maximum displacement are the load and displacement at failure point. Stiffness is slope of the linear portion of the curve. Energy is area under the load-displacement curve. Young’s modulus of elasticity (modulus) = stress/strain. Stress = load/cross-sectional area.

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**Fig. 3.** Time course of the Achilles Functional Index in the exercise and nonexercise groups. Data are expressed as mean (standard error of mean). N = 10 for each group. The exercise effect was not significant. The effect of time was statistically significant, and linear \( P < 0.0001 \) (repeated measures ANOVA).
DISCUSSION

We have previously shown that rigid immobilization inhibits the functional and mechanical recovery of injured rat Achilles tendons.17 The results of this experiment indicate that a rehabilitation program enforcing exercise over and above motion ad libitum neither enhances nor inhibits the functional and mechanical recovery of the injured Achilles tendon in the rat.

The rat is a small quadruped with a more developed plantaris muscle than the human. In this, as in previous studies, the plantaris tendon was removed so that the rat anatomy would be closer to that of the human. No form of external splint was provided during the postoperative period. The animals were, however, able to "protect" the injured limb with the other three limbs, a situation most akin to touchdown or partial weightbearing in a biped. The rat has a more rapid rate of metabolism, with an associated increase in rate of repair and a shorter life span, than the human. We have previously found differences between experimental groups despite the rat's rapid rate of metabolism and propensity to heal.16-18 The model of clean transection of the midsubstance of the Achilles tendon is not a direct imitation of Achilles tendon rupture, because in the clinical setting, the tendon does not rupture cleanly but pulls apart with frayed ends. In this model the pseudosheath was essentially intact, and the distance between the divided tendon ends was approximately 3 mm (the length of the tendon is 10 mm). Another difference between humans with Achilles tendon ruptures and the rats used in the experiment is age. Achilles tendon rupture classically occurs in middle-aged individuals,10 where the vascular impairment7 and degenerative changes of the Achilles tendon are usually more prominent. The animals used in this study were relatively immature and were chosen because they were easy to handle and demonstrated highly reproducible walking tracks.

The model for the functional evaluation of Achilles tendon healing16 was based on the work of Hruska et al.,13 de Medinaceli et al.,9 and Lowdon et al.14 Hruska et al.13 showed that the locomotor pattern in normal rats was constant for a range of speeds, sizes, and ages of rats. De Medinaceli et al.9 went on to use measurements from the hindpaw prints of rats to develop a formula which reflected the functional state of damaged sciatic nerves. His method of recording prints was improved by Lowdon et al.14 We adapted these methods to assess objectively the function of walking rats with Achilles tendon injuries, and we developed the AFI.16 In this study, the AFI followed a pattern similar to our previous investigations.4,15,16

The morphological and mechanical properties of the healing tendons were also similar to previous studies.4,15 Healing tendons were approximately 20% longer, had 3-4 times the cross-sectional area, 80% of the failure load, 50% of the stiffness, 150% of the energy, and 15% of the modulus of uninjured constructs.

In this study, enforced exercise was achieved by compelling the rats to swim daily for 15 minutes. Burroughs and Dahners5 used the same rehabilitation program in rats with divided medial collateral ligaments (MCL). They found that simple mobilization (normal cage activity) had a beneficial effect on MCL strength as compared with immobilization,15 a finding similar to the Achilles tendon research results.17 Burroughs and Dahners5 found that enforced exercise had a further beneficial effect on MCL strength. Daily swimming for 5 minutes was as effective as daily swimming for 15 minutes on MCL strength at day 14. We found that enforced exercise for 15 minutes did not affect Achilles tendon healing. These results support the concept that motion is valuable for MCL and Achilles tendon healing, but that motion beyond a certain threshold is of no further benefit to healing. Burroughs and Dahner's and our studies suggest that...
either the threshold for the maximum benefits of motion are soft tissue specific, with the threshold for the maximum beneficial effects of motion on Achilles tendon healing being lower than the threshold for MCL healing; or the nature of the motion seen by the healing MCL may be different from that seen by the healing Achilles tendon subject to the same exercise program.

In summary, we have previously shown with this model that in regard to the functional and mechanical outcome of divided rat Achilles tendons, surgical repair has no beneficial effect on them; however, immobilization with an external fixator has had a detrimental effect on both the functional and mechanical outcomes of divided rat Achilles tendons. This current study was designed to determine whether enforced exercise, more than normal cage activity, has a beneficial effect on tendon healing. The results indicate that exercise, in the form of 15 minutes of daily swimming, did not have any additive beneficial effects on the healing of rat Achilles tendons. It is possible, therefore, that a threshold of minimal Achilles tendon motion, which in the rat is achieved by normal cage activity, is beneficial for tendon healing, but that additional motion is of no additional benefit. This threshold for maximum benefit of motion for healing may be soft tissue specific.

ACKNOWLEDGMENTS

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Original Research Papers

Modulation of tendon healing by nitric oxide

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Abstract. Nitric oxide (NO) is a small, diffusible free radical that is generated from L-arginine by a family of enzymes, collectively termed the nitric oxide synthases. We investigated the role of NO in tendon healing. NO synthase activity and immunoreactivity was absent in un-injured rat Achilles tendon. After surgical division there was a five-fold increase in NO synthase activity and immunoreactivity within the healing tendon at day 7, with a return to near baseline levels at day 14. Inhibition of NO synthase activity with oral administration of Nω-nitro-L-arginine methyl ester (L-NAME) resulted in a significant reduction in cross-sectional area (30% at day 7, p < 0.01, 50% at day 15, p < 0.001) and failure load (24% at day 7, p < 0.01) of the healing Achilles tendon constructs. Rats fed the enantiomer of L-NAME, (D-NAME) had inhibition of nitric oxide synthase inactivation during tendon healing and inhibition of nitric oxide synthase inhibits this tendon healing.

Key words: Nitric oxide – Tendon healing – Free radical – Wound healing – Nitric oxide synthase

Introduction

Nitric oxide (NO) is a small, free radical synthesized from the amino acid L-arginine by a family of enzymes, the nitric oxide synthases. Nitric oxide’s size and reactivity are important in its role as a multifunctional intra- and intercellular messenger molecule. NO is involved in many physiological and pathological processes (for review see [1]). Examples include the formation of memory [2, 3], olfaction [4], and the regulation of blood pressure [5], erection [6] and uterine relaxation [7]. NO is produced in large amounts by an inducible isoform of NO synthase in macrophages [8, 9], neutrophils, lymphocytes and peripheral-blood monocytes [10] during host defense and immunological reactions [11], and septic shock [12]. Constitutive, Ca2+-dependent isoforms of the enzyme are found in the brain, and the endothelial layer of blood vessels and release NO at low, relatively stable concentrations [1]. Recently, NO has been shown to be induced in inflammation [13] and ileitis [14] and to be important in fetal development [15]. We wondered whether NO may also play a role in soft tissue healing. The aim of this study, therefore, was to determine if NO was important in tendon healing.

Methods

Materials

The reduced form of β-Nicotineamine adenine dinucleotide phosphate (NADPH), calmodulin, valine, Nω-nitro-L-arginine methyl ester (L-NAME), Nω-nitro-D-arginine methyl ester (D-NAME), L-arginine, L-citrulline, β-NADPH, nitroblue tetrazolium, triton X and Dowex 50W anion exchange resin were obtained from Sigma Chemical Co., St. Louis, MO, USA. Nω-methyl-L-arginine monacetate (L-NMMA) was obtained from Calbiochem (Nottingham, UK). L-(2,3,4,5-3H) arginine hydrochloride was obtained from Amersham (Buckinghamshire, UK). Tetrhydrobiopterin was obtained from Schirks Laboratories (Basel, Switzerland). NO synthase antibodies to endothelial cell NO synthase (anti EC-NOS) and macrophage NO synthase (anti mc-NOS) were purchased from Transduction Laboratories, Lexington, KY. For anti EC-NOS a 20.4 kDa protein fragment corresponding to the amino acids 1030-1209 of human EC-NOS was used as an immunogen. The
monoclonal antibody was purified from mouse ascites using chromatographic techniques. The antibody cross-reacts with rat, rabbit and human EC-NOS. For anti mac-NOS a 21 kDa protein fragment corresponding to the amino acids 961-1144 of mouse mac-NOS was used as an immunogen. The monoclonal antibody was purified from mouse ascites using chromatographic techniques. The antibody cross-reacts with rat, mouse and human mac-NOS. Fluorescein-conjugated goat IgG fraction to mouse immunoglobulin (IgG, IgA, IgM) was purchased from Cappel Research Products, Durham, NC, USA.

**Experimental groups**

One hundred and eight outbred male Sprague-Dawley rats (Harlan Sprague Dawley Inc, Indianapolis, IN, USA), weight 280–320 g, were utilized for NO synthase activity (n = 32 rats), histological and immunohistochemical evaluation (n = 24 rats) and NO synthase inhibition experiments (n = 52 rats). NO synthase activity was determined at 6 h (n = 8), 1 day (n = 8), 7 days (n = 8) and 14 days (n = 8) in both the surgically divided and un-injured Achilles tendon. Immunohistochemistry and histological evaluation of un-injured and surgically divided Achilles tendons was performed at day 1 (n = 3), day 4 (n = 2), day 7 (D-NAME, n = 10; L-NAME, n = 6) and day 14 (n = 3). Cross-sectional area and biomechanical properties were evaluated in the same specimens, otherwise all other evaluations (immunohistochemical, histological, NO synthase activity) were made on separate specimens.

In the following NO synthase inhibition experiments animals were fed L-NAME, D-NAME and L-arginine at 1 mg/ml (=300 mg/kg/day) in their drinking water ad libitum for four days prior to surgical division of the right Achilles tendon and throughout the post-operative experimental period: (a) 1 week D-NAME (n = 11) vs L-NAME (n = 8); biomechanical analyses at day 7. (b) 2 weeks water (n = 4) vs L-NAME (n = 4); morphological and histological analyses at day 14. (c) 3 weeks D-NAME (n = 8), L-NAME (n = 8), L-arginine (n = 8), biomechanical assessment and autopsy at day 22. L-NAME (Nω-nitro-L-arginine methyl ester) is a structural analogue of L-arginine and is a
Nitric oxide synthase assay

Specimens from the left (un-injured) and the right (previously surgically divided) Achilles tendons were harvested at 6 h, 24 h, 7 and 14 days after surgery (n = 8 for each group) and quickly frozen in liquid nitrogen. Samples were homogenized on ice using an Ultra-Turrax T25 homogenizer (Janke & Kunkel, IK Labortechnik, Staufen, Germany) in a homogenization buffer composed of: 50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM EGTA, 12 mM 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (pH 7.4). Conversion of \(^{3}\)H-L-arginine to \(^{3}\)H-L-citrulline was measured in the homogenates as previously described [12]. Briefly, cell homogenate (40 μl) was incubated in the presence of \(^{3}\)H-L-arginine (10 μM, 5 kBq/μl), NADPH (1 mM), tetrahydrobiopterin (5 μM), valine (50 mM), calcium (2 mM) and calmodulin (30 μM) for 30 min at 37 °C in HEPES buffer (pH 7.5). The reaction was stopped by dilution with 1 ml of ice cold HEPES buffer (pH 7.5) containing EGTA (2 mM) and EDTA (2 mM). Reaction mixtures were applied to Dowex 50W (Na\(^{+}\) form) columns and the eluted \(^{3}\)H-L-citrulline activity was measured by scintillation spectroscopy (Beckman, LS3801; Fullerton, CA, USA). Experiments performed in the absence of calcium and in the presence of EGTA (5 mM) determined the calcium-independent NO synthase activity. Parallel experiments in the presence of calcium and the NO synthase inhibitor L-NMMA (3 mM) determined the extent of detection and/or formation of \(^{3}\)H-L-citrulline that was independent of specific NO synthase activity, and subtracted from all values. The data represent means of two independent determinations, each of them performed in quadruplicates using pooled tendon samples from four rats for each experimental group in the first determination and in quadruplicates using pooled tendon samples from four rats for each experimental group in the second determination.

Statistical analysis

All values in the text and figures are expressed as mean ± SEM of n observations. Statistical analysis between experimental groups was performed using un-paired two-tailed Student’s t-tests and ANOVA. Statistical analysis between the right surgically divided and the left un-injured Achilles tendons were performed using paired two-tailed Student’s t-tests. Variables with heterogenous variances were analyzed by signed rank tests. The confidence limit was predetermined at an alpha level of 0.05.

Results

Effects of surgery

The macroscopic appearance of un-injured tendons was that of glistening, pearly-white, longitudinally arranged
also became more organized, with fewer fibroblasts and negligible Ca++ independent increase in Ca++ dependent and Ca++ independent determinations. At 6 h and 24 h post surgery there was a slight increase in NO synthase activity (Fig. 2). At day 7, there was a five-fold increase in Ca++ dependent NO synthase activity (Fig. 2). At the same time point, no Ca++ independent NO synthase activity was found. At day 14, NO synthase activity had returned to near normal levels. The majority of NO synthase activity at day 14 was, however, Ca++ independent. There were no changes in NO synthase activity at any of these time points in the un-injured contralateral side (not shown).

In un-injured tendon samples, very low Ca++ dependent and negligible Ca++ independent NO synthase activity was detected. At 6 h and 24 h post surgery there was a slight increase in Ca++ dependent and Ca++ independent NO synthase. By day 7, there was a five-fold increase in Ca++ dependent NO synthase activity (Fig. 2). At the same time point, no Ca++ independent NO synthase activity was found. At day 14, NO synthase activity had returned to near normal levels. The majority of NO synthase activity at day 14 was, however, Ca++ independent. There were no changes in NO synthase activity at any of these time points in the un-injured contralateral side (not shown).

**Fig. 2.** Time course of total and Ca++ independent nitric oxide synthase activity in surgically divided rat Achilles tendons. There was no detectable nitric oxide synthase activity in un-injured tendons at any of the time point (not shown). Mean ± SEM of two independent determinations, each performed in quadruplicates using pooled tendon samples from 4 + 4 rats in each experimental group. **p < 0.01 when compared with the un-injured side using signed rank tests and paired Student’s t-tests, and p < 0.01 when compared with the other time points using ANOVA.

structures 10 mm long and 2.5–3.0 mm² in cross-section. In contrast, all tendons previously subjected to surgical division were opaque structures 10–17 mm long and 6–10 mm² in cross-sectional area. At the microscopic level, un-injured tendons consisted of dense, parallel bundles of homogenous eosinophilic substance aligned with the long axis of the tendon. Interspersed within this matrix were a few longitudinally arranged spindle and oval shaped fibroblasts with prominent nuclei and sparse cytoplasm (Fig. 1a). In the divided Achilles tendons, the area between the cut ends of the tendon initially was filled with a loose connective tissue matrix containing numerous inflammatory cells (neutrophils, basophils, macrophages and lymphocytes) and erythrocytes (Fig. 1b). Over 15 days this loose connective tissue matrix was replaced by a more homogeneous, denser eosinophilic matrix. Plump mesenchymal cells with large nuclei were the predominant cell type, and were aligned in the longitudinal axis of the tendon (Fig. 1c). With time, the tendon-new matrix interface became less distinct. The collagenous tissue also became more organized, with fewer fibroblasts and more matrix (Fig. 1d).

**Nitric oxide synthase activity**

In un-injured tendon samples, very low Ca++ dependent and negligible Ca++ independent NO synthase activity was detected. At 6 h and 24 h post surgery there was a slight increase in Ca++ dependent and Ca++ independent NO synthase. By day 7, there was a five-fold increase in Ca++ dependent NO synthase activity (Fig. 2). At the same time point, no Ca++ independent NO synthase activity was found. At day 14, NO synthase activity had returned to near normal levels. The majority of NO synthase activity at day 14 was, however, Ca++ independent. There were no changes in NO synthase activity at any of these time points in the un-injured contralateral side (not shown).

**Immunohistochemistry**

The specificity of EC-NOS and mac-NOS antibodies was confirmed on western blots of mouse macrophage lysate and rat aorta homogenates (not shown). Sections of tendon prepared with the secondary antibody alone, or after 24 h formaldehyde fixation showed no immunofluorescence (inset Fig. 3a), confirming that there was no non-specific binding of the secondary antibody.

The tendon fiber bundles and fibroblasts within the tendon matrix of un-injured tendons showed minimal immunofluorescence to EC-NOS and mac-NOS (Fig. 3a). This contrasted with the tissue that filled the defect of the cut Achilles tendon. This tissue reacted strongly to both EC-NOS and mac-NOS. The intensity and number of cells reacting to NO synthase antibodies was far greater at day 4 (Fig. 3b and c) and day 7 (Fig. 3d and e) than at day 14 (Fig. 3f). The intensity and distribution of immunofluorescence to mac-NOS and EC-NOS in this healing tissue were slightly different. EC-NOS was distributed more diffusely throughout the all the cells within the healing tendon tissue, often with the greatest intensity at the edges of the cells (Fig. 3b and d). Cells reacting to mac-NOS had a more heterogeneous pattern of immunofluorescence with some cells staining very brightly and others less so (Fig. 3c and e). At day 14 there was minimal immunofluorescence to both EC-NOS and mac-NOS (Fig. 3f). The un-injured tendon fibers had minimal immunofluorescence to EC-NOS and mac-NOS through the whole healing period.

**Nitric oxide synthase inhibition**

**Tendon morphology.** Inhibition of NO synthase by the L-arginine analogue Nω-nitro-L-arginine methyl ester (L-NAME) given orally four days prior to surgery (to ensure pre-injury NO synthase inhibition) and throughout the experimental period inhibited and cross-sectional area of healing Achilles tendon by 30% at day 7 (p < 0.01), 50% at day 15 (p < 0.001) and 17% at day 22 (p < 0.05) (Fig. 4). NO synthase inhibition did not alter the cross-sectional area of un-injured tendon at day 7 or day 15. On day 22 there was a slight reduction in cross-sectional area of un-injured tendons in L-NAME fed rats (Fig. 4). Within the healing tendons the cut ends of the tendon were separated by 5–8 mm, a distance that did not change with healing time or with the administration of L-NAME or L-arginine (Table 1).

**Light microscopy.**

There was an overall decrease in the cross-sectional area of healing Achilles tendon in rats fed the nitric oxide synthase inhibitor L-NAME, compared with sections from rats fed D-NAME, as illustrated in Figure 4. There were, however, no qualitative differences in the microscopic appearance of the healing tissue in L-NAME versus D-NAME fed rats, i.e. the morphological characteristics of the cells and the proportion of matrix was similar in both groups. Furthermore, there were no differences in the vascularity of the healing tendon.
Fig. 3. Direct immunofluorescence of healing rat Achilles tendon to anti-human endothelial nitric oxide synthase (EC-NOS) and macrophage nitric oxide synthase (mac-NOS). There was no immunofluorescence in any section when using secondary antibody alone (inset a). (a) Un-injured Achilles tendon. Note the immunofluorescence of muscle (M), with minimal activity in normal tendon (T). EC-NOS, x 640. (b) Day 4 healing Achilles tendon. EC-NOS, x 640. (c) Day 4 healing Achilles tendon. mac-NOS, x 640. (d) Day 7 healing Achilles tendon. Note the diffuse reactivity in the healing tendon. EC-NOS, x 640. (e) Day 7 healing Achilles tendon. Note the more heterogeneous immunofluorescence pattern. Some cells are very reactive, while others are much less so. mac-NOS, x 640. (f) Day 14 healing Achilles tendon. Note the overall lack of immunofluorescence. mac-NOS, x 640.

Biomechanics

All load-deflection curves exhibited a typical response for soft tissue structures. There was a small non-linear response in the toe region (i.e. increasing stiffness or slope of the load-displacement curve) which was followed by a linear response up to failure (Fig. 5). The failure site of all un-injured tendons was at the distal tendon-calcaneus interface. In the previously divided tendons, failure occurred at both the proximal clamp-tendon interface, the
portion of the curve. Stiffness = slope of the linear portion of the curve. Energy = area under the load-displacement curve. Strain = change in length/initial length. Young's modulus of elasticity (modulus) = stress/strain.

mid-substance and the tendon-calcaneal interface. The majority of failures at day 7 were in the mid-substances (13 mid-substance, 7 clamp-tendon interface). At day 22 the majority (14 of the first 15 tested) of failures in the healing tendon occurred by avulsion or slippage of the tendinous fibers from the proximal fixation. For this reason the failure load data for the healing tendons at day 22 was disregarded. The healing Achilles tendon constructs had a greater toe displacement, maximum displacement, were less stiff and had a lower ultimate failure load at day 7 than un-injured tendon constructs (Table 1).

Inhibition of NO synthase activity with L-NAME caused a reduction in failure load of healing tendon constructs (24% reduction at day 7, \( p < 0.001 \)). The un-injured Achilles tendon constructs were un-affected by L-NAME ingestion (Table 1). Inhibition of NO synthase did not affect toe displacement, maximum displacement, stiffness, energy, modulus or maximum stress of the healing tendons of the surgically divided or un-injured Achilles tendon constructs (Table 1).

**Weight**

Rats fed the NO synthase inhibitor L-NAME either failed to gain weight or lost weight over the first 15 days of treatment, and by day 22 the rats were 30% lighter than their initial pre-operative weight. This contrasted to rats fed the same dose of D-NAME or L-arginine, who all increased their weight by 30% over 22 days (Fig. 6).

**Other systemic effects**

From day 15, L-NAME fed rats became progressively unwell. Three out of the eight rats in this group developed motor palsies of either the upper limbs \( (n = 1) \) or lower limbs \( (n = 2) \). All L-NAME fed rats lost coat texture and developed peri-orbital rubor. 5/8 developed severe dyspnea. Two died on day 21, and the whole experiment was
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Table 1. Effects of nitric oxide synthase inhibition on the morphological and biomechanical properties of healing rat Achilles tendon constructs seven days after surgical division of the (R) Achilles tendon. Rats were fed with nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) or its inactive enantiomer Nω-nitro-D-arginine methyl ester (D-NAME). Dose = 1 mg/ml in drinking water ad libitum four days prior to surgery and post-operatively. See Fig. 5 for explanation of terms.

<table>
<thead>
<tr>
<th></th>
<th>D-NAME</th>
<th>L-NAME</th>
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<tbody>
<tr>
<td></td>
<td>un-injured (L)</td>
<td>surgically divided (R)</td>
</tr>
<tr>
<td>Cross-sectional area (mm²)</td>
<td>2.6 (0.1)</td>
<td>7.3 (0.3)***</td>
</tr>
<tr>
<td>Tendon length (mm)</td>
<td>6.0 (0.4)</td>
<td>11 (0.4)***</td>
</tr>
<tr>
<td>Gap between tendon ends (mm)</td>
<td>N/A</td>
<td>5.9 (0.3)</td>
</tr>
<tr>
<td>Toe displacement (mm)</td>
<td>1.5 (0.4)</td>
<td>2.5 (0.3)*</td>
</tr>
<tr>
<td>Maximum displacement (mm)</td>
<td>4.8 (0.5)</td>
<td>7.6 (0.3)***</td>
</tr>
<tr>
<td>Failure load (Newtons)</td>
<td>83 (2.5)</td>
<td>35 (1.2)***</td>
</tr>
<tr>
<td>Stiffness (Newtons/m)</td>
<td>31000 (2300)</td>
<td>7600 (410)***</td>
</tr>
<tr>
<td>Energy (Newton-meters)</td>
<td>0.26 (0.03)</td>
<td>0.28 (0.02)</td>
</tr>
<tr>
<td>Modulus (MegaPascals)</td>
<td>65 (5.0)</td>
<td>11 (0.6)***</td>
</tr>
<tr>
<td>Maximum stress (MegaPascals)</td>
<td>32 (1.7)</td>
<td>5 (0.2)***</td>
</tr>
</tbody>
</table>

Mean (SEM), n = 11 (D-NAME), n = 9 (L-NAME). *** p < 0.001, * p < 0.05 when compared with the un-injured side; paired two-tailed Student's t-tests and signed rank tests. ++ p < 0.01 when compared with the control (D-NAME) group; un-paired two-tailed Student's t-tests.

Table 2. Effects of nitric oxide synthase inhibition and of L-arginine on the morphological properties of healing rat Achilles tendon constructs twenty-two days after surgical division of the (R) Achilles tendon. Rats were fed the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) or its inactive enantiomer Nω-nitro-D-arginine methyl ester (D-NAME) or the substrate for nitric oxide synthase (L-arginine). Dose = 1 mg/ml in drinking water ad libitum four days prior to surgery and post-operatively.

<table>
<thead>
<tr>
<th></th>
<th>D-NAME</th>
<th>L-NAME</th>
<th>L-arginine</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>un-injured (L)</td>
<td>surgically divided (R)</td>
<td>un-injured (L)</td>
</tr>
<tr>
<td>Cross-sectional area (mm²)</td>
<td>2.9 (0.1)</td>
<td>7.5 (0.4)***</td>
<td>2.7 (0.1)</td>
</tr>
<tr>
<td>Tendon length (mm)</td>
<td>6.6 (0.7)</td>
<td>12.5 (0.5)***</td>
<td>6.5 (0.3)</td>
</tr>
<tr>
<td>Gap between tendon ends (mm)</td>
<td>N/A</td>
<td>6.1 (0.3)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Mean (SEM), n = 8 for each group. *** p < 0.001 when compared with the un-injured side; paired two-tailed Student's t-tests and signed rank test. + p < 0.05, ++ p < 0.01 when compared with the control (D-NAME) group; un-paired two-tailed Student's t-tests.

shortened to 22 days rather than the planned 28 days. The D-NAME and L-arginine fed rats displayed none of the above changes.

Discussion

Here we demonstrate that NO· synthase activity was induced during rat Achilles tendon healing. The increased activity was predominantly within the healing tissue, was maximal at day 7, and returned to baseline activities at day 14. Inhibition of NO synthase prior to injury inhibited the magnitude of the healing response (tendon cross-sectional area and failure load).

To our knowledge, this is the first time that the biochemical and immunohistochemical activity of NO· synthase has been evaluated in healing tendon or ligament. There was minimal activity of NO· synthase in normal tendon, and small amounts at 6 h and 24 h. At 7 days, however, there was a significant increase in Ca²⁺ dependent NO· synthase activity in the healing tendon. This activity returned to near baseline levels by day 14. While the Ca²⁺ dependency of the NO· synthase activity points to a constitutive isoform of NO· synthase, all isoforms of NO· synthase have a requirement for Ca²⁺ and chelation of all the Ca²⁺ can lead to inactivation of the inducible isoform of NO· synthase [19]. Thus further studies are necessary to determine the isoform(s) of NO· synthase in healing tendon. The immunolocalization
studies were consistent with the biochemical studies, in so far as there was little immunofluorescence of NO synthase in normal tendon and at day 14, while there was considerable immunofluorescence in the healing tendon at day 4 and day 7. This time course of NO synthase activity implies that normal Achilles tendon did not express NO synthase, but that (a) during the early phase of tendon healing the tendon cells were induced to express NO synthase, or (b) the healing tendon was populated by cells with NO synthase activity, and that these cells were later replaced by fibroblasts that did not express NO synthase. The immunolocalization studies favor the latter as there was minimal NO synthase immunoreactivity in the original tendon, more in the tendon fibroblasts near the cut edge and considerable activity throughout the healing tendon. Further studies are necessary to determine the types of cells which express NO synthase in this healing tissue. The coronal sections of healing Achilles tendon at day 4 and day 7 suggested that there was more migration of cells from the paratenon (the pseudosheath surrounding the tendon) into the defect than from the ends of the cut tendon. By day 14 the morphological appearance of the cut tendon ends was less clear and was consistent with migration of tendon cells from the Achilles tendon proper. This sequence of events is consistent with previous observations [16, 17].

A role for NO in tendon healing was supported by our experiments in which the magnitude of Achilles tendon healing was reduced by the systemic administration of a NO synthase inhibitor (L-NAME). A similar result was obtained with the inactive enantiomer of L-NAME, D-NAME. L-arginine did not enhance tendon healing. One concern that we had when evaluating the results of NO synthase inhibition on tendon healing was that with such impressive systemic effects from NO synthase inhibition, it may be difficult to "sift out" the effects on tendon healing in an animal model (weight loss, muscle mass loss, loss in walking speed and eventually paralysis). Furthermore, any changes in tendon healing may be secondary to systemically induced immobilization [20]. Many of the experiments have addressed this issue and pointed toward an independent role for nitric oxide in tendon healing. Specifically, (1) the NO synthase activity was elevated in the healing tendon at day 7 and returned to normal by day 14, (2) NO synthase activity was strongly localized to the healing within the healing tendon, (3) reduction in tendon cross-sectional area and failure load occurred as early as day 7, seven days prior to evidence of the systemic effects of NO synthase inhibition, (4) cross-sectional area changes of healing tendons were greatest at day 7 and day 14, and were actually less obvious at day 22 when the maximal effects of immobilization should have been apparent.

NO synthase inhibition affected the volume of healing tissue, rather than the morphological or material properties of the healing Achilles tendon constructs. This was reflected in a decrease in failure load and tendon cross-sectional area without a significant change in maximum stress (failure load/ cross sectional area) of Achilles tendon constructs in rats fed with nitric oxide synthase inhibitor, L-NAME. The effects of NO synthase inhibition on the volume of the healing tissue were most pronounced at day 14. The mechanism(s) whereby NO modulates these effects on tendon healing require further investigation.

In summary, the studies outlined in this paper suggest that a NO synthase is induced during the early phases of tendon healing in rats and that systemic inhibition of this enzyme reduces the magnitude of the healing response. These results have potential clinical significance, since if NO is a modulator of tendon healing, then the addition of agents which spontaneously release NO might enhance or initiate soft tissue healing, especially in situations when soft tissue healing is impaired (e.g. ischemia, diabetes). The reverse may also hold true: selective local inhibition of NO synthase may inhibit fibroblast proliferation when it is unwanted, e.g. in arthrofibrosis, Dupuytren's contracture and keloid formation.

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References


Temporal expression of nitric oxide synthase isoforms in healing Achilles tendon

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Abstract

We investigated the temporal expressions of the three nitric oxide synthase (NOS) isoforms by semi-quantitative polymerase chain reaction (PCR) assays and by immunoblot analysis, following Achilles tendon transection in rats. Four days after injury, there were increases in the steady-state levels of mRNA for all three NOS isoforms, with peaks for the inducible isoform (iNOS) (23-fold increase) at day 4, the endothelial isoform (eNOS) (24-fold increase) at day 7 and the neuronal isoform (bNOS) (seven-fold increase) at day 21. The temporal expression of NOS isoforms at a protein level was consistent with the results at the mRNA level. We have previously shown a five-fold increase in the NOS activity, as detected by 3H-arginine to 3H-citrulline conversion, at day 7 post-injury. These findings indicate that all three NOS isoforms are expressed during tendon healing with differential expression patterns during the various phases of tendon healing. These findings may prove clinically relevant with respect to strategies for regulating tendon healing. © 2001 Orthopaedic Research Society. Published by Elsevier Science Ltd. All rights reserved.

Introduction

Nitric oxide (NO) is a short-lived free radical with biological functions in nervous, cardiovascular and immune systems, and tissue healing [5,12,20,22,25,35,44]. NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). Three isoforms of NOS have been identified, all requiring reduced nicotinamide adenine dinucleotide phosphate (NADPH) as the electron donor: the neuronal isoform (bNOS, NOS-I), constitutively expressed in discrete neuronal populations [4,42,43,50], the endothelial isoform (eNOS, NOS-II), present in endothelial cells of blood vessels [10,30], and the inducible isoform (iNOS, NOS-III), expressed in various cell types including macrophages when activated [26,46].

NOS is upregulated following injury to tissues, and most evidence indicates that this increased local NO production promotes the normal healing process. For example, NOS inhibitors delay the healing of excisional skin wounds while application of NO via donors accelerates skin wound healing [34,37]. Mice deficient in iNOS exhibit impaired skin wound healing that is reversible by iNOS gene transfer [49]. Two conditions associated with poor wound healing (diabetes and corticosteroid use) are also associated with reduced iNOS expression [6,36]. It is likely that NO contributes to wound healing by promoting collagen synthesis [13,34] and angiogenesis [17]. In the gastrointestinal tract, NO dilates blood vessels and increases mucosal blood flow [19,29]. Inhibition of NOS activity delays gastric ulcer healing, whereas exogenous NO donors reverse this effect [16]. Reduction of constitutive NOS expression and activity is suggested to be involved in ulcerogenic processes [18]. NO also has a role in tendon healing. In previous reports we have presented evidence that NOS is induced during tendon healing and inhibition of NOS resulted in a significant reduction in cross-section area and failure load of the healing Achilles tendon constructs [25]. However as yet, relatively little is known about the isoforms of NOS which are expressed during tendon healing. Since different NOS isoforms may have different functions in tendon healing and since isoform selective inhibitors of NOS are being developed for therapeutic use, it is important to define the patterns of expression for NOS isoforms in tendon healing. In this study, we used a combination of western blot and semi-quantitative polymerase chain reaction (PCR) to assess...
the temporal expression of NOS isoforms in rat Achilles tendon healing. Healing tissue was investigated at 4, 7, 14 and 21 days post-injury, as these time points represent the inflammatory phase, granulation phase and remodelling phase for the healing process in this model.

Materials and methods

Materials

Polyvinylidene difluoride membrane was obtained from NEN Life Science Products (Boston, MA, USA). Monoclonal primary antimurine iNOS, bNOS and eNOS antibodies were purchased from Transduction Laboratories (Lexington, KY, USA). The enhanced chemiluminescence reagent was purchased from Pierce (Rockford, IL, USA) and the horseradish peroxidase conjugated sheep anti-mouse antibody was obtained from Silenus Laboratories (Melbourne, Australia). Moloney murine leukemia virus reverse transcriptase (M-MLV) was obtained from Promega Life Science (Madison, WI, USA), and ribonuclease inhibitor (RNase inhibitor) was purchased from NEN Life Science Products (Lexington, KY, USA). iNOS, bNOS and eNOS isoforms in rat Achilles tendon were investigated at the remodelling phase for the healing process in this model.

Animal model

All procedures and protocols were approved by the Committee on Animal Research of the University of New South Wales, Sydney. The rats were housed in beta-chip-lined plastic cages, two animals per cage, with a light, dark cycle in our animal care facility and fed rat chow and water ad libitum. Anesthesia was achieved by intraperitoneal injection of 0.5 mg/kg ketamine and 5 mg/kg xylazine. Surgical division of the right Achilles tendon and the Achilles tendon and plantaris were dissected free from the surrounding fascias. The Achilles tendon was transected with a scalpel in an axial fashion. 0.5 cm from its calcaneal insertion. The tendinous portion of the plantaris was removed to prevent any possible action as an internal splint. The skin was then sutured with two simple nylon sutures of 4-0 ethilon monofilament nylon on a PC-1 cutting needle. No operation was performed on the left uninjured hind limb. No cast or dressings were applied and the animals were unrestricted during the healing phase. The animals were sacrificed by CO2 inhalation.

Forty-four male Sprague-Dawley rats, weighing approximately 350 g each, were utilized for Western blot (n = 16 rats) and semi-quantitative PCR (n = 28). Specimens for Western blot and semi-quantitative PCR were harvested at 4, 7, 14 and 21 days following the surgery. The uninjured left Achilles tendons were used as controls.

RNA extraction and cDNA synthesis

RNA was isolated using Trizol reagent following manufacturer's instructions. Samples containing 1 μg of total RNA per time point were reverse transcribed with 400 U of M-MLV RT in 50 μl of reaction volume, containing 2 μmol/l of each dNTP, 50 μmol/l Tris-HCl (pH 8.3), 75 mmol/l KCl, 3 mmol/l MgCl2, 10 mmol/l dithiothreitol and 500 ng of random primers to prime cDNA synthesis. Samples were then incubated at 37°C for 1 h.

Semi-quantitative PCR

Construction of internal DNA control. A competitive internal DNA control for each NOS type was prepared by amplifying a non-homologous 574 base pair (bp) BamHI/EcoRI v-erb B fragment (38, 39, 48) (commercially available from Clontech, Palo Alto, CA, USA), using three pairs of composite primers in a manner similar to that designed by Clontech Laboratories (Protocol #PT1521-1 of competitive PCR reaction kit, Cat. 1700-1). Briefly, the composite primers were constructed in such a manner as to contain the target DNA primer sequence, i.e. the NOS of interest, followed by a short stretch of nucleotides which would hybridize to the neutral-behaving 574 bp DNA fragment. The sequences of the primers of NOS isoforms are listed in Table 1. To construct each internal DNA control, two rounds of PCR amplification were performed. The first amplification contained composite primers and the neutral DNA fragments. The second amplification contained the NOS isoform-specific primers and the diluted PCR products from the first amplification. All PCR reactions were performed in a thermocycler with reactions carried out for 16 cycles of 94°C denaturation for 30 s, 55°C annealing for 45 s and 72°C elongation for 45 s, followed by a final extension at 72°C for an additional 10 min for appropriate cycles. PCR products were electrophoresed on a 1.2% (w/v) agarose gel to confirm the presence of an intense band of expected molecular weight product. The second round PCR products were purified using QIAquick PCR Purification Kit (QIAgen) and were determined by measuring the absorbance at 260 nm. A portion of the final purified internal DNA control was diluted to 100 amol/ml and used as a stock solution for all dilutions used in subsequent PCR experiments. The amplicons generated by PCR were sequenced and compared to known gene bank sequences to ensure that the proper product was being amplified.

Competitive PCR and Quantitation. Serial dilutions of known quantities of internal DNA control were added to PCR reactions containing constant amounts of sample target cDNA using the same NOS isoform-specific primers. The PCR reaction cycling conditions were exactly the same as described above. 10% portions of the PCR products were electrophoretically fractionated on a 1.5% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide. Each gel was transilluminated and photographed using ultraviolet light and polaroid photography. The intensities of PCR products were analyzed by an image quantitation system (Quantity One, Bio-Rad, Hercules, CA, USA). The intensity of the sample PCR product was compared to that of the internal DNA control. By knowing the concentration of the internal DNA control, the amount of the sample cDNA could be evaluated, and thus the relative amount of initial sample mRNA could be calculated.

To verify the accuracy and reproducibility of competitive PCR, we synthesized cDNA from both 0.5 and 2 μg of rat macrophage cells total RNA and then performed competitive PCR. Four identical experiments were performed, each with the reverse transcription.
Samples of healing tendon tissue were isolated from the four groups of rats as described above and homogenized in an ice-cold homogenization buffer that contained 50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM EGTA, 500 µg/ml AEBSF, 2 µg/ml leupeptin, 5 µg/ml pepstatin A, 5 µg/ml of aprotinin and 0.1% (w/v) β-mercaptoethanol. The homogenates were centrifuged at 14,000 g for 30 min at 4°C to remove cell debris. The homogenated proteins were separated by 7.5% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were then transferred to a PVDF membrane at 100 V for 2 h. After the transfer, membranes were blocked with 0.1% (v/v) Tween-20 in Tris-buffered saline (TTBS) containing 5% (w/v) non-fat dry milk overnight at 4°C, and subsequently incubated with primary anti-iNOS, anti-bNOS and anti-eNOS mAb diluted with 1% (w/v) bovine serum albumin (BSA) in PBS, for 1 h. After washing in TTBS, the membranes were incubated with a horseradish peroxidase-conjugated sheep anti-mouse Ab in 1% (w/v) BSA/PBS for 1 h before being developed with the ECL substrate. The immunoreactive bands of NOS were quantitated by densitometry.

The specificity of the antibodies for each of the three different NOS isoforms was tested by incubating the antibody with authentic samples of rat iNOS, eNOS and bNOS obtained from interferon-γ and lipopolysaccharide stimulated RAW 264.7 murine macrophage cell lysate, rat brain and aorta lysate, respectively [9,38].

Statistical analysis

All values in the text and figures are expressed as mean ± SE. Statistical analyses between experimental groups were performed using unpaired two-tailed Students t tests and analysis of variance (ANOVA). Statistical analyses between the right surgically divided and the left uninjured Achilles tendon were performed using paired two-tailed Student's t tests. Significance was accepted at the 5% level.

Results

NOS mRNA expression by RT-PCR

There was a negative linear relationship between target/competitor signal density and competitor concentration in the competitive PCR assay. This relationship was sensitive to initial total RNA concentration (Fig. 1), indicating that competitive PCR could be used to accurately measure small changes in the concentration of iNOS, eNOS and bNOS.

The time-course study indicated that the mRNA for all three NOS isoforms were significantly increased in the healing tendon (Fig. 2), iNOS mRNA concentrations were significantly increased 4 days post Achilles tendon injury (23-fold of control values, P < 0.05) and then decreased to near non-injured control values by 14 days post-injury. The increase in eNOS mRNA occurred later, compared to iNOS. eNOS mRNA levels which were low at day 4, increased at 7 days (34-fold of control value, P < 0.05), and then returned to basal levels by 21 days post-injury. bNOS mRNA was also upregulated during healing tendon. There was a slow steady rise which increased to maximal expression at 21 days post-injury (7.5-fold of control, P < 0.05). In contrast, uninjured tendon had very low amounts of all three NOS isoforms as determined by RT-PCR.
Fig. 2. Time course of NOS isoform mRNA expression following rat Achilles tendon division using competitive PCR. Values shown are mean ± SE for seven rats at each time point after Achilles tendon division. * = P < 0.05, ** = P < 0.01, *** = P < 0.001 vs. controls, using unpaired two-way Student's t tests.

Fig. 3. Time course of NOS isoform protein expression following rat Achilles tendon division. (a) Example of immunoreactive bands of NOS isoform at different time points post-injury. (b) Summary data for quantitative densitometry. Values shown are mean ± SE for four rats at each time point. * = P < 0.05, ** = P < 0.01, *** = P < 0.001 vs. controls using unpaired two-way Student's t tests.

Discussion

To obtain a better insight into the potential role of NO radicals in tendon healing, the temporal expressions of all three NOS isoforms in concert with NOS activity [25] were studied in an experimental rat Achilles tendon healing model. In this model, four time points were chosen as they likely represent the early inflammatory phase, the matrix synthesizing phase and the matrix remodelling stage. We found that all the three NOS isoforms were upregulated post-injury at the mRNA level, the protein level, and functional activity [25] in a complex but consistent pattern during the course of tendon healing.

In this model of tendon healing there was no or minimal NOS mRNA, protein or enzyme activity [25] in uninjured normal Achilles tendons. Following Achilles tendon division, however, there was an early increase in the expression of mRNA for the inducible NOS gene and a corresponding increase in iNOS protein at day 4 and 7, followed by a reduction in iNOS mRNA and protein. Mononuclear macrophages are one of the more
prominent cells 4 days following tendon injury [33]. It is possible, therefore, that these cells were responsible for the expression of iNOS, as in other models. iNOS has been shown to be expressed in large amounts by macrophages.

Our findings are in contrast to those of Reno et al. [31], who reported that, in a rabbit ligament healing model, iNOS mRNA was unchanged early after injury, but was elevated at 6 weeks post-injury. It is possible that a rise in iNOS mRNA prior to 3 weeks was missed in their study, since the earliest time point chosen for investigation in their study was 3 weeks post-injury. Species’ differences in NOS expression may also be responsible, as well as potential differences between ligament and tendon healing.

eNOS mRNA was expressed in the highest concentrations on day 7 following Achilles tendon division, with relatively little expression on days 4, 14 and 21. Similarly at a protein level, the highest concentration of eNOS was on day 7. This increase in eNOS may reflect an increased vascularization of the healing tendon on day 7. eNOS has been shown to be important in vascular dilation, in inhibiting platelet aggregation and in angiogenesis [21,23,43]. After tendon injury, overexpression of eNOS may act by these mechanisms to promote an increased oxygen supply to the tendon healing site.

One of the most interesting findings of our study was an increase in bNOS mRNA and protein in the healing tendon that began and steadily increased from day 4 to 21. To our knowledge, bNOS expression has not been reported in a healing wound before. Further investigations are necessary to determine which cells are expressing bNOS. Two possibilities include (1) cells of a neurogenic origin, and (2) fibroblasts, since the major cell types in healing wounds at days 14 and 21 are fibroblasts. Nitric oxide generated by bNOS is thought to act in the central nervous system as a retrograde messenger in certain synapses, where it is possibly involved in potentiation and memory. In the peripheral nervous system, NO acts much like a classical neurotransmitter in regulating gastrointestinal motility, regional blood flow and neuroendocrine function [11,14]. Recent studies have shown that NO biosynthesis in excitable tissues is not restricted to neurons. bNOS is expressed in skeletal muscle [15,27] and bronchial epithelial cells [3,47].

In a previous study, we found that NOS synthase activity was detectable during the early phases of tendon healing [25]. At 14 days post-injury, NOS activity had returned to low or undetectable amounts. In this study, mRNA transcripts of bNOS and bNOS protein were still present at 14 and 21 days post-injury. This discrepancy in NOS activity versus bNOS mRNA and protein level may be due to the limited sensitivity of the NOS activity assay, coupled with the fact that the amounts of NO synthesized by constitutive NOS isoforms are approximately a thousand times lower than those synthesised by the inducible isoform [2,14,22,28].

With respect to the cellular localization of the expression of NOS isoforms, we have preliminary results that indicate that all three NOS isoforms were expressed in fibroblasts, whereas iNOS and eNOS also were expressed in macrophages and endothelial cells, respectively. Further investigations are necessary to fully define the cellular localization of NOS isoforms in healing tendon.

NO may be involved in several aspects of the normal Achilles tendon healing process. First, large quantities of NO produced by inducible isozyymes may act as a killer molecule. This antimicrobial potency may influence healing by preventing infection and enhancing any degradation of the denatured material [8]. NO generated from iNOS can induce apoptosis in inflammatory cells. Apoptosis plays a major role in promoting resolution of acute inflammation by the clearance of inflammatory cells and remodelling of the inflamed site, thus preventing the acute inflammation from persisting as chronic inflammation [32,1]. Second, endogenous NO produced constitutively from endothelium has angiogenic and vasodilatory functions [29,51]. By increasing the local blood flow and promoting the formation of new microvessels from preexisting blood vessels, NO may play a significant role in wound healing and tissue regeneration [41]. Adequate tissue blood flow is essential for tendon healing. Third, nitric oxide is critical to wound collagen accumulation and acquisition of mechanical strength [25]. In vivo inhibition of NO synthesis has been shown to decrease wound mechanical strength and new collagen deposition [13,25,34].

Exactly which isoform of NOS is beneficial in tendon healing is yet to be determined. We have previously shown that inhibition of NOS by a relatively non-specific inhibitor, N-o-nitro-L-arginine methyl ester (L-NAME), reduced Achilles tendon cross-sectional area and load to failure by 30–50% [24]. We have also found that NOS is important for fracture healing. Specifically, we have shown that NOS is induced during the healing process of a fractured rat femur, and that inhibition of NOS with L-NAME in this model inhibits cross-sectional area and failure load of the healing rat femur. The addition of nitric oxide in the form of an NO donor reversed the detrimental effects of NOS inhibition in the rat fracture healing model [7].

In conclusion, these studies show that all three NOS isoforms are expressed in tendon healing, each with its own unique temporal expression. These findings confirm the likely importance of NO as a physiological mediator of tendon healing and raise the possibility that modulation of tendon healing may be achievable in vivo by differential targeting of constitutive and inducible NOS isoforms with selective NOS inhibitors [40] or by the use of NO donors [45].
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References


The cell specific temporal expression of nitric oxide synthase isoforms during Achilles tendon healing

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Abstract. Objective and design: We have previously shown that nitric oxide synthase (NOS) activity is upregulated following tendon injury, and that this activity is important to Achilles tendon healing. The aim of this study was to identify the cellular distribution of nitric oxide synthase isoforms during tendon healing.

Material or subjects: Surgical division of the right Achilles tendon was performed in eighty-five male Sprague-Dawley rats. Healing Achilles tendons were harvested at 4, 7, 14 and 21 days following the surgery. The un-injured left Achilles tendons were used as controls. Using RNase protection assays, in situ hybridization and immunohistochemistry, mRNA and protein of NOS isoforms were evaluated.

Results: Minimal NOS expression was found in un-injured tendon. A cell specific temporal pattern for the mRNA and protein for all three NOS isoforms was found following injury to the Achilles tendon. iNOS was maximal on day 4 in macrophages and fibroblasts. eNOS was maximal on day 4 in endothelial cells and fibroblasts. bNOS expression gradually increased up to day 21 and was found only in fibroblasts.

Conclusions: These results suggest that all three nitric oxide synthase isoforms are expressed by fibroblasts in a coordinated temporal sequence during tendon healing. The sequential pattern of NOS expression in healing fibroblasts suggests that each NOS isoform may play a different role in the healing process and provides opportunities to modify tendon healing in the clinical setting.

Key words: Inducible nitric oxide synthase – Constitutive nitric oxide synthase – Nitric oxide – Tendon repair – Rat

Introduction

Tendon healing is a well-ordered and coordinated process involving inflammation, cell proliferation, matrix deposition, and tissue remodelling. After injury, new tissue generation starts with clot formation and is followed by granulation tissue formation. The latter process encompasses macrophage accumulation, fibroblast ingrowth, matrix formation, and angiogenesis. Inflammation and granulation tissue formation are driven by a complex mixture of growth factors, which are released coordinately into the area of injury. Besides these protein factors and mitogens, evidence is emerging for an important role of small diffusible molecules in wound repair. One of them is nitric oxide (NO), a free radical gas.

Nitric oxide (NO) is a short-lived free radical with biological functions in nervous, cardiovascular, immune systems, and tissue healing [1–6]. NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). At least three isoforms of NOS have been identified. The neuronal isoform (bNOS, type I) is constitutively expressed in discrete neuronal populations, including a subpopulation of dorsal root ganglion neurons [7–9]. The endothelial isoform (eNOS, type III) is present in endothelial cells of blood vessels [10, 11], and the inducible isoform (iNOS, type II), is expressed in various cell types when activated, including macrophages, glial cells [12, 13] and chondrocytes [14].

NOS is upregulated following injury to tissues, and most evidence indicates that this increased local NO production promotes the normal healing process. For example, NOS inhibitors delay the healing of excisional skin wounds while provision of NO via donors accelerates skin wound healing [15, 16]. Mice deficient in iNOS exhibit impaired skin wound healing that is reversible by iNOS gene transfer [17]. The conditions associated with poor wound healing (e.g. diabetes and corticosteroid use) are also associated with reduced iNOS expression [6, 18]. It is likely that NO contributes to skin wound healing by promoting collagen synthesis [15, 19] and angiogenesis [20]. In the gastrointestinal tract, NO dilates blood vessels and increases mucosal blood flow [21, 22]. Inhibition of NOS activity delays gastric ulcer healing, whereas exogenous NO donors, reverse this effect [23, 24]. NO also has a role in tendon healing. In previous reports, we have presented evidence that NOS is induced during tendon healing and inhibition of NOS resulted in a significant reduction in cross-section area and failure load of healing Achilles tendon constructs [2]. Although it is now well established that many effects of tissue healing are mediated by the NOS pathway, relatively little is known about the isoforms of
NOS expressed in tendon healing, and their cellular distribution.

Since different NOS isoforms may subserve different functions in tendon healing and since isoform selective inhibitors of NOS are currently being developed for therapeutic use, it is clearly important to define the patterns of expression for NOS isoforms in tendon healing. The purpose of the present investigation, therefore, was to examine the cellular distribution of NOS isoform expression. In this study, healing tendon was investigated at 4, 7, 14 and 21 days post injury, as these time points represent the inflammatory phase, granulation phase and remodelling phase for the healing process in this model.

Materials and methods

Animal model

Eighty-five male rats of the Sprague-Dawley strain were used in the study. The animals were 11–12 weeks old, and their body weight ranged from 300 to 350 g. Two rats were housed per cage, and the rats received laboratory chow and water ad libitum. Anaesthesia was achieved by intraperitoneal injection of 80 mg/kg ketamine and 5 mg/kg xylazine. Surgical division of the right Achilles tendon was performed as previously outlined [2, 25, 26]. Briefly, the Achilles tendon was transected in an axial fashion 0.5 cm from its calcaneal insertion. The tendinous portion of the plantaris was removed to prevent any possible action as an internal splint. No cast or dressings were applied and the animals were unrestrained during the healing phase. The animals were sacrificed by CO2 inhalation and were utilized for western blot (n = 20 rats) and RNase protection assays (n = 25 rats), in situ hybridization (n = 20 rats) and immunohistochemistry (n = 20 rats). Specimens were harvested at 4, 7, 14 and 21 days following the surgery. The uninjured left Achilles tendons were used as controls. All procedures and protocols were approved by the Committee on Animal Research of the University of New South Wales, Sydney.

RNA extraction and RNase protection assay

Total RNA was extracted according to Trizol reagent protocol (Life Technologies, Inc, Cergy Pontoise, France). Purified RNA was dissolved in RNase-free water. RNA concentration and quality was assessed spectrophotometrically at wavelengths 260 and 280 nm.

Thirty micrograms of total RNA from injured and uninjured tendon were used for RNase protection assays. RNase protection assays were carried out as described [27]. Briefly, RNA samples were hybridized at 42°C overnight with 100,000 cpm of the labelled antisense transcript. Hybrids were digested with RNase A/T1 mixture (dilution, 1:100, Ambion, Austin, TX, USA) for 30 minutes at 37°C. Under these conditions, every single mismatch is recognized by the RNases. Protected fragments were separated on 5% (w/v) acrylamide/8 M urea gels. A probe for β-actin was included as a control for the amount of input total RNA and the recovery of protected probe fragments.

RNA probe synthesis

The rat bNOS clone was made from a 544 base pair (bp) EcoRI-Kpn I fragment of full-length rat nNOS cDNA clone (a kind gift from Dr. A.M. Snowman of Johns Hopkins University, Baltimore, MD). Bluescript II KS+ (Stratagene, La Jolla, CA, USA) plasmid was used for B-nos subclone. Both rat iNOS and eNOS clones were generated by polymerase chain reaction as previously described [28]. The amplified iNOS cDNA fragment corresponded to nucleotides 3191–3584 of the published sequence (Genebank accession #U02534). pGET-Easy plasmid (Promega, Madison, WI, USA) was used for both iNOS and eNOS subclones. Antisense and sense cRNA probes were synthesized by in vitro transcription with the relevant RNA polymerases (Boehringer-Manheim, Mannheim, Germany). RNA probes labelled with 32P-UTP were used for RNase protection assays. RNA probes labelled with digoxigenin-UTP were used for in situ hybridization.

In situ hybridization

Paraffin-embedded sections of 5 μm thickness were deparaffinized in xylene and dehydrated through graded ethanol concentrations, then treated with proteins K (1 μg/ml), fixed with 4% (w/v) paraformaldehyde in PBS (pH 7.4), and acetylated in 0.25% (w/v) acetic anhydride containing 0.1 M triethanolamine. The sections were then covered with hybridization solution (50% deionized formamide, 1X Denhardt's solution, 10% (w/v) Dextran sulphate and 0.5% (w/v) sodium dodecyl sulphate (SDS), 200 μg/ml salmon sperm DNA, 5 × SSC (sodium chloride, trisodium, citrate, 50 μg/ml sodium pyrophosphate and 1mM levamisole) and prehybridized for 1 h at 55°C. The digoxigenin-UTP labelled antisense or sense probes were applied at a concentration of 5ng/ml to the hybridization solution, and the sections were then hybridized for 12–18 h at 55°C. After hybridization, the sections were washed in 2× SSC then in 0.2× SSC, all for three times for 10 minutes each at 55°C. The final wash was in 0.1× SSC for 10 minutes while the ambient temperature dropped from 55°C to room temperature. The immunodetection of digoxigenin-labeled transcripts was performed according to the manufacturer's protocol (Boehringer-Manheim, Mannheim, Germany). Briefly, sections were incubated with alkaline phosphate conjugated with anti-digoxigenin polyclonal sera diluted 1:250 for 2 h at room temperature. The hybridization products were visualized with 5-bromo-4-chloro-3-iodolyl-phosphate (BCIP) and nitroblue tetrazolium chloride (NBT). Finally, the sections were counterstained lightly with Mayer's hematoxyline and mounted.

For negative controls, in situ hybridization with a sense probe was performed. Sections pre-tREATED with 100 μg/ml RNase A before incubation with the labeled probe were also used as negative controls to evaluate probe specificity.

Immunohistochemistry

The longitudinally orientated paraffin-embedded sections were cut to 5 μm thickness. Sections were deparaffinized in xylene and dehydrated through graded ethanol concentrations. After the antigen retrieval, the sections were blocked by 3% (v/v) H2O2, followed by 10% (w/v) non-fat dry milk, then incubated with one of the following NOS antibodies (dilution, 1:50 to 1:300), Transduction Laboratories, Lexington, KY, USA): polyclonal anti-iNOS, monoclonal anti-eNOS, or monoclonal anti-NOS. After unbound primary antibodies were washed off with PBS, the sections were incubated with biotinylated anti-IgG secondary antibody (LSAB+ kit (DAKO)), followed with streptavidin peroxidase. Antigenic sites were visualized using diaminobenzidine (DAB) as the chromagen. Slides were then counterstained with Mayer's hematoxyline, dehydrated, cleared, and mounted with mounting medium. A similar protocol was used for negative control sections except that anti-NOS antibody was replaced by mouse or rabbit IgG.

Double-labeling immunohistochemistry for phenotypic markers

To determine whether NOS-expressing cells were of fibroblastic or monocyte/macrophage lineage cells, sections of healing tendon on days 4 and 7 after injury were subjected to double-immunofluorescence staining, using a monoclonal mouse anti-rat ED-1 (Serotec Ltd., Oxford, UK) as a monocyte/macrophage phenotypic marker [30] and using monoclonal mouse anti-rat proliferating cell nuclear antigen (PCNA) (Fujiga Chemical Industries Ltd., Toyama, Japan) as a marker for fibroblasts [31].
After blocking with 10% (w/v) nonfat dry milk and goat anti-rat IgG (dilution, 1:50, Zymed Laboratories Inc., San Francisco, CA, USA), the longitudinally orientated sections were incubated with anti-ED-1 antibodies for 1h, incubated with goat anti-mouse IgG HRP conjugate and developed for 1–3 minutes in DAB. Tissue sections were then thoroughly washed in PBS and re-blocked with goat anti-rat IgG, and then incubated with anti-rat prolyl 4-hydroxylase (β) antibody for another 1h, followed with biotinylated anti-mouse IgG serum and streptavidin-alkaline phosphatase. For the antigenic site of fibroblast, AP fast red reagent was used as the chromagen.

Statistical analysis

All values in the text and figures are expressed as mean ± SE of observations. Statistical analysis between experimental groups was performed using unpaired two-tailed Student’s t tests and analysis of variance (ANOVA). Statistical analysis between the right surgically divided and the left uninjured Achilles tendon were performed using paired two-tailed Student’s t tests. Significance was accepted at the 5% level.

Results

NOS isoforms mRNA are highly induced during tendon healing

To determine a possible role of NOS isoforms in Achilles tendon healing, we first analyzed the time course of mRNA expression for the NOS isoforms during this healing process. We isolated total RNA from healing tissue at different intervals after tendon injury and performed RNase protection assays. This time-course study indicated that the mRNA for all three NOS isoforms were significantly increased in the healing tendon (Fig. 1). iNOS mRNA was significantly increased 4 days post injury (3.7 fold of control values, p < 0.05) and then decreased to near non-injured control values by 14 days post injury. The increase in eNOS mRNA occurred later, compared to iNOS. eNOS mRNA levels were lower at 4 days, increased at day 7 (2.4 fold of control value, p < 0.05), and then returned to basal levels by 21 days post injury. iNOS mRNA was also upregulated in healing tendon. BNOS mRNA exhibited a slow steady rise with a maximal signal 21 days post injury (4.2 fold of control, P < 0.05). In contrast, uninjured tendon had a very low amount of mRNA for all three NOS isoforms.

NOS isoforms were expressed in a cell-type specific manner

Monocytes and fibroblasts are the two major cell types in healing tendon tissue. We used serial sections stained for NOS isoform and monoclonal antibodies directed against cell lineage-specific markers, to evaluate the cellular distribution of NOS isoform mRNA and protein expression. The results were consistent with the patterns of mRNA and protein expression in tendon healing tissue noted using RNase protection assay (RPA) and immunoblotting (49).

iNOS mRNA and protein expression were simultaneously detected in serial sections of Achilles tendon day 4 post injury (Fig. 2). At this time point, there was maximal expression of iNOS mRNA and protein. Comparisons with serial sections stained for cell-type-specific markers suggested that
Fig. 2. iNOS expression in healing rat Achilles tendon day 4 post injury. Immunohistochemistry revealed: (A) iNOS-immunoreactivity was localized in cells morphologically identified as macrophages (arrows), and fibroblasts (arrowheads). (B) There was no iNOS immunoreactivity in the serial section which used rabbit IgG (negative control). In situ hybridization revealed: (C) iNOS mRNA in both macrophage like cells (arrows) and fibroblast like cells (arrowheads). (D) There was no signal detected in a serial section hybridized with sense probe (negative control). (E) Immunohistochemical double staining with rat macrophage (arrows) and fibroblast (arrowheads) specific cell markers, in a serial section, to confirm that both fibroblasts and macrophages expressed iNOS. Black scale bar represents 20 μm.
most iNOS protein expressing cells were of the macrophage like age. Healing tendon fibroblasts also expressed iNOS, however the intensity of staining of iNOS was stronger among macrophages. Endothelial cells were negative or faintly stained for iNOS. When the primary antibodies were replaced by non-immune mouse IgG, very little or no staining was present in the cells in tendon healing tissue. In situ hybridization demonstrated a similar cell expression pattern of iNOS mRNA as that of iNOS protein found during immunohistochemistry staining. No signal was evident in segments hybridized to sense probe.

Sections of healing Achilles tendon day 7 post injury were used to evaluate the cellular distribution of eNOS, as this time point represented maximal eNOS mRNA and protein expression. We found that eNOS was located mainly in the endothelial cells and in a few fibroblast-like cells in the healing tendon tissue (Fig. 3).

For bNOS localization, sections of day 21 samples were used for immunohistochemistry and in situ hybridization. At this time point, bNOS protein and mRNA were abundantly expressed (Fig. 4). Fibroblasts were the major cells of healing tissue at day 21 post Achilles tendon division. At this time point, fibroblasts were easily identified as longitudinal cells within a collagenous matrix. We found that bNOS was expressed exclusively in the fibroblasts.

No positive signals for any of the three NOS isoforms were found in uninjured left tendon using in situ hybridization and immunohistochemistry.

Discussion

In the present study, an increased NOS expression was demonstrated at both protein and mRNA levels during Achilles tendon healing in macrophages and fibroblasts as well as in the vascular endothelial cells. All three NOS isozymes were expressed in a temporal manner in fibroblasts at the healing tendon.

We have previously found that NO synthase activity was induced during the early phases of tendon healing in rats and that systemic inhibition of this enzyme reduced the magnitude of the healing response [2]. These results imply that NO
produced by the three NOS isozymes has important physiological roles in tendon healing.

In the present data, one of the most interesting findings was that fibroblasts expressed all three NOS isoforms during tendon healing. The reason for this is not clear. For iNOS, it can be speculated that following tendon injury, the fibroblasts were stimulated by a complex composition of inflammatory cytokines and growth factors [32, 33] to express iNOS. This higher level of NO might play a role in maintaining the blood supply to the healing site and in controlling bacterial infection during the early stages of wound healing [34]. Moreover, the slightly higher NO level inside and adjacent to the fibroblasts might protect the cells from damage caused by oxygen-free radicals released by activated infiltrating leukocytes such as neutrophils and macrophages, because NO can neutralize both oxygen and hydroxyl radicals [35]. NO released from fibroblasts by the constitutive nitric oxide synthases (bNOS and eNOS) may have regulatory roles in tendon healing. NO generated within a cell also has the potential to modify the biological activities of adjacent cells because of the ease with which this gaseous molecule can pass through cell membranes [36]. Mathematical evidence suggests that NO generated from a point source may be active over distances up to and beyond 0.2 mm within a few seconds [37]. The range of NO action is governed by the rate of NO formation that, in turn, is based on the number of cells producing NO and the type of NOS isoform present [38]. All these findings imply that expression of eNOS in fibroblasts may be beneficial for tendon healing by augmenting granulation tissue blood flow and angiogenesis at the injury site.

Fig. 4. bNOS expression in healing rat Achilles tendon day 21 post injury. Immunohistochemistry (A) and in situ hybridization (C) revealed the signals for bNOS protein and mRNA were in fibroblasts (arrows). There was no bNOS immunoreactivity in the negative controls (B); or when hybridized with the sense RNA probe (D). Black scale bar represents 20 μm.
have been identified in skeletal muscle [40, 41] and bronchial epithelial cells [42, 43]. This work presented here is the first time that bNOS has been found in healing wounds and in fibroblasts. The roles of bNOS expression in healing tendon are yet to be determined. However, it is likely that its major role occurs after the inflammatory phase and during the remodelling phase as this is when bNOS was most highly expressed.

Fibroblasts are the major cell population in healing tendon tissue. These cells may be important in maintaining an adequate NO concentration in tissue. Historically, the fibroblast has been considered to be a rather inert collagen synthesizing cell. Recent data from other laboratories has shown that fibroblasts can be activated to produce cytokines and chemokines [44, 45]. Rodent fibroblasts have been shown to produce NO on stimulation with cytokines and lipopolysaccharide [46]. Human dermal fibroblasts also have been shown to express both constitutive and inducible NO synthase isozymes [47]. In the present study, we found fibroblasts were the only type of cell, which had the capability to express all three NOS isozymes during the phases of tendon healing.

Overuse activity has been implicated as an etiologic factor in injury to the Achilles tendon. Since we used the contralateral uninjured tendon as control, it is possible these control tendons were potentially overused during this experiment. However, we found no evidence for inflammatory or degenerative processes in histologic examination of these control tendons, and their gross morphologic characteristics were normal.

In this study the temporal expression of the NOS isoform mRNA synthesis detected by RNase protection assay, a semiquantitative method, corresponded well with our previous quantitative method, and also was consistent with the intensity of the immunoreactivity for the corresponding NOS isoform proteins [49].

It is interesting to note that the temporal expression pattern of nitric oxide synthase isozymes in healing tendon is very similar to that of healing bone [48–50]; yet the cells that express the NOS isozymes are different in the two healing tissues. In both tissues, macrophages and endothelial cells express iNOS and eNOS respectively. In healing tendon, fibroblasts express iNOS, eNOS and bNOS in a sequential fashion, while in healing bone chondrocytes express iNOS, eNOS and bNOS in a sequential fashion [51].

We have previously shown that inhibition of NOS is detrimental to healing tendon [2] and to healing bone [48, 52], and that addition of NO may enhance fracture healing [48]. Our data here show that all three NOS isozymes are strongly induced in specific cells within healing tendon in a temporal fashion that is unique to each isoform. This information may be important in designing strategies to enhance or inhibit tendon healing.

**Acknowledgements**

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**References**


Spontaneous recovery of injured Achilles tendon in inducible nitric oxide synthase gene knockout mice

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Abstract. Objective and Design: To determine if inducible nitric oxide synthase (iNOS) gene could affect Achilles tendon healing using iNOS gene knockout mice.

Methods: 21 iNOS knockout (iNOS⁻⁻) mice and 8 of the wild type (iNOS⁺⁺) mice were utilized in this study. Group 1: iNOS⁻⁻ mice (n = 8), group 2: iNOS⁻⁻ mice (n = 11) and group 3: iNOS⁺⁺ with a NOS inhibitor, (aminoguanidine, 500 mg/kg/day, via an intraperitoneal mini-osmotic pump for 7 days, n = 10). The right Achilles tendon was transected in all mice and harvested on day 7 for cross-sectional area and biomechanical properties. Serum nitrate concentration of the mice was measured by gas chromatography mass spectrometry (GC/MS).

Results: A significant reduction in cross-sectional area of the healing Achilles tendon was observed in group 1 mice compared to group 2 mice (p < 0.01). The serum nitrate concentration in both group 2 and group 3 mice was lower than that in group 1 mice (p < 0.01) iNOS gene deletion and inhibition of NOS did not affect the biomechanical properties of the healing tendons.

Conclusions: iNOS gene is not solely responsible for the beneficial effects of nitric oxide (NO) on tendon healing.

Key words: Nitric oxide synthase - tendon healing - knockout mice - NOS inhibition

Introduction

Tendon healing is a well-ordered and coordinated process involving inflammation, cell proliferation, angiogenesis, matrix deposition, and tissue remodeling [1, 2]. These processes are driven by a complex mixture of growth factors, which are released coordinately into the area of injury. Besides these protein factors and mitogens, evidence is emerging for an important role of small diffusible molecules in wound repair. One of them is nitric oxide (NO), a free radical gas.

NO is produced in higher organisms by the oxidation of one of the terminal guanidonitrogen atoms of L-arginine. This process is catalyzed by the enzyme nitric oxide synthase (NOS). The enzyme NOS exists in three isoforms, neuronal NOS (nNOS, type I), inducible NOS (iNOS, type II), and endothelial NOS (eNOS, type III). The isoforms nNOS and eNOS are constitutively expressed, and their activity is regulated by intracellular calcium concentration. The isoform iNOS is located at chromosome 17 (17cen-q11.2), contains 4.1 kb mRNA encoded a 131 kDa size protein [3–5]. iNOS is inducibly expressed in macrophages and other cells after stimulation by cytokines, lipopolysaccharides, and other immunologically relevant agents. Expression of iNOS is regulated at the transcriptional and posttranscriptional level by signaling pathways that involve agents such as the redox-responsive transcription factor NF-κB or mitogen-activated protein kinases (MAPKs) [6–8].

NO is involved in many physiological and pathological processes, including regulation of blood flow and pressure via endothelial-dependent relaxation of blood vessels, neuronal transmission with activation of guanylylcy clase pathways in target neurons, host defense immune response via cytokotoxie, antimicrobial and tumoricidal activity, and inflammation [9–13]. There is a growing body of evidence that suggests that NO is important in the healing of soft tissues. For example, NO inhibitors delay the healing of excisional skin wounds while provision of NO via donors accelerates skin wound healing [14, 15]. Mice deficient in iNOS exhibit impaired skin wound healing that is reversible by iNOS gene
transfer [16]. The conditions associated with poor wound healing (e.g., diabetes and corticosteroid use) are also associated with reduced iNOS expression [17, 18]. Inhibition of NOS activity delays gastric ulcer healing, whereas exogenous NO donors, reverse this effect [19, 20]. Our previous reports have presented evidence that NOS is upregulated during tendon healing and inhibition of NOS resulted in a significant reduction in cross-section area and failure load of healing rat Achilles tendon constructs [21–23]. We have also confirmed that NO has a positive effect on bone healing [24]. Our studies support the hypothesis that NO is important for tendon healing, but which isoform of NOS, and particularly if iNOS is important for tendon healing are not yet clear. The aim for this study, therefore, was to evaluate the effects of deleting the iNOS gene on Achilles tendon healing in mice.

**Material and methods**

**Materials**

Wizard Genomic DNA Purification Kit (Cat. #A1120) was purchased from the Promega Corporation (Madison, Wisconsin, USA). GeneAmp PCR System 2400 is the product of PERKIN ELMER (Norwalk, CT, 06859 USA). Aminoguanidine (hydrochloride) (Cat. No. 81530) was purchased from Cayman Chemical (Ann Arbor, MI, 48108, USA). Mini-osmotic pumps were from ALZET Technical Information Services (DURECT Corporation Cupertino, CA, USA). *N*-labeled potassium nitrate (98%+, Cambridge Isotope Laboratories, Inc.) was obtained from NovaChem Pty Ltd. (South Yarra, VIC 3141, Australia). o, m-, and p-nitroanilines and trifluoroacetic anhydride were obtained from Sigma-Aldrich Pty Ltd. (Castle Hill, NSW 2154, Australia). Hewlett-Packard 5989B mass spectrometer/ Hewlett-Packard 5971A MSD were the products of Hewlett-Packard, Meriden (CT, USA). Reagent grade organic solvents were obtained from commercial suppliers and were used without treatment. Acids, bases, and other chemicals were analytical grade and were obtained from commercial suppliers.

**Animals**

iNOS gene knockout (iNOS−/−) mice and their wild-type (iNOS+/+) (C57BL/6) mice were the gifts from Dr Gunasegaran Karupiah, Sydney University of Australia, which was authorized to breed by Cornell University of USA, the original developers of iNOS− and iNOS+/+ mice embryos. iNOS− mice were generated as described [25]; the gene replacement vector, pNOS-SV1, was designed to delete the proximal 505 bases of the iNOS promoter, a region required for iNOS expression in macrophages, plus exons 1-4, including the ATG translation start site in exon 2. iNOS− and iNOS+/+ littermates (129/SvEv X C57BL/6 Fl), were bred at the specific pathogen-free unit, Biological Resources Centre, University of New South Wales, Australia. Experiments were performed according to each institution's guidelines for animal use and care.

**iNOS PCR Genotyping**

iNOS genotype was confirmed by PCR as previously outlined [26]. Briefly, mice tail biopsy DNA was prepared as described by Laird et al [27] using Wizard Genomic DNA Purification Kit (Promega). The 400-600bp of genomic DNA was analyzed by PCR in a 50-μl reaction volume containing 10mM Tris- HCl (pH 8.3), 50mM KCl, 1.5mM MgCl2, 200μm dNTPs, and 2.5 units of Taq polymerase (Sigma). Primer pairs (200mM each) were as follows: 5’-ATACACGTTCCTCTCCTCCTCC-3' (3', primer (5'-GGCTTTCTGTCTGTCTGCTCT-3')

**Animal model and experimental groups**

Twenty-one 8-12 weeks old male iNOS− mice and eight 8-12 weeks old male iNOS+/+ mice were utilized for this study. Group 1: iNOS−/− mice (n = 8), group 2: iNOS−/− mice (n = 11) and group 3: iNOS−/− mice treated with a systemic NOS inhibitor, amino-guanidine (AG), 500mg/kg/day, via an intraperitoneal mini-osmotic pump for 7 days (n = 10).

All procedures and protocols were approved by the Animal Care and Ethics Committee of the University of New South Wales, Sydney, Australia. The mice were housed at the specific pathogen-free unit, Biological Resources Centre, University of New South Wales, Australia. Anesthesia was achieved by intraperitoneal injection of Nembutal (10 times dilution in 0.5% saline, 40mg/kg). Surgical division of the right Achilles tendon was performed as previously outlined on rats with some modification [28, 29]. Briefly, a 0.5 cm midline incision was made over the right Achilles tendon, and the Achilles tendon and plantaris were isolated from the surrounding fascia. The Achilles tendon was transected with a scalpel in an axial fashion 0.2-0.5 cm from its calcaneal insertion. The tendinous portion of plantaris was removed to prevent any possible action as an internal split. The skin was then sutured with one or two simple nylon suture of 5-0 ethion monofilament nylon on a PC-1 cutting needle. No operation was performed on the left uninjured hind limb. No cast or dressings were applied and the animals were unrestricted during the healing phase. For group 3, the animals were treated by continuous infusion of AG 500mg/kg body weight/day using intraperitoneally implanted mini-osmotic pumps (Alzet Osmotic Pumps, Model 2001, DURECT Corporation, Cupertino, CA USA). The laparotomy wounds were closed in two layers with running 4-0 nylon sutures. After operation, daily clinical observation was made. Observation included an evaluation of the behaviour (eating, drinking and moving) of the mice, any sign of infection at the operation site and pain that may need postoperative analgesia.

All of the animals were sacrificed on day 7 following the surgery by an overdose intraperitoneal Nembutal. The Achilles tendons were harvested and the uninjured left Achilles tendons were collected as controls. 0.4-1ml of blood was taken from the mouse heart before sacrificing them. Serum was separated from the clotted blood and stored in −20°C for nitrate estimation (see below).

**Cross-sectional area of tendon measurement**

A constant gap was created between two glass slides using standard shims placed at each end (0.27 mm for uninjured tendon, 0.53 mm for injured tendon). The tendon was placed between the two slides causing the tissue to flatten. The constrained tendon created a shadow when viewed through a transmission microscope and this was used to determine the tendon width. The cross-sectional area was calculated by multiplying the shim thickness by the tendon width.

**Biomechanical testing**

Biomechanical assessment was carried out on an in-house mini tensile testing system. The muscle and intramuscular tendinous fibres were compressed between one set of serrated aluminium grips that were attached to the lead screws of the testing system. A block of dry ice was placed against the side of the grips to freeze the compressed tissue. The calcarious was fixed to a second pairs of grips (set 7.5 mm from the first grips), which were attached to a 50 N load cell. The specimens were...
kept moist throughout the entire testing procedure by constant irrigation with 0.9 % saline. Each specimen underwent a constant velocity ramp to failure (2.5 mm/sec). Voltage-time and displacement-time histories for each test were collected using a dedicated computer and software for subsequent data analysis.

Nitrate level of serum measurement via Gas Chromatography-Mass Spectrometry (GC/MS) methods

Nitrate level of serum was measured by gas chromatography mass spectrometry (GC/MS) following preparation of samples by nitration of toluene as described [30, 31]. Nitration of toluene was done by using trifluoroacetic anhydride (TFAA) as a catalyst. An aliquot of an aqueous solution of [15N] potassium nitrate internal standard (200 μM, 50 μl) was mixed in a screw-top glass vial (13 x 100 mm) with either serum samples (50 μl) or calibrated dilutions of native sodium nitrate for the standard curve. The mixture was then dried under a stream of nitrogen (or SpeedVac rotary evaporator, Savant Instruments, Thermo Scientific, Clayton VIC Australia 3168). TFAA (200 μl) and toluene (1 ml) were added to the residue and the tubes were capped and heated at 70 °C for 60 min. After cooling to room temperature, the toluene solution was washed sequentially with water (1 ml), aqueous sodium bicarbonate (1%, 1 ml), and water (1 ml). The toluene solution was separated, dried over anhydrous sodium sulfate (500 mg), and removed to a gas chromatography (GC) autosampler vial for GC/MS analysis.

Gas chromatography mass spectrometry (GC/MS) was performed on a Hewlett-Packard 5890 gas chromatograph interfaced to either a Hewlett-Packard 5973B mass spectrometer or a Hewlett-Packard 5971A MSD. Chromatographic separations were performed in split less mode using an HP-5MS capillary column (30 m x 0.25 mm i.d. with 0.25 μm stationary phase film thickness, Hewlett-Packard Australia) with the following temperature program: 70 °C constant for 2 min and then 20 °C per minute to 150 °C. The GC/MS interface heater, the ion source, the quadrupole, and the injection port temperatures were maintained at 280, 250, 100, and 240 °C, respectively. Full scan mass spectral data were obtained using the HP 5973B. Nitrate analyses were performed in the selected ion monitoring mode and ions were generated by electron ionization. The molecular ions (M+; m/z 137 for the unlabeled form and m/z 138 for the 15N-labeled isotope) of the α-isomer of nitrotoluene were monitored. The (m/z) peak areas were determined and the area ratios of the ion pairs calculated. Calibration curves were generated for the assay after correction for (a) the contribution to the [15N] nitrotoluene peaks at m/z 138 and m/z 121 due to the natural abundance of 15N present in endogenous or unlabeled nitrotoluene and (b) the contribution to the peaks at m/z 137 and m/z 120 arising due to the small amount of unlabeled nitrate present in the original [15N] nitrate used as internal standard. These corrections were made for each analysis by the analysis of separate samples containing unlabeled and 15N-labeled nitrate alone. Nitrate levels were reported as mmol/ml.

Statistical analysis

All data are presented as mean ± SD. Differences among experimental groups were assessed using unpaired two-tailed Student’s t-tests and one-way analysis of variance (ANOVA). The level of statistical significance was accepted at p < 0.05.

Results

iNOS PCR genotyping

To confirm the iNOS genotype of the mice, the genomic DNA was extracted from mice tail tips, and analyzed by PCR. One pair of primers, for the wild type alleles, was fo-

Fig. 1. Cross-sectional area of un-injured and healing Achilles tendons on day 7 post-division of the Achilles tendon in mice. iNOS\(^{-/-}\): n = 8; iNOS\(^{+/-}\): n = 11. The values are presented in the figure as mean ± SD.

Cross-sectional area of the Achilles tendon

As in our previous experiments in rats [28], healing mouse Achilles tendons were much thicker than control, un-injured Achilles tendon (Table 1). No significant differences were noted in cross-sectional area of the healing Achilles tendon or un-injured tendon when comparing the iNOS\(^{+/-}\) group and the iNOS\(^{-/-}\) group (Fig. 1/Table 1). A significant reduction, however, in cross-sectional area of the healing Achilles tendon was observed in iNOS\(^{+/-}\) mice treated with the nitric oxide synthase inhibitor, AG, 500 mg/kg body weight/day for 7 days. The reduction of the cross-sectional area of healing Achilles tendon in iNOS\(^{+/-}\) mice treated with AG was 20% (p < 0.01) compared with the iNOS\(^{-/-}\) group mice (Fig. 2). NO synthase inhibition did not alter the cross-sectional area of un-injured tendons at day 7.

Biomechanics

To investigate the biomechanical properties of mice Achilles tendon, both of un-injured and healing tendons, we designed a mini tensile testing system for mice Achilles tendon. All load-deflection curves exhibited a typical response for soft tissue structures. The failure site of all un-injured tendons was at the distal tendon-calcaneus interface. Failure sites of injured Achilles tendons at day 7 were in the mid-substance.
Achilles tendon healing in iNOS knockout mice

Table 1. Effects of iNOS gene deletion and nitric oxide synthase inhibition on cross-sectional area and biomechanical properties of mice Achilles tendon seven days after surgical division of the right Achilles tendon, Mean (SD). **: p < 0.01 when compared with the iNOS+/− (R) group using one-way analysis of variance (ANOVA).

<table>
<thead>
<tr>
<th>Assessments</th>
<th>iNOS+/− (n = 8)</th>
<th>iNOS+/− (n = 11)</th>
<th>iNOS+/− + AG (n = 10)</th>
</tr>
</thead>
</table>
| Cross-sectional Area (mm²) | 0.4 (0.1) | 1.6 (0.4) | 0.4 (0.1) | 1.8 (0.2) | 0.4 (0.1) | 1.4 (0.2)
| Failure load (Newton) | 12 (2.4) | 1.7 (0.3) | 12 (2.1) | 2.8 (1.6) | 12 (1.8) | 2.1 (0.3)
| Stress (Newton/mm²)  | 29 (6.3) | 1.1 (0.2) | 30 (8.2) | 1.7 (0.9) | 33 (10) | 1.5 (0.2)
| Stiffness (Newton/mm) | 17 (3.0) | 0.8 (0.3) | 15 (5.5) | 1.2 (0.8) | 16 (4.0) | 0.9 (0.3)
| Young modulus (Mpa)  | 165 (27) | 2.0 (0.7) | 149 (60) | 2.7 (1.4) | 183 (61) | 2.5 (1.0)
| Energy (Joules)      | 5.1 (1.3) | 0.7 (0.3) | 7.2 (3.6) | 0.9 (0.5) | 7.6 (2.6) | 1.0 (0.3) |

Fig. 2. Effects of aminoguanidine (AG) on the cross-sectional area of Achilles tendon of iNOS−/+ mice. iNOS+/− (uninjured): uninjured Achilles tendon of iNOS+/− mice with (n = 10) or without (n = 11) AG, 500 mg/kg/day. iNOS−/+ (injured): injured Achilles tendon of iNOS+/− mice with (n = 10) or without (n = 11) AG, 500 mg/kg. **: p < 0.01 (Mean ± SD) using un-paired Student’s t-test.

Fig. 3. Nitrate concentration of mice sera on day 7 following tendon injuries was measured by GC/MS methods. iNOS+/−: wild type mice, n = 8. iNOS+/−: iNOS gene knockout mice, n = 11. iNOS+/− + AG: iNOS gene knockout mice treated with NOS inhibitor, AG, 500 mg/kg/day via intraperitoneal mini osmotic pumps for 7 days, n = 10. **: p < 0.01 (Mean ± SD) using un-paired Student’s t-test.

No significant differences in failure load or stress (failure load/cross-sectional area) of the healing Achilles tendon constructs were found among the three groups. The uninjured Achilles tendon constructs were unaffected by iNOS gene deletion or NOS inhibitor either (Table 1). iNOS gene deletion and inhibition of NO synthase did not affect maximum displacement, stiffness, energy, modulus or maximum stress of the healing tendons of the surgically divided or uninjured Achilles tendon constructs (Table 1).

Serum nitrate

The serum nitrate concentrations of both the iNOS+/− mice group (n = 11) and the iNOS+/− mice with AG group (n = 10) were significantly lower than that in iNOS+/+ mice group, 62% reduction in iNOS+/− group and 58% reduction in iNOS+/− with AG group compared with iNOS+/+ group (p < 0.001). However, there was no significant difference of nitrate concentrations between iNOS+/− group mice and iNOS+/− with AG group mice (Fig. 3).

Discussion

To our knowledge, this is the first investigation of the Achilles tendon healing in inducible nitric oxide synthase deficient (iNOS+/−) mice.

Systemic inhibition of nitric oxide synthase by aminoguanidine significantly reduced the cross-sectional area of the healing Achilles tendon in iNOS+/− mice. However, no significant difference in cross-sectional area or biomechanical properties of the healing Achilles tendon was found between the iNOS+/+ group and the iNOS+/− group.
Systemic NOS inhibition resulted in a reduction in cross-sectional area of the healing Achilles tendon, which is consistent with healing Achilles tendons in rats [21-23]. However, deletion of the iNOS gene (iNOS<sup>-/-</sup>) did not affect tendon cross-sectional area or biomechanical properties. This suggests that the iNOS gene may not be the only important contributor to the beneficial effects of NO on tendon healing and other NOSs (eNOS and nNOS) may also play a role in tendon healing. There are some reports that indicate that eNOS may play a significant role in regenerating endothelium, in cell migration, in growth factor-stimulated angiogenesis and in diabetes-impaired healing [32-34].

Aminoguanidine is a competitive NOS inhibitor. In normal mice, aminoguanidine has a partial selectivity for iNOS versus eNOS, and has little selectivity for iNOS versus nNOS [7]. For iNOS gene deficiency mice, however, aminoguanidine is likely to inhibit eNOS and nNOS, as there is no iNOS expression. The serum nitrate levels in iNOS<sup>-/-</sup> mice were 2.5 time of that in iNOS<sup>+</sup> mice, with no significant difference between iNOS<sup>-/-</sup> group and iNOS<sup>+</sup> + AG group. The explanation for this controversy may be that iNOS is the predominant isoform for NO production after tendon injury, while eNOS and nNOS produce smaller amounts of NO after tendon injury. Our findings are consistent with the results of other researchers: iNOS (inducible NOS) can synthesize prodigious amounts of NO for extended periods of time, whereas both eNOS and nNOS (constitutive NOS) produce NO in relatively low amounts of NO [35-37]. The results from our serum nitrate experiments further confirmed that iNOS<sup>-/-</sup> mice we used were iNOS gene deficient.

We chose an intraperitoneal mini-osmotic pump for delivering a NOS inhibitor to mice. We performed similar experiments twice before, giving the NOS inhibitors orally in the drinking water, but did not find any effect on cross-sectional area of healing Achilles tendon or serum nitrate (data not shown). Schaffer MR et al. [38] also found that NOS inhibitors given orally in the drinking water or by daily intraperitoneal injection had no effect on wound nitrite/nitrate concentrations or deposition of collagen in mice skin wounds. When given continuously through intraperitoneally placed osmotic pumps, aminoguanidine (500 mg/kg/day) significantly reduced wound fluid nitrite/nitrate concentrations and lower accumulation of collagen in wounds. The diurnal drinking pattern of rodents may, in a fashion similar to intermittent intraperitoneal injection, fail to maintain the plasma or wound fluid concentrations of inhibitor at concentrations effective at inhibiting NOS activity. These findings could reflect the short biological half-life of NOS inhibitors in mice [39, 40].

Taken together, the results of this study indicate that nitric oxide (NO) is beneficial in mouse tendon healing; but the iNOS gene is not solely responsible for the beneficial effects of NO on tendon healing.

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Addition of nitric oxide via nitroflurbiprofen enhances the material properties of early healing of young rat Achilles tendons

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Abstract: Objective and design: To determine if the addition of nitric oxide (NO) via nitroflurbiprofen (NO-flurbiprofen) would enhance rat Achilles tendon healing.

Materials and methods: Sixty-five male Sprague-Dawley rats were randomly divided into NO-flurbiprofen, flurbiprofen and vehicle groups, given drugs or vehicle subcutaneously, and their right Achilles tendon divided. Histological assessment was carried out at day 5, 10, and 15 post-operation. Healing tendon biomechanical properties and hydroxyproline content were measured at day 10.

Results: The healing Achilles tendon from the NO-flurbiprofen and flurbiprofen groups showed a better organization of extracellular collagenous matrix than that from the vehicle group. Flurbiprofen and NO-flurbiprofen decreased healing tendon cross-sectional area by 30% and 20%. This reduction was accompanied by a decreased failure load in the flurbiprofen group, but not the NO-flurbiprofen group. NO-flurbiprofen prevented the reduction of body weight gain observed in the flurbiprofen group.

Conclusion: Both flurbiprofen and NO-flurbiprofen promoted better collagen reorganization during tendon healing. NO-flurbiprofen further improved tendon healing by increasing tendon stress and reducing the side effects (body weight loss) of flurbiprofen. The enhanced tendon healing by NO-flurbiprofen is likely due to the release of NO from the compound.

Key words: Nitric oxide (NO) – Tendon healing, non-steroid anti-inflammatory drug (NSAID) – Nitroflurbiprofen

Introduction

Rupture of the Achilles tendon has been documented in the literature for several centuries [13]. It is a particularly debilitating injury for which the current treatment strategies are limited and frequently debated. Tendon healing is a complex and multi-factorial process. The processes of tendon healing have been divided into an inflammatory phase, a reparative phase or collagen producing phase and finally a remodeling phase [54], in which the major physiological events are fibroblast migration and proliferation, collagen synthesis and collagen fibril reorganization [15]. Numerous endogenous and exogenous factors including inflammatory cytokines [16, 32, 53], postaglandins [31, 40] and antioxidants [39] are involved in this process.

Nitroflurbiprofen (NO-flurbiprofen) is a novel non-steroidal anti-inflammatory drug (NSAID) that has greatly reduced ulcerogenic activity in the stomach and small intestine by releasing nitric oxide (NO) [14]. The enhanced anti-inflammatory effect of NO-flurbiprofen has been widely reported in various animal models [9, 21, 48, 50]. This drug was produced by the incorporation of a nitric oxide (NO) moiety through an ester linkage to the carboxyl group of the common anti-inflammatory drug flurbiprofen. It has been shown that cellular esterases release the parent NSAID, and cytochrome oxidases transform the nitrite moiety into NO [1, 41, 50].

NO is a multifunctional intra- and inter-cellular messenger molecule involved in many physiological and pathological processes. Examples include the formation of memory [5], the regulation of blood pressure [12, 26, 28], and the acceleration of wound healing [42, 43, 55]. We have previously shown that nitric oxide synthase activity (catalyzing L-arginine to generate NO) was enhanced in rat healing Achilles tendon [29, 30, 34]. When nitric oxide synthases were inhibited, healing tendon cross-sectional area and fail-
Curure load were inhibited by 50% and 25% respectively [34]. These findings suggest that NO also plays an important role in tendon healing. However, we do not know if the addition of NO would accelerate tendon healing.

Based on our previous findings and the hypothesis that the NO level could be manipulated to enhance tendon healing, we designed the present study to determine if the addition of NO via a novel NO-flurbiprofen compound would enhance tendon healing.

Materials and methods

Animal Model

All procedures and protocols were approved by the Committee on Animal Research of the University of New South Wales, Sydney. A rat healing Achilles tendon model was used as previous described [33]. Rats were housed in beta-chip-lined plastic cages, three animals per cage, with 12 hr light: dark cycle in a central animal care facility. They were fed rat chow and water ad libitum. All rats were given health checks and were given at least two weeks to adapt to their new environment prior to surgery. Anaesthesia was achieved by intraperitoneal injection of 80 mg/kg ketamine and 1 mg/kg xylazine. Surgical division of the right Achilles tendon was performed under sterile conditions as outlined [33]. Briefly, a 1.0 cm-midline incision was made over the right Achilles tendon, and the Achilles tendon and plantaris tendon were isolated free from the surrounding fascia. The Achilles tendon was transected with a scalpel in an axial fashion 0.5 cm from its calcaneal insertion. Care was taken not to disrupt the surrounding tissues. The tendinous portion of plantaris tendon was removed to prevent any possible action as an internal splint. The skin was then sutured with two simple nylon sutures of 4-0 ethilon monofilament nylon on a PC-1 cutting needle. No cast or dressings were applied and the animals were unrestricted and fed a normal diet after surgery. Anaesthesia was achieved by intraperitoneal injection of 1.0 mg/kg ketamine and 0.05 mg/kg xylazine. Healing Achilles tendons for biomechanical testing and collagen content assay were harvested at day 10 post-operation.

Administration of drugs

NO-flurbiprofen and flurbiprofen were provided by NiCoX, Paris, France. The compounds were dissolved in a vehicle of 5% (v/v) dimethyl sulfoxide (DMSO) and 0.5% (w/v) methylcellulose (CMC, Sigma, St Louis, MO). Rats were daily injected subcutaneously with NO-flurbiprofen (15 mg/kg/day), or flurbiprofen (10 mg/kg/day) from two days before surgery to the day of tissue harvesting. Control animals received the same volume of vehicle only. NO-flurbiprofen at 15 mg/kg/day provides the same amount of flurbiprofen at 10 mg/kg/day, as the NO-releasing moiety of NO-flurbiprofen accounts for approximately 33% of the molecular weight of NO-flurbiprofen [50,51].

Histological assessment

Histological assessment was carried out to evaluate the cellularity, vascularity, extracellular-matrix collagen density and collagen fibril organization of the healing Achilles tendon. Specimens were harvested at day 5, day 10 and day 15 post-operation and were immediately fixed in buffered formaldehyde saline (pH 7.4), dehydrated, and embedded in paraffin wax. Coronal sections of the mid-substance of the Achilles tendons were cut at 5 μm thickness and stained with hematoxylin and eosin. Tissue sections were examined under a Leica DMLB microscope (Leica, Postfach, Germany) using both transmitted and polarized light [11,36].

To semi-quantify tendon collagen organization, tendon sections were examined by two blinded investigators using polarized light and scored “1–5” representing from “relatively immature” to “relatively mature” tendon organization. The scores were based on the orientation and network structure of collagen fibers, the formation of collagen fiber bundles, and the length and diameter of the bundles. Score 1. No birefringence of collagen fiber bundles formed. Collagen fibers showed no unique orientation. Score 2. Collagen fibers formed collagen bundles. Areas of collagen bundles were less than 50% with loose network structure. Length and diameter of these bundles were less than 100 μm and 15 μm. Score 3. Less than 50% of collagen fiber bundles formed but with light network structure. Length and diameter of these bundles were over 100 μm and 15 μm. Score 4. Over 50% of collagen bundles formed in healing site. The orientation of these bundles were along the axis of the tendon. Score 5. Normal tendon structure. Figure 2A, B, C and D represent score 5, 3, 3 and 2.

Measurement of healing tissue dry weight and collagen content

Healing Achilles tendons were harvested at day 10 post-operation for the measurement of collagen content and sample weight. After removing all extraneous soft and hard tissues, the healing tendon samples were freed from muscle and the calcaneus. Wet tissue specimens were weighed (wet weight) immediately, dried overnight in a freeze-drier (RV1-100, Savant, Farmingdale, NY) and weighed again (dry weight). Collagen contents of tendons were determined by measuring the concentration of hydroxyproline in each tissue specimen as previously described [45,52]. Briefly, 5 mg of dried tendon tissue was hydrolyzed with 1 M HCl at 105 °C for 12 h. HCl was evaporated at 60 °C and the hydrolysed tissues were reconstituted in 20 ml H2O, and neutralised with sodium hydroxide (NaOH) to pH 7.0. Fifty microliters of chloramine T solution (60 mM; Sigma, St Louis, MO) was mixed with 100 μl standard solutions at concentrations of 0, 20, 10, 5, 2.5, 0 μg/ml of hydroxyproline (Sigma, St Louis, MO) or samples in a 96-well plate to initiate hydroxyproline oxidation. After 20 min incubation at room temperature, the chloramine T was destroyed by adding 50 μl perchloric acid (HClO4) to each well. The mixture was allowed to stand for 5 min. Color was developed by adding 50 μl p-dimethylaminobenzaldehyde (p-DMAAB, Sigma, St Louis, MO) solution to each well. The plate was incubated in a water bath (60 °C) for 20 min, and then cooled in tap water for 5 min. Absorbance was read at 550 nm using a spectrophotometer (SPECTRAMAX3403, Molecular Devices, Sunnyvale, CA). The hydroxyproline concentration was calculated from the standard curve. The total collagen content of each sample was calculated based on the hydroxyproline concentration and hydrolysed sample volume. Results are expressed as microgram hydroxyproline per milligram dry tendon tissue. A normal Achilles tendon sample was included in each assay as a quality control. All assays were performed in triplicates, and both intra-assay and inter-assay co-efficients of variation (CV) were calculated.

Biomechanical testing

Rat healing Achilles tendon cross-sectional areas and failure load were assessed at day 10 following the surgery. The cross-sectional areas of healing Achilles tendons were measured with an area microometer (Mitutoyo, Kawasaki, Japan) similar to that described previously [33,47]. After measurement, the harvested tissues were kept in 0.9% (w/v) saline and proceeded to mechanical testing. The failure load of healing tendon was assessed using an Instron tensile testing system (Instron Test Equipment Ltd, England) as shown in Fig. 1. Briefly, the muscle and intramuscular tendinous fibers were compressed between two serrated aluminum grips which were attached to the lead
Fig. 1. Schematic illustration of the method for healing Achilles tendon biomechanical assessment. Biomechanical assessment was carried out on a Hounsfield tensometer testing machine. The muscle and intramuscular tendinous fibers were compressed between two serrated aluminum grips which were attached to the lead screw of the testing system. A block of dry ice was placed against the side of one grip to freeze the compressed tissue. The calcaneus was fixed to a second grip which was attached to a load cell. Care was taken throughout the testing procedure to ensure that the tendon was kept moist by continuous irrigation with 0.9% saline. The in-situ testing length of the tendon was maintained at 10 mm. Force and displacement was collected using a dedicated computer and software. Failure load was expressed as maximum force to tendon rupture (N).

Statistical Analysis

All values in the text and figures are expressed as mean ± SEM of n observations. Inter-observer reliability for collagen organization between the two blinded investigators was examined using intraclass correlation coefficient (ICC). Statistical analyses among multiple experimental groups was performed using analysis of variance (ANOVA) and unpaired two-tailed Student's t-tests. The confidence limit was predetermined at an alpha level of 0.05.

Results

Histological appearance

Hematoxylin and eosin stained healing Achilles tendon sections were observed under transmitted light microscopy. At day 5, the area between the cut ends of the tendon was filled with a loose connective tissue matrix containing numerous inflammatory cells and blood vessels. At day 10, the loose connective tissue matrix had been replaced by a more homogenous, denser eosinophilic matrix. Plump fibroblasts with large nuclei were the predominant cell type, and generally they were aligned in the longitudinal axis of the tendon. The junction between the old tendon and the new matrix was characterized by dense band of oval shaped fibroblasts with little organization. With time, the tendon-new matrix interface and the band of disorganized fibroblasts became less distinct. At day 15, the collagenous tissue became more organized, with fewer fibroblasts and more matrix. No significant difference in the cellularity and vascularity of healing tendons was found among NO-flurbiprofen, flurbiprofen and vehicle groups (data not shown).

When viewed with polarized light, tendon collagenous fibres with common alignment displayed bright birefringence. In normal Achilles tendons, tendon fibers were incorporated within large fibre bundles (Fig. 2A). The birefringence of bundles of collagen fibers was longer, wider, and in better longitudinal alignment than that of the healing tendons. Figure 2B–D represent the birefringence appearance of healing Achilles tendon from NO-flurbiprofen, flurbiprofen and vehicle group. Extracellular collagenous organization was semi-quantified based on the orientation and network structure of collagen fibers, the formation of collagen fibre bundles, the length and diameter of the bundles by two blinded observers. As shown in Fig. 3, there was a significant increase in the collagen organization of healing tendon tissue from NO-flurbiprofen and flurbiprofen groups compared to vehicle group. Inter-observer reliability between the two blinded investigators was graded as excellent (ICC=0.92) using intraclass correlation coefficient (ICC).

Collagen content of healing tendon

Collagen content of the tendon specimens are expressed as micrograms of hydroxyproline per milligram of dry tissue. As shown in Fig. 4, the collagen content of non-divided tendon was approximately twice as that of the healing Achilles tendon at day 10 post-surgery. There were no statistically significant differences in collagen content of healing tendon among NO-flurbiprofen, flurbiprofen and vehicle groups. The intra-assay co-efficient of variation was 7%, and the inter-assay co-efficient of variation was 4%, demonstrating the reproducibility of the hydroxyproline assay.

Biomechanical properties of healing Achilles tendons

There were significant differences in cross-sectional area of healing Achilles tendons between NSAID (NO-flurbiprofen and flurbiprofen) rats and vehicle rats. As shown in Table 1, the NO-flurbiprofen and flurbiprofen groups had decreased healing tendon cross-sectional areas by 30% and 20% compared to that of vehicle group (NO-flurbiprofen vs. vehicle, 5.3 ± 0.1 mm² vs. 6.9 ± 0.2 mm², p < 0.001; flurbiprofen vs. vehicle, 4.7 ± 0.2 mm² vs. 6.9 ± 0.2 mm², p < 0.001). There was no significant difference in cross-sectional areas between the NO-flurbiprofen and flurbiprofen group.

Following cross-sectional area measurement the failure load was tested on the same healing Achilles tendons. The failure site of the healing tendon was in the mid-substance in
Fig. 2. Rat healing Achilles tendon collagen organization at day 10 post operation. Tissue sections were stained with H&E, observed under polarized light microscopy. A shows a normal Achilles tendon. B, C and D show the birefringence appearance of healing Achilles tendon from NO-flurbiprofen, flurbiprofen and vehicle group. The whitened bar corresponds to 50 μm.

Fig. 3. Semi-quantification of healing Achilles tendon extracellular collagen organization. The healing Achilles tendon from NO-flurbiprofen, flurbiprofen and vehicle group were harvested at day 10 post-operation. Tissue sections were stained with H&E, observed under polarized light microscopy. Extracellular collagenous organization was semi-quantified based on the orientation and network structure of collagen fibers, the formation of collagen fiber bundle, the length and diameter of the bundle. Scores "1-5" represent from "relatively immature" to "relatively mature" tendon organization. There was a significant increasing in the collagen organization of healing tendon tissue from NO-flurbiprofen and flurbiprofen groups compared to vehicle group. Values are mean ± SEM, n = 4 for each group. * P<0.05 when compared with vehicle group. Un-paired two-tailed Student's t-tests and ANOVA.

Fig. 4. Total collagen content of healing Achilles tendons in rats treated with vehicle, flurbiprofen, or NO-flurbiprofen at day 10 post-operation. Sham-operated Achilles tendons were used as internal control. Values are mean ± SEM, n = 4 for sham operated group, n = 12, 9 and 11 for NO-flurbiprofen, flurbiprofen and vehicle group. *** p<0.001 when compared with normal Achilles tendons. Un-paired two-tailed Student's t-tests and ANOVA.
all samples. In contrast, the left, uninjured Achilles tendons failed at the tendon-calcaneus interface in all samples. The failure load of uninjured Achilles tendon was approximately twice as that of healing Achilles tendon. As shown in Table 1, there was a significant decrease in failure load in flurbiprofen group compared with that in the vehicle group (30 ± 2.6 N vs. 39 ± 0.2 N, p < 0.05). However, no significant difference in failure load was found between the NO-flurbiprofen and vehicle group (36 ± 1.1 N vs. 39 ± 0.2 N). These results indicate that NO-flurbiprofen promoted a stronger healing tendon than flurbiprofen.

Failure stress is an expression of the material properties of a construct and is defined as the maximum failure load per unit cross-sectional area of the tendon (failure load divided by cross-sectional area). There was a significant increase (improvement) in healing tendon stress in NO-flurbiprofen group over the vehicle group (6.9 ± 0.3 N/mm² vs. 5.6 ± 0.2 N/mm², p < 0.05, Table 1). No significant difference in healing Achilles tendon stress was found between flurbiprofen and vehicle group. Both NO-flurbiprofen and flurbiprofen had decreased healing tendon cross-sectional area when compared with the vehicle group. However, this reduction in tendon cross-sectional area was accompanied by a decreased failure load in the flurbiprofen group, but not the NO-flurbiprofen group, suggesting additional NO via NO-flurbiprofen had a protective or beneficial effect on the healing tendon mechanical properties.

Healing tendon wet weight and water content

To compare extracellular matrix water content of healing tendon, harvested tendon tissues were measured for their wet weight, dry weight and length. Water content of healing tendon was calculated by subtracting dry weight from wet weight and the results were normalized by length of healing tendons. As shown in Table 2, the wet weight and water content per unit length of repaired tendons were greater than that from the sham animals. Both NO-flurbiprofen and flurbiprofen treatment significantly reduced the wet weight and water content per unit length of healing tendon when compared to the vehicle. There was no statistically significant difference in wet weight/length and water content/length of healing tendon between NO-flurbiprofen and flurbiprofen groups.

Table 1. The biomechanical properties of healing Achilles tendons in rats treated with NO-flurbiprofen, flurbiprofen, or vehicle at day 10 post-operation.

<table>
<thead>
<tr>
<th>Cross-sectional area (mm²)</th>
<th>Failure load (N)</th>
<th>Failure stress (N/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>6.9 ± 0.2</td>
<td>39 ± 1.9</td>
</tr>
<tr>
<td>Flurbiprofen</td>
<td>4.7 ± 0.2**</td>
<td>30 ± 2.6*</td>
</tr>
<tr>
<td>NO-flurbiprofen</td>
<td>5.3 ± 0.1**</td>
<td>36 ± 1.1†</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>2.3 ± 0.1</td>
<td>64 ± 1.3</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, n = 5 for each group. *Significantly different from vehicle at the 0.05 level; **Significantly different from vehicle at the 0.01 level; †Significantly different from flurbiprofen at the 0.05 level. Un-paired two-tailed Student’s t-tests and ANOVA.

Discussion

The present study has shown that addition of NO via NO-flurbiprofen improved the rat Achilles tendon healing process by enhancing healing tendon material properties, promoting better collagen reorganization, and preventing body weight loss. Both NO-flurbiprofen and flurbiprofen significantly decreased cross-sectional area and markedly increased tendon failure stress. But more important, NO-flurbiprofen did not affect failure load of healing tendon and rats body weight gain compared with vehicle in this rat model.

Flurbiprofen is a non-steroidal inflammatory drug widely used clinically for its analgesic and anti-inflammatory properties. However, it has certain, sometimes severe side effects, especially to the gastro-intestinal [2, 7, 8, 22]. These side effects of flurbiprofen are most likely related to its ability to block prostaglandin synthesis through the inhibition of cyclo-oxygenase (COX) and particularly COX-1. Prostaglandins have a well-known role in the remodelling of wound healing. Exogenous prostaglandins can accelerate wound healing and the recovery of gastrointestinal ulcers [20, 46]. When endogenous prostaglandins synthesis is blocked, the wound healing process can be delayed [17, 37]. Flurbiprofen has exhibited inhibitory effects on skin wound healing, possibly through the inhibition of prostaglandins [17, 37]. It is likely that prostaglandins also play a similar role in tendon healing. In our study, cross-sectional area of healing tendon in the flurbiprofen group decreased 30% compared with that of the vehicle group. However, flurbiprofen also caused a significant decrease in failure load (25%) and body weight gain (25%).

The novel compound, NO-flurbiprofen, was designed to reduce the side effects of flurbiprofen by releasing nitric oxide. In previous animal experiments [3, 19, 24, 25, 44, 50, 51] and clinical trials [35], NO-flurbiprofen has been shown to be successful in reducing the side effects of flurbiprofen. However, NO-flurbiprofen has not been tested on tendon healing. Results from our study have demonstrated that NO-flurbiprofen enhanced healing tendon biomechanical properties. When compared with flurbiprofen, NO-flurbiprofen showed similar effects on cross-sectional area, and more importantly, it significantly improved the failure load and stress of healing Achilles tendon compared with flurbiprofen. The beneficial effects of NO-flurbiprofen in the very early healing of the young rat Achilles tendon are likely attributed to the NO that is released from this compound. Other studies have shown increased plasma nitrate/nitrite levels in rats treated with NO-flurbiprofen compared to the vehicle group. These results suggested that the addition of NO via NO-flurbiprofen prevented the reduction of body weight gain observed in flurbiprofen injected rats.

Rat body weight gain

Following tenotomy, all animals showed good recovery. During the experimental period, the rat body weight was recorded daily. At day 10 post-operation, the rat body weight gain was calculated. As shown in Fig. 6, the rats in the NO-flurbiprofen and vehicle groups gained weight at the same rate. However, administration of flurbiprofen resulted in a decrease in rat body weight gain (flurbiprofen vs. vehicle, 31 ± 3 g vs. 41 ± 3 g, p < 0.05). These results suggested that the addition of NO via NO-flurbiprofen prevented the reduction of body weight gain observed in flurbiprofen injected rats.
levels after administration of NO-flurbiprofen while they were unaffected by flurbiprofen [49, 50]. NO, acts as a messenger molecule in physiological and pathological conditions, increasing blood flow and reducing neutrophil adherence to the vascular endothelium.

In this rat Achilles tendon healing model, we first conducted morphological and histological studies to compare the healing tissue cellularity, vascularity, matrix collagen density and orientation among flurbiprofen, NO-flurbiprofen and vehicle groups. Although there were no significant differences in cellularity and vascularity found among these experimental groups, the NSAID’s, especially NO-flurbiprofen, had a positive impact on collagen organization of the healing rat Achilles tendon at day 10 post-operation. Collagen re-organization is an important phase of tendon healing [4, 15, 23]. We further assessed the biomechanical properties of healing tendon at day 10 post-operation. Agents that could strengthen healing tendon at this early stage would be beneficial tendon healing. Other investigators have also suggested to therapeutic interventions should be modified to address the specific events of each stage [15]. It has been shown that initial inflammation, fibroplasia and fibrillogenesis, and progressive alignment and organization of the collagen fibrils into bundles that were oriented in the longitudinal axis of the tendon occurred at the early stages of healing. We have found the failure load of the operated tendon was more than half of the normal tendon signifying that significant repair had taken place at day 10. Day 10 is a critical point during healing when the tendon has achieved significant strength (failure load, 50–61% of intact tendon) but is still susceptible to retear. Results from our biomechanical tests demonstrated that NO-flurbiprofen significantly enhanced healing tendon biomechanical properties (failure load/ cross-sectional area) at day 10 after surgery.

The tendons from the vehicle and NO-flurbiprofen groups had the same failure load which would signify equivalent strength. However, a reduced size in cross-sectional area but with the same failure load in the NO-flurbiprofen group indicates better material properties of the healing tendon in the NO-flurbiprofen group. It has been suggested that the production of collagen [27] and collagen cross-links [10, 18] are directly associated with the biomechanical properties of the tissue. Our results on collagen content measured by hydroxyproline concentration showed no statistical differences among the NO-flurbiprofen, flurbiprofen and vehicle groups. The slightly enhanced material properties (stress) in NO-flurbiprofen rats maybe due to enhanced collagen fibril alignment in the longitudinal axis of the repairing tendon.

In this study, rats were given either 10 mg/kg of flurbiprofen, 15 mg/kg of NO-flurbiprofen or vehicle. The higher dose of NO-flurbiprofen was used because the NO-releasing moiety of the compound accounts for approximately 33% of the molecular weight. Thus, the 15 mg/kg delivered a comparable amount of flurbiprofen to the animals as a 10 mg/kg of the native NSAID [50, 51]. Single daily dosage of each compound was chosen in our study, as these dosages have already been shown to have maximal pharmacological actions with lowest toxicity in vivo [6].

While the rats treated with NO-flurbiprofen continued to gain weight at the same rate as the vehicle treated rats, those treated with flurbiprofen either did not gain weight, or lost weight. By the end of the study period, the body weights of the flurbiprofen treated rats were significant lower than those of the other two groups. These results were consistent with the reports by other investigators [51].

In summary, our study has shown that both flurbiprofen and NO-flurbiprofen promoted better collagen reorganization during tendon healing. NO-flurbiprofen further improved tendon stress performance minimally (about 20% compared with vehicle alone, and 8% compared with flurbiprofen controls) and the animals had less weight loss.
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ADDICTION OF NO VIA NO-PARACETAMOL ENHANCES COLLAGEN CONTENT AND ORGANIZATION OF HEALING RAT ACHILLES TENDON
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** Orthopaedic Research Institute, St George Hospital, University of New South Wales, Australia

Introduction:
Nitric oxide (NO) is an important messenger molecule in many physiological processes. Our previous study has shown that the addition of NO via NO-flurbiprofen, a non-selective cyclo-oxygenase (COX) inhibitor, enhanced the material properties of healing tendon. NO-paracetamol is a derivative of paracetamol which selectively inhibits COX-3, a recently identified COX localized in the central nervous system. The aim of this study was to investigate the effects of nitro-paracetamol (NO-paracetamol) on tendon healing.

Methods:
All procedures and protocols were approved by the Committee on Animal Research of the University of New South Wales, Sydney. A rat healing Achilles tendon model was used as previously described. Seventy outbred male Sprague-Dawley rats, weight 250-300g (6-8 weeks), were included in the study for tendon histological assessment (n=18), biomechanical testing (n=18), and collagen content measurement (n=34). NO-paracetamol and paracetamol (Nicox, Paris, France) were dissolved in a vehicle of 5% (v/v) DMSO and 5% polyethylene glycol. Rats were assigned to one of two treatment groups: standard paracetamol (Para) or NO-paracetamol (NO-para). Seven rats were included in each group for tendon histological assessment (n=18), biomechanical testing and collagen content assays. Harvested tendons were harvested at days 5, 10 and 15 post-operation. Healing tendons were harvested at day 10 post-operation for cross-sectional area assessment, biomechanical testing and collagen content assays. Harvested tendons for collagen content assessment were weighed immediately (wet weight), dried overnight in a freeze-drier and weighed again (dry weight). Tendon in NO-paracetamol group (NO-para) was significantly higher than that of the vehicle and paracetamol groups, observed under the polarized light microscope (200X).

Results
Collagen content of healing tendon: The collagen content of healing tendon in NO-paracetamol group (NO-para) was significantly higher than that in vehicle group (Veh) and paracetamol groups (Para) (Fig 1). The NO-paracetamol administered rats also had a higher ratio of tendon dry weight to wet weight, suggesting higher protein content in tendons from NO-paracetamol group.

Stiffness (N/mm)

<table>
<thead>
<tr>
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<th>Veh</th>
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<th>NO-para</th>
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<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
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</tr>
<tr>
<td>Para</td>
<td>0.00</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>NO-para</td>
<td>0.00</td>
<td>0.05</td>
<td>0.05</td>
</tr>
</tbody>
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Fig 3: Effect of NO-paracetamol on cross-sectional area of healing tendon

Fig 4: Effect of NO-paracetamol on stress of healing tendon

Histological Assessment: The collagen organization of healing tendon tissue in NO-paracetamol group was greater than that of the vehicle and paracetamol groups, observed under the polarized light microscope (Fig 6).

Discussion and Conclusion
The present study has provided evidence for the first time that NO-paracetamol increased total collagen content and dry weight, material properties and stiffness and decreased the cross-sectional area of healing rat Achilles tendon. These results indicate that NO-paracetamol can improve tendon healing and hold promise for the treatment of damaged tendons.

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** NicOx SA, Sophia Antipolis, France
Overexpression of Nitric Oxide Synthases in Tendon Overuse

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ABSTRACT: Tendon disorders with a chronic nature, including the rotator cuff, are extremely common, and represent a major clinical problem. Mechanical overload has been proposed as an important etiologic factor in tendinopathies. Nitric oxide (NO), a free radical produced by nitric oxide synthases (NOSs), is a potent regulator and stimulator of biological processes including tendon degeneration and healing. It is also involved in response to mechanical stimuli in different tissues. In an animal model of acutely injured tendon healing temporal and differential expression of NOS isoforms has been demonstrated, suggesting that different patterns of NOSs expression may have different biological functions. Therefore, we hypothesized that tendon overuse may result in a differential upregulation of NOSs, particularly iNOS. An animal model of supraspinatus tendon overuse was utilized, which consisted of treadmill running. A group of animals of the same strain and age subjected to normal cage activity were used as controls. Following a 4-week exercise protocol supraspinatus tendons were harvested, RNA was extracted, and subjected to competitive reverse transcription and polymerase chain reaction (RT-PCR) to determine the expression levels of inducible-, endothelial-, and neuronal-NOS isoforms (i-, e-, and nNOS). The mRNA expression of all three NOS isoforms increased in the supraspinatus tendons as a result of overuse exercise. iNOS and eNOS mRNA expression increased fourfold ($p < 0.01$), and there was an increase, but statistically not significant, in nNOS mRNA expression in the overused tendons when compared with the controls. This study is the first to show that NOS isoforms are upregulated in rotator cuff tendon as a result of chronic overuse, and suggests the involvement of nitric oxide in the response of tendon tissue to increased mechanical stress. © 2005 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 24:80–86, 2006

Keywords: tendon; overuse; nitric oxide; expression; rotator cuff

INTRODUCTION

Mechanical overload, as a result of repetitive microtrauma from overuse, has been proposed as the primary etiologic factor in tendinopathies, including tendinopathy of the rotator cuff.1–6 At the histological level, two contrasting processes in the overused tendons at the same time have been described. Evidence of tissue degeneration include disruption and microtears of collagen bundles, appearance of fibrinoid, hyalin, and mucoid degeneration, fibrocartilagenous metaplasia, hypo- and acellularity, and necrosis. In contrast, other areas of tendons show signs of repair: hypercellularity, fibrosis, and proliferation of fibroblastic, fibrovascular tissue.7–9 Similarly, at the molecular level, there is evidence to support the presence of degenerative changes as well as repair. Increased activation of matrix metalloproteinases, collagen degradation,10–12 and increased rate of apoptosis13 are contrasted by increased synthesis of type III collagen and proteoglycans,14,15 and increased number of cells expressing smooth muscle actin.16,17

Nitric oxide (NO) is a diatomic, highly reactive, free radical. It is produced by a family of enzymes called nitric oxide synthases (NOSs). Three
isoforms of NOS have been described. The inducible form (iNOS) produces relatively high concentrations of NO in response to stimuli, while endothelial (eNOS) and neuronal (nNOS) isoforms are expressed constitutively and produce relatively low, stable amounts of NO. Nitric oxide is an important messenger molecule that plays a crucial role in a wide variety of physiological functions.

It is important to consider that NO may induce contrasting effects both at the cellular and molecular levels, depending on NO concentration and source. High levels of NO are often associated with degradative processes,18,19 including modulation of the activation of metalloproteinase enzymes,20 cytotoxicity,21 and are induced by pro-inflammatory cytokines.20,22 On the other hand, there is a growing body of evidence that suggests that NO is important in the healing of soft tissues including gastric ulcers,23 anal fissures,24 tendon,25,26 skin,27,28 and bone.29,30 Furthermore, NO promotes processes crucial to wound healing such as angiogenesis,31,32 cell proliferation,33,34 and collagen synthesis.27,28

In an acutely injured tendon-healing animal model a temporal and differential expression of all three NOS isoforms has been demonstrated.25 Using the same animal model, it was also shown that systemic NOS inhibition diminished tendon healing.26 Another important aspect of NO in relation to musculoskeletal tissue is that mechanical stress is a potent stimulator of NO synthesis in many cell types including endothelial cells,35 osteoblasts,36 and chondrocytes.37 Static and dynamic compression forces have been shown to induce NO production in cartilage38 and meniscal tissue.39

Based on the observations that rotator cuff tendon overuse results in increased load and that tissue response to mechanical load has included activation of NO pathways, we hypothesized that NOS isoforms, particularly iNOS would be upregulated in rotator cuff tendons in response to overuse exercise.

An experimental animal model of overuse of the rotator cuff by treadmill running has been developed recently that results in significant morphological, histological, biomechanical, and biochemical changes in the overused rat supraspinatus tendons, which are similar to those observed in tendinopathies.10,41

The aim of our study was to investigate whether NOS isoforms are expressed and in what relative quantities in the rotator cuff as a result of overuse.

### MATERIALS AND METHODS

#### Rotator Cuff Overuse Animal Model

Supraspinatus tendon overuse was modeled by using an established repetitive exercise protocol, which consists of treadmill running.41 All procedures and protocols were approved by the Animal Care and Ethics Committee of the University of New South Wales under ACEC No. 99/101. Thirty male Sprague–Dawley rats (Biological Resources Centre, Sydney, Australia) with an average weight of 480 g (±20 g) were used. All rats were housed in an air-conditioned animal house under 12-h day/12-h night lighting condition with 60% humidity at 21°C. The animals were acclimatized for at least 2 weeks prior to exercise.

Twelve rats were used for the rotator cuff tendon overuse experiments. Initially, a 2-week treadmill pre-training period was conducted to acclimatize the rats to the running at the desired final speed and duration. Two animals not cooperating during early stages of pretraining were replaced by animals from the remaining group. Following pretraining, the rats were subjected to overuse exercise running at 17 meters per minute (1 km/h), on a 10 degree decline, for 1 h per day, 5 days a week for 4 weeks.40 This exercise protocol results in approximately 7,500 strides per day, which is consistent with the number of strokes an elite swimmer may perform during a typical training protocol.42 A group of 12 rats of the same strain and gender with similar age and weight subjected to normal cage activity were used as control animals for comparison to the overuse group. Following the treatment, the animals were sacrificed by carbon dioxide inhalation. Direct measurement of NO in biological tissues is very difficult due to its extremely short half-life and does not discriminate between the activities of different NOS isoforms. Measurement of NOS protein levels by enzyme activity assay or immunoblotting requires relatively large quantities of tissue; therefore, estimation of NOS isoform expression at the transcriptional level, as being accurate and reproducible, best suited our aims.

#### RNA Extraction and cDNA Production from Overused Supraspinatus Tendons

Supraspinatus tendons were harvested using an aseptic technique immediately after euthanasia of the animals, cleaned from any muscle contamination, snap frozen in liquid nitrogen, and stored at −80°C. Supraspinatus tendons from both sides of the animals were collected, and tendons from two animals were combined for RNA extraction using the TRIzol reagent43 (Life Technologies, Grand Island, NY), with a total yielding of 2–15 μg RNA (n = 6). The OD260/280 ratios were maintained between 1.7–1.9, confirming that the quality of the RNA was satisfactory. To synthesize cDNA 1 μg total RNA and 0.5 μg random hexamer primers (Promega, Madison, WI) were added to RNase-free
water for a final volume of 13.4 μL, then incubated at 70°C for 30 min on a GeneAmp 2400 thermocycler (Perkin-Elmer Applied Biosystems, Branchburg, NJ). A master mix of 5 μL 5× concentrated reverse transcription reaction buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl2, and 50 mM dithiothreitol), 5 μL 10 mM dNTPs (mixture of 2.5 mM dATP, dGTP, dCTP, and dTTP), 2.5 units of Recombinant RNasin ribonuclease inhibitor and 200 units of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase DNA polymerase (Promega) were added to each reaction tube. Then, the RT mixture was heated and incubated at 37°C for 60 min on the thermocycler for reverse transcription. For each tested RNA sample, a control reaction was run with MMLV reverse transcriptase omitted from the reaction mixture to exclude false positive results from genomic DNA contamination.

Detection of NOS mRNA Isoforms by Competitive RT-PCR

Competitive PCR is a very sensitive and specific, semiquantitative method to measure quantities of a target cDNA sequence. A serial dilution of a known amount of internal control cDNA is mixed to the target cDNA in the same PCR reaction, which then amplified in a competitive manner resulting in two PCR products with different sizes. The ratio of these two PCR products measured by densitometry allows to calculate the original quantity of the target cDNA. The accuracy and reproducibility of this competitive PCR method has been verified and published previously by our laboratory. Two microliters of these diluted control DNA with known quantities and 2 μL of sample target cDNA were added to a PCR reaction mixture containing 4 μL 10 mM dNTPs, 5 μL 10× PCR reaction buffer, 2 units REDTaq DNA polymerase, and 1 μL of 0.5 μM NOS isoform-specific upstream and downstream primers (Table 1), for a final reaction tube volume of 50 μL. The amplification was performed in the GeneAmp 2400 thermocycler. The PCR conditions were initial denaturation at 94°C for 5 min, amplification 35 cycles (denaturation at 94°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 45 s), and a final extension at 72°C for 7 min. PCR products were fractionated, visualized, photographed, and the images were subjected to densitometric analysis as described earlier. The intensity of the sample PCR product was compared to that of the internal control DNA. There was a linear relationship between the ratio of sample/internal control DNA signal intensity and the internal control concentration (protocol PT1521-1 of Competitive PCR reagent kit, Clontech, Palo Alto, CA). By knowing the concentration of the internal control DNA, the amount of the sample cDNA, and, from the start, the relative amount of initial sample mRNA could be calculated.

Data are presented as mean ± SEM. Differences among experimental groups were assessed by unpaired Student’s t-test. The level of statistical significance was set at p < 0.01.

RESULTS

All animals selected for the exercise regimen completed the 4-week protocol. There was no significant body weight difference between the exercised animals when compared to the cage activity group at the beginning and end of the study, and there was no visible macroscopic difference between supraspinatus tendons in the overuse animals when compared to the tendons of control animals.

The mRNA expression of all three NOS isoforms increased in the supraspinatus tendons. Inducible NOS mRNA expression demonstrated a fourfold (p < 0.01) increase in the overused supraspinatus tendons (4.2 × 10⁻⁵ ± 1.3 × 10⁻⁵ amol) when compared with the controls (1.1 × 10⁻³ ± 0.5 × 10⁻⁵ amol) (Fig. 1). Endothelial NOS mRNA

| Table 1. Rat NOS Isoform Specific Primer Sequences for PCR Reactions |
|-----------------|-----------------|-----------------|
| Product Size (bp) | Primer Sequence (5′ → 3′) | Primer Sequence (5′ → 3′) |
| iNOS | 223 | Sense | TGCATGGAAACGTATAAGGCAAA |
| | | Antisense | GTTTCTGGTCATGCTAGAAC |
| eNOS | 553 | Sense | ATATCTTCAGCCCCAACG |
| | | Antisense | AACCCTTCCATTTGATCAG |
| bNOS | 280 | Sense | TACTCCATCGTCCCTTCCA |
| | | Antisense | GGAATGTCATATTGTCG |

Oligonucleotide primer sequences were designed using “primers on the World Wide Web” and synthesized by Life Technologies, Melbourne, VIC, Australia.
expression also increased significantly \((p < 0.01)\) by nearly fourfold in the overused supraspinatus tendons \(152 \times 10^{-6} \pm 33 \times 10^{-6} \text{ amol}\) when compared with supraspinatus tendons from the cage-confined controls \(45 \times 10^{-6} \pm 15 \times 10^{-6} \text{ amol}\) (Fig. 2). Also, there was a nearly threefold increase in nNOS mRNA expression in the overused rat tendons \(6.0 \times 10^{-3} \pm 3.8 \times 10^{-3} \text{ amol}\) compared with the controls \(2.0 \times 10^{-3} \pm 0.4 \times 10^{-3} \text{ amol}\) (not statistically significant, \(p = 0.08\)) (Fig. 3). There was a low but detectable level of mRNA expression of all three NOS isoforms in the control supraspinatus tendons of the cage activity group.

**DISCUSSION**

Overuse tendon disorders are very common and typically effect manual laborers and athletes. It has been reported that the prevalence of rotator cuff impingement is 5% among slaughterhouse workers,\(^3\) Achilles tendon overuse injuries range between 7 and 9% in top-level runners,\(^1\) and lateral epicondylitis effects 1.2% of the general population.\(^6\)

Our current study is the first to show that NOS isoforms are upregulated in the rotator cuff and suggest the involvement of NO in the tendon tissue response to overuse. These findings raise the important question: what is the function of NO in the overused tendon? There are several pathways whereby NO may contribute as a messenger agent or by direct action to the cascade of molecular events that occur in rotator cuff tendon during normal function and during pathological conditions.

In a normal tendon exposed to physiological mechanical stimuli baseline level of NOS expression, especially the constitutive eNOS and nNOS isoforms, may be mediators of tenocyte metabolism and turnover of tendon matrix components.

**Figure 1.** iNOS mRNA expression in rat supraspinatus tendons following 4-week treadmill running exercise protocol (Overuse) compared with cage-activity controls (Control). Mean ± SEM, \(n = 6, **p < 0.01\).

**Figure 2.** eNOS mRNA expression in rat supraspinatus tendons following 4-week treadmill running exercise protocol (Overuse) compared with cage-activity controls (Control). Mean ± SEM, \(n = 6, **p < 0.01\).

**Figure 3.** nNOS mRNA expression in rat supraspinatus tendons following 4-week treadmill running exercise protocol (Overuse) compared with cage-activity controls (Control). Mean ± SEM, \(n = 6, \) No statistically significant difference.
The beneficial biological effects of induced level of NO include vasodilation, increase collagen synthesis and other matrix proteins, and stimulation of cell proliferation. These processes may be part of the adaptation of the tendon to load or a repair response to submicroscopic injuries that occurred in the tendon matrix. Our previous experiments using an acutely injured rat Achilles tendon healing model demonstrated a temporal and differential expression of NOSs with an early (first week) peak of iNOS expression was followed by an increase in eNOS and finally in nNOS expression at 2 and 3 weeks. In our current study we found all three NOS isoforms are over-expressed.

Nitric oxide has been demonstrated to contribute to angiogenesis and cyclooxygenase-2 (COX2) induction by mechanical stimuli. Vascular endothelial growths factor and COX2 have been noted to be induced in this overexercise animal model. One of the limitations of our study is that NOSs expression was evaluated at a single time point. The NOS expression pattern we detected at 4 weeks may not be uniform for the whole duration of the tendon overuse exercise.

A fine balance may exist between physiologic mechanical stimulus, resulting in tendon matrix turnover and adaptation and mechanical overload leading to tendon damage. In a theoretical model NO may play a double act; depending on the local concentration it may contribute to healing and repair or degeneration of the injured tendon tissue (Fig. 4).

We hypothesized that iNOS may be induced in overused rat supraspinatus tendons. Our study has shown that all three isoforms of nitric oxide synthase were induced during overuse of rat supraspinatus tendons.

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Nitric Oxide Enhances Collagen Synthesis in Cultured Human Tendon Cells

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ABSTRACT: Collagen deposition is an important process that occurs during wound healing. We and others have shown that nitric oxide (NO) is important in tendon healing. The mechanisms whereby healing is enhanced are, however, undetermined. The aim of this study was to investigate whether NO could enhance collagen synthesis in cultured human tendon cells via exogenous NO and via an adenovirus containing the gene for inducible nitric oxide synthase (Ad-iNOS). Tendon cells from the torn edge of the tendons of patients undergoing rotator cuff repair surgery were cultured following collagenase digestion, and stimulated with exogenous NO (SNAP), transfected with Ad-iNOS, and treated with the NOS inhibitor, L-NMMA. Total protein and collagen synthesis were evaluated by 3H-proline and collagenase sensitive 3H-proline incorporation in human tendon cells. High doses of exogenous NO (SNAP) inhibited collagen synthesis. Lower doses enhanced total protein and collagen synthesis of the tendon cells. Ad-iNOS successfully transfected active iNOS into human tendon cells in vitro and also enhanced total protein and collagen synthesis of the tendon cells. The NOS inhibitor, L-NMMA, inhibited the effects of iNOS on the cells. Our studies show for first time that nitric oxide can enhance collagen synthesis in human tendon cells in vitro. These results may explain, in part, at least, the beneficial effects of NO donors in animal models and during the treatment of tendinopathies in human clinical trials.

Keywords: nitric oxide; collagen synthesis; tendon cells; adenoviral vector; gene transfection

INTRODUCTION

Increasing collagen deposition is very important for enhancing wound strength and integrity, especially for tendon healing, because in tendon, the type I collagen-containing fibril is the major element responsible for structure stabilization and the mechanical attributes of this tissue.¹

Tendon healing is a complex process driven by a number of growth factors released into the area of injury. Evidence is emerging for an important role of a small diffusible molecule in wound repair, nitric oxide (NO), a free radical gas.

As one of the smallest molecules in nature, NO can rapidly diffuse through membranes and biological structures within the cell and its surrounding matrix. In biological fluids, NO has a half-life of only a few seconds because it readily reacts with other atoms or molecules. NO has a high affinity for heme and non-heme iron, sulphhydril or thiol groups, superoxide anion, and molecular oxygen.² In the presence of oxygen, NO rapidly metabolizes to nitrite (NO₂⁻), which is further oxidized by oxyhemoglobin in the blood to nitrate (NO₃⁻). Under culture conditions, nitrite and nitrate are stable metabolites of NO. Nitrite and nitrate are often measured as marker molecules for the production of NO.³

NO is produced in higher organisms by the oxidation of one of the terminal guanidinonitrogen atoms of L-arginine. This process is catalyzed by
the enzyme nitric oxide synthase (NOS). The enzyme NOS exists in three isoforms, neuronal NOS (nNOS, type I), inducible NOS (iNOS, type II), and endothelial NOS (eNOS, type III). The isoforms nNOS and eNOS are constitutively expressed, and their activity is regulated by intracellular calcium concentration. The isoform iNOS is located at chromosome 17 (17cen-q11.2), contains 4.1 kb mRNA encoded a 131-kDa size protein.4–6 iNOS is inducibly expressed in macrophages and other cells after stimulation by cytokines, lipopolysaccharides, and other immunologically relevant agents.

The link between nitric oxide (NO) and collagen synthesis has been studied both in vivo and in vitro. In most studies, treatment with NO donors, dietary L-arginine, or iNOS overexpression via gene therapy, increases collagen content of experimental wounds.7–10 NOS inhibition has also been found to decrease collagen and granulation tissue formation in experimental wounds.11,12 Our previous reports also confirmed that NOS is upregulated during tendon healing and inhibition of NOS resulted in a significant reduction in cross-section area and failure load of healing Achilles tendon constructs.13–15 We have also noted in our recent clinical trials, that application of topical nitric oxide improved early pain with activity, late functional measures, and outcomes of patients with extensor tendinosis, tendinosis of the extensor mechanism in the elbow, the Achilles and the supraspinatus in the shoulder.16–18

Most of the findings, however, are from the studies on cutaneous wound healing. Little is known about the relationship between NO and collagen synthesis in tendon cells. The aim for this study, therefore, was to investigate whether NO could enhance collagen synthesis in human tendon cell.

METHODS

Human Tendon Tissue Collection and Tendon Cell Isolation

Human rotator cuff tendon samples were collected aseptically from the torn edge of the tendons of patients undergoing rotator cuff repair (Human Ethics Committee approval No. 96/55). The tendon samples were from 14 patients. The ages of the patients were from 46 to 79 years with a median age of 55 years, including 10 female patients and 4 male patients. The durations of symptoms were from 1 to 43 months and median duration was 5 months. During rotator cuff repair, the edges of the torn tendon are usually debrided and discarded. For these experiments, these pieces of tendon were collected, put in normal saline [0.9% (w/v) NaCl] on ice, and transferred in a sterile fashion from the operating room to the laboratory.

Tendon samples were washed in sterile Dulbecco’s phosphate-buffered saline (D-PBS) (0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, and 1.4 mM KH2PO4, pH 7.4) three times at 37°C and then were cut into small (about 1 mm3) pieces. Diced tendon tissue samples were washed in PBS again, then placed into a 75 cm2 culture flask in 50 ml of collagenase digestion solution containing 0.25 mg/ml type I A Clostridium histolyticum bacterial collagenase, 2% (v/v) HEPES [N-(2-hydroxyethyl) piperazine-N’-(2ethanesulfonic acid)], 2% (v/v) Antibiotic-Antimyotic (ABAM) (GIBCO™, Invitrogen Corporation, 100×, Cat# 15240-062, Grand Island, NY) in 1 x concentration of Hank’s Balanced Salt Solution. The tendon pieces were digested at 37°C under constant agitation in a hybridization orbital shaking water bath for 24–30 h. The tendon digest was transferred to a 50-ml conical tube and the cells were pelleted at 1,500 g for 10 min at room temperature in a Beckman GS-6 benchtop centrifuge using a GS-GH 3.8 swinging bucket rotor (Gladesville, NSW, Australia). The cell pellet was recovered in 20 ml PBS, and pelleted again at 1,500g for 10 min. The cell pellet was then resuspended in 10 ml of the culture media, including 10% (v/v) fetal bovine serum (FBS), 1% (v/v) ABAM, and 2 mM L-Glutamine (100×, Cat# 25030-081, GIBCO BRL, Life Technologies, Grand Island, NY) in Dulbecco’s modified Eagle’s medium (DMEM), transferred into a new 25 cm2 cell culture flask and incubated constantly at 37°C, 5% CO2 and humidified atmosphere for 10–14 days.

Tendon Cell Primary Cultures, Passage, and Routine Culture

Primary tendon cell cultures were grown for 10–14 days with the culture medium changed every 2–3 days. After confluence in 25 cm2 flasks, the tendon cells were passaged: the cells were washed with PBS for two to three times and incubated in 2–3 ml of 1 × trypsin/EDTA solution [0.05% (w/v) Trypsin, 0.53 mM EDTA 4Na] (Cat.# 15400-054, Gibco) at 37°C for 5–10 min to detach the adherent tendon cells from the flask. The cell suspension was diluted with 10 ml serum-free DMEM, transferred into a 50-ml conical tube and pelleted by centrifuging at 1,500g for 10 min. The cell pellet was resuspended in the culture media, and a 20 μl of aliquot was transferred to a 1.5 ml tube for cell counting. The aliquot was mixed with an equal volume of 0.4% (w/v) trypan blue dye, and then applied to a hemocytometer (Fisher Scientific, Pittsburgh, PA) covered with a coverslip. The viable cells that excluded trypan blue uptake were counted under a light microscope. Aliquots were counted in triplicate and averaged. The cell suspension was diluted to the required volume and cell density. Appropriate volume of cell suspensions was plated in a new 75 cm2 flask with 12 ml of the culture media.
media and continued to be incubated at 37°C, 5% CO₂ and humidified atmosphere. The culture media was changed one time per week. The tendon cells from passages 2–4 were used for all subsequent experiments.

Adenovirus

Adenoviral constructs containing inserts of the iNOS isoform gene (Ad-iNOS), as well as an “empty” virus without insert (Ad-Empty), were purchased from the University of Iowa Gene Transfer Vector Core. Adenoviral constructs containing inserts of the iNOS isoform gene had full-length (3,690 bp) mouse iNOS cDNA

(Gene Bank accession #M87039) inserted into an AdCMV vector following a CMV promoter between Not I and XhoI restriction sites. The stock viral concentrations were as follows: Ad5CMV-empty, 5.5 × 10⁸ PFU/ml; and Ad5CMViNOS, 2 × 10⁸ PFU/ml. The viruses were suspended in phosphate-buffered saline containing 3% sucrose, aliquoted to 20 ml portions and stored at −80°C for further use.

Tendon Cells Transfected with Adenovirus or Treated by SNAP or L-NMMA

Trypsinized tendon cells were counted, diluted to the desired cell density, and seeded either in a 96-well cell culture plate (10⁵ cells/well/200 μl culture media), or a 6-well cell culture plate (10⁶ cells/well/1 ml culture media) overnight prior to experimental treatment. The cells were transfected/treated the next day, with each of the treatment groups containing 5 or 6 wells of cells being treated identically. All experiments were validated in at least two independent trials.

The desired concentrations of either viruses (Ad5CMV-empty and Ad5CMViNOS), nitric oxide donor, SNAP (S-nitroso-N-acetylpenicillamin), or the nitric oxide synthase inhibitor, L-NMMA (N⁰-monomethyl-L-arginine) were prepared freshly in cell culture media, pre-warmed to 37°C, and applied to the tendon cells. The tendon cells were then incubated under regular culture conditions for 24 h to allow viral transfection or SNAP/L-NMMA treatment. Finally, nontransfecting virus was removed and the media was replaced with fresh, virus-free media at 24 h. In the SNAP/L-NMMA treatment, the media were further supplemented with SNAP/L-NMMA at the time of the viral transfection and an every subsequent change of the culture media.

Assessment of Cell Viability

Cell viability was evaluated using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega; Amandale, NSW, Australia). This method is based on the conversion of a tetrazolium salt into a formazan product by viable cells. The formazan absorbs at a characteristic wavelength of 490 nm, and the production of formazan and absorbance at this wavelength is directly proportional to the number of living cells in the culture. This method is referred as a MTS assay.

Tendon cells were seeded in 96-well culture plate at 10⁵ cells/well cell density and incubated overnight. The following morning, the cells were transfected with Ad-iNOS and with Ad-Empty or treated by SNAP/L-NMMA. At the end of the experiment, 40 μl of the MTS [5-(3-carboxymethoxyphenyl)-2/(4,5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium, inner salt] reagent (provided in the kit) was added into each well, the cells were incubated at 37°C for 30 min. One hundred microliters of the supernatant were transferred to a reading plate of Spectra-MAX 250 spectrophotometer (Molecular Devices, California, USA) and 490 nm absorbance was read. Complete culture media containing the MTS reagent without cells was used as the blank. The results of six repeats of this experiment were averaged.

Measurement of Nitrite Level

Nitrite (NO₂⁻), the stable end product of nitric oxide, was measured in the culture media of cultured cells utilizing the spectrophotometric method based on the Greiss reaction. The Greiss reagent consisted of a freshly made 1:1 solution of 0.1% (w/v) N-(1-Naphthyl) ethylenediamine (Sigma, N-9125, Sigma-Aldrich Chemical, Castle Hill, NSW, Australia) in water and 0.1% (w/v) sulphanilamide (Sigma, S-9251) in 5% (v/v) phosphoric acid. One hundred microliters of the Greiss reagent was added to 100 μl of sample media in a 96-well microtiter plate and the OD absorbance at 550 nm was read. For nitrite quantitation, serial dilutions of NaNO₂ dissolved in DMEM were used to create a standard curve. DMEM was used as a blank. The SOFTmax PRO software (Jandel Scientific, California, USA) calculated the standard curve and interpolated the OD readings to give nitrite values for samples.

Total Protein and Collagen Synthesis Assay of Human Tendon Cells

Human tendon cells (1 × 10⁵/well) were seeded into 6-well culture plates and grown for 24 h to confluence. Next morning, the cells were transfected with adenoviruses for 24 h or treated with SNAP/L-NMMA as described above. Then, the cells were labeled with 10 μCi L-[2,3-³H]-proline (Perkin-Elmer Life Sciences Inc., Boston, MA) containing 50 μg/ml ascorbic acid in 1 ml of the fresh media per well, and incubated 18–20 h. The supernatant of the culture was collected, the monolayer of cells was washed twice with PBS. The cells were scraped in 2 ml of PBS and sonicated briefly on ice. Total protein of the sonicated cells and their supernatant was precipitated with 10% (v/v) TCA (trichloroacetic acid). After centrifugation at 3,000–5,000g for 15 min, the precipitate was washed several times with 5% TCA (v/v) containing 1 mM proline and finally dissolved in 0.2 M NaOH. Following neutralization with 0.15 M HCl and 1 M HEPES, 100 μl of the
neutralized solubilized protein was counted in a liquid scintillation counter (LS 6500 Scintillation System, Beckman Instruments, Inc). These aliquots were used to calculate total protein synthesis (TP). For assessment of absolute collagen synthesis (ACS), 500 µl of the neutralized solubilized protein was incubated with 200 µl collagenase buffer (20 mM Tris-HCl, pH 7.6, containing 50mM CaCl$_2$) and 100 units of collagenase (Worthington Biochemical Corp., Freehold, NJ) at 37°C overnight. Subsequently proteins were precipitated with 10% TCA (v/v) and 3 mM tannic acid, and finally 1 ml of the supernatant was counted in a liquid scintillation counter. Samples treated with H$_2$O instead of collagenase were run in parallel as controls for non-specific breakdown of collagen. Subtraction of counts released in this blank incubation from those obtained from the collagenase digestion yielded collagen-specific incorporation, ACS.

Statistical Analysis

All data are presented as mean ± standard deviation (SD). Differences among experimental groups were assessed using unpaired two-tailed Student’s $t$-tests and one-way analysis of variance (ANOVA). Dose responses of SNAP and Ad-iNOS were analyzed by using analysis of linear regression. The level of statistical significance was accepted at $p < 0.05$.

RESULTS

Cell Viability

The effects of the NO donor, SNAP, and iNOS gene transfection on cell viability of the human tendon were investigated by using the MTS assay. The cells ($1 \times 10^5$) were treated with SNAP for 24 h. Untreated cells served as controls. Results were normalized to untreated cells and expressed as percentage (%) of control. No significant effect on cell viability of human tendon cells was detectable following SNAP stimulation, at 100 µM and 1,000 µM (1 mM) doses (Fig. 1a).

The iNOS gene was transfected into $1 \times 10^5$ of the cells by using 100 pfu/cell adenoviruses containing iNOS gene (Ad-iNOS) or without gene insert (Ad-Empty) for 48 h. Untreated cells served as controls. Results were normalized to untreated cells and expressed as percentage (%) of control. No significant effect on cell viability of human tendon cells was detectable following SNAP stimulation at 100 µM and 1,000 µM (1 mM) doses (Fig. 1a).

Nitrite Production

The NO release into cell culture media by SNAP treatment and iNOS gene transfection were measured by using the Greiss reaction assay.

Increasing doses of SNAP (from 10 to 800 µM) to treat the cells ($1 \times 10^5$) resulted in a dose-dependent increase of nitrite release from the
human tendon cells at 24 h. There was minimal nitrite production by the untreated cells (2.97 ± 0.33 μM, at 24 h). After 24 h incubated with SNAP, the nitrite productions in the media were significantly increased, which was SNAP dose dependent (Fig. 2a). The nitrite level of the culture media after 48 hs stimulated with SNAP was also measured. The results were similar to the results of 24 h stimulation (p > 0.05) (data not shown).

When 1 × 10⁵ of the cells was exposed to different doses (10, 50, and 100 pfu/cell) of Ad-iNOS, the release of nitrite into the culture media was measured to monitor a functional iNOS gene. The cells only group served as control.

Two time points of adenovirus transfection, 24 h and 48 h, were chosen to measure the nitrite production of the culture media. After 24 h transfection with adenovirus, there was minimal nitrite production by the cells only group (3.28 ± 0.27 μM) and by the empty adenovirus-transfected cells group (2.93 ± 0.61 μM), and no significant difference was found between above two groups. Significant increases (p < 0.001) in nitrite production were observed in 50 and 100 pfu/cell of iNOS gene transfected cells groups when compared to the baseline nitrite levels in the cells only group. After 48 h, there were little changes in the cells only group and the empty adenovirus group compared with 24 h transfection. Significant increases of nitrite production were observed in 50 and 100 pfu/cell of iNOS gene transfected cells groups (p < 0.001). Ten pfu/cell of Ad-iNOS had no effects on nitrite release from the cells at both of 24 and 48 h transfection (Fig. 2b). The nitrite productions of the culture media released from iNOS gene transfected human tendon cells at 48 h were Ad-iNOS doses dependent (Fig. 2c).

Total Protein Synthesis

Total ³H-proline incorporated protein synthesis of human tendon cells was measured by ³H-proline incorporation into the human tendon cells as a nonspecific estimate of collagen synthesis.

The cells (1 × 10⁵) were treated with SNAP at 100 μM or 1,000 μM and labeled with 10 μCi L-[2,3-³H]-proline for 24 h, then total protein of the cells was measured by ³H radioactivity counting. SNAP at 100 μM significantly increased total protein synthesis of human tendon cells compared with the untreated cells. However, 1,000 μM of SNAP had no significant effects on total protein synthesis of human tendon cells (Fig. 3a). The cells (1 × 10⁵) were transfected with Ad-iNOS at 10, 50, or 100 pfu/cell concentration for 48 h and labeled with 10 μCi L-[2,3-³H]-proline for 24 h. Total protein of the cells was measured by ³H radioactivity counting. Fifty and 100 pfu/cell of Ad-iNOS increased total protein synthesis of human tendon cells compared with Ad-Empty transfected cells. Ten pfu/cell of Ad-iNOS had no significant effects on total protein synthesis of human tendon cells (Fig. 3b).

Collagen Synthesis

The collagen syntheses in the cells were measured by ³H-proline incorporation into collagenase sensitive fraction of the cells.

Incubation of the cells in the presence of 100 or 400 μM SNAP significantly increased absolutely collagen synthesis by 20% and 15% of the control level compared with the control group (Fig. 4a). Lower concentrations (10 and 50 μM) of SNAP had no effects on collagen synthesis and higher concentrations (800 μM) inhibited cellular collagen synthesis to about 70% of the control levels.

A series of doses (10, 50, or 100 pfu/cell) of Ad-iNOS were tested on collagen synthesis on human tendon cells. Incubation of human tendon cells in the presence of 50 and 100 pfu/cell of Ad-iNOS significantly increased collagen synthesis (ACS) compared to the Ad-Empty group, and increased about 10% and 20% of the control level, respectively. A lower concentration (10 pfu/cell) of Ad-iNOS had no effects on collagen synthesis (Fig. 4b).

Effects of NOS Inhibitor on iNOS Transfected Human Tendon Cells

Cell Viability and Nitrite Production

Effects of the NOS inhibitor, the L-arginine analogue L-NMMA, on cell viability of human tendon cells transfected with adenovirus-iNOS gene were measured by using MTS assay. The cells (1 × (10⁵)) were exposed to 100 pfu/cell of Ad-iNOS with or without L-NMMA (0.5 mM) for 48 h. No inhibition effect of L-NMMA could be found on iNOS gene transfected human tendon cells (Fig. 5a).

An inhibition effect of nitrite production in the iNOS gene transfected tendon cells was achieved by L-NMMA. L-NMMA was added to 10⁵ cells transfected with Ad-iNOS at 50 and 100 pfu/cell. Nitrite released into the culture media was measured at 48 h following transfection in the presence of 0.5 mM concentration of the L-NMMA.
Figure 2. Dose-dependent nitrite production in media of the human tendon cells following SNAP stimulation at 24 h and iNOS gene transfection at 24 and 48 h. (a) Control: tendon cells only without treatment with SNAP. SNAP: $10^5$ tendon cells treated with SNAP in series doses (10, 50, 100, 400, and 800 μM) for 24 h. ***: $p < 0.001$ compared with control group (Mean ± SD, $n = 6$). (b) Cells only: tendon cells cultured without transfecting adenovirus. Ad-Empty: tendon cells transfected with 100 pfu/cell adenovirus without gene insert. Ad-iNOS: tendon cells transfected with 10, 50, or 100 pfu/cell adenovirus containing iNOS gene insert. The time points of transfection are shown in the figure. ***: $p < 0.001$ compared with cells only and Ad-Empty groups for each time point; +++: $p < 0.001$ compared to 24 h time point (Mean ± SD, $n = 6$), by using the statistical methods of unpaired two-tailed Student’s $t$-tests. (c) Nitrite productions of Ad-iNOS dose response on human tendon cells. Cells only group served as 0 pfu/cell of Ad-iNOS dose. Other groups were transfected with different Ad-iNOS doses (10, 50, and 100 pfu/cell). ***: $p < 0.001$ compared with 0 pfu/cell of Ad-iNOS dose group (Mean ± SD, $n = 6$), by using the statistical methods of one-way analysis of variance (ANOVA). At 48 h culture, the nitrite productions of the culture media released from the tendon cells were Ad-iNOS doses dependent, by using the analysis of linear regression, $R^2$ (the Regression coefficient) = 0.983 ($p < 0.01$).
The amounts of nitrite produced by the iNOS gene-transfected cells were significantly decreased \((p < 0.001)\) by adding 0.5 mM of L-NMMA into both of 50 pfu/cell and 100 pfu/cell Ad-iNOS groups (Fig. 5b). The reductions from 50 and 100 pfu/cell Ad-iNOS with L-NMMA groups were about 35% and 50%, respectively, compared with iNOS gene-transfected (50 or 100 pfu/cell) cells without L-NMMA groups.

**Total Protein and Collagen Synthesis**

The effects of L-NMMA on total protein and collagen synthesis of iNOS gene transfected human tendon cells were investigated by the methods of \(^3\text{H}\)-proline incorporation into the cells and incorporation into the collagenase sensitive fraction of the cells. A significant inhibitory effect of L-NMMA was found on total protein synthesis of iNOS gene transfected human tendon cells \((p < 0.001)\). The reduction of total protein synthesis in the L-NMMA group was about 30% when compared with the without L-NMMA group (Fig. 6a).

There was a significant inhibitory effect of L-NMMA on collagen synthesis in 50 and 100 pfu/cell of Ad-iNOS groups \((p < 0.05\) and \(p < 0.01\)), the reductions of collagen synthesis in L-NMMA groups was about 9% and 16% of without L-NMMA group (Fig. 6b).

**DISCUSSION**

Total protein synthesis of human tendon cells was measured by \(^3\text{H}\)-proline incorporation into the human tendon cells as a nonspecific estimate of collagen synthesis. In the presence of collagenase, the experiments were repeated for a more specific index of collagen synthesis, i.e., assessing the collagenase sensitive \(^3\text{H}\)-proline incorporation into the cells. The NO donor S-nitroso-N-acetylpenicillamine (SNAP) was used initially. Our results showed that total \(^3\text{H}\)-proline protein synthesis and collagen synthesis of the cells from injured human tendon tissue were increased in the presence of 100–400 \(\mu\text{M}\) SNAP. High doses of SNAP \((\geq 800 \mu\text{M})\), however, had no effect on total \(^3\text{H}\)-proline protein synthesis and even inhibited collagen synthesis of the human tendon cells. Furthermore, total \(^3\text{H}\)-proline protein and collagen synthesis of the human tendon cells could also be enhanced by active iNOS via adenoviruses containing iNOS gene (Ad-iNOS) compared with the adenovirus without gene insert (Ad-Empty) group. The specific effect of NO on collagen synthesis was confirmed when the NOS inhibitor, L-NMMA, inhibited the effects of iNOS on the cells.

Our studies revealed that both exogenous NO (SNAP) and active iNOS could enhance total \(^3\text{H}\)-proline protein and collagen synthesis in
Figure 3. Effects of SNAP treatment and iNOS gene transfection on total protein synthesis in human tendon cells via $^3$H-proline incorporation into the cells. (a) Control: the human tendon cells without SNAP treatment. SNAP: the human tendon cells treated with SNAP at 100 $\mu$M or 1,000 $\mu$M concentration. **: $p < 0.01$ (Mean ± SD, $n = 6$). (b) Control: cells cultured without transfecting adenovirus. Ad-Empty: tendon cells transfected with 100 pfu/cell adenoviruses without gene insert. Ad-iNOS: tendon cells transfected with 10, 50, or 100 pfu/cell adenoviruses containing iNOS gene insert. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ compared with Ad-Empty group (Mean ± SD, $n = 6$), by using the statistical methods of one-way analysis of variance (ANOVA).
Figure 4. The effects of SNAP and iNOS gene transfection on collagen synthesis in human tendon cells measured by [\(^{3}\text{H}\)] proline incorporation into collagenase sensitive fraction of the cells. (a) Control: untreated human tendon cells. SNAP: the human tendon cells treated with different doses of SNAP, 10, 50, 100, 400, and 800 µM. *: \(p < 0.05\), **: \(p < 0.01\), ***: \(p < 0.001\) compared with control group (Mean ± SD, \(n = 6\)). (b) Ad-Empty: human tendon cells transfected with adenovirus 100 pfu/cell without gene insert. Ad-iNOS: human tendon cells transfected with adenovirus at 10, 50, or 100 pfu/cell concentration with iNOS gene insert. *: \(p < 0.05\), **: \(p < 0.01\) compared to Ad-Empty group (Mean ± SD, \(n = 6\)).
human tendon cells and inhibition of NOS inhibited the effects of iNOS on the cells. To our knowledge, this is the first report about the relationship between NO and collagen synthesis in tendon cells. Our results were consistent with the findings of Witte et al., who showed that low concentrations of the NO donor SNAP enhanced collagen protein synthesis in rat dermal fibroblasts. Shi et al. showed that the fibroblasts from iNOS KO mice proliferated more slowly, synthesized less collagen, and contracted fibroblast-populated collagen lattices more slowly than wild-type fibroblasts. Collagen synthesis was restored to normal in KO fibroblasts in response to NO donors (SNAP). Similarly, increases in collagen content in experimental

Figure 5. The effect of L-NMMA on cell viability and nitrite production of iNOS gene transfected human tendon cells. (a) Ad-iNOS: human tendon cells transfected with adenovirus 100 pfu/cell with iNOS gene insert. Ad-iNOS + L-NMMA: human tendon cells transfected with adenovirus 100 pfu/cell with iNOS gene insert and treated by 0.5 mM L-NMMA. Values of the figure present as mean ± SD, n = 6 for each group. (b) Ad-iNOS 50 pfu/cell: tendon cells transfected with 50 pfu/cell adenovirus containing iNOS gene insert with or without 0.5 mM of L-NMMA. Ad-iNOS 100 pfu/cell: tendon cells transfected with 100 pfu/cell adenovirus containing iNOS gene insert with or without 0.5 mM of L-NMMA. ***: p < 0.001 (Mean ± SD, n = 6).
wounds was found when the wounds were treated with other NO donors, active iNOS via gene therapy or dietary L-arginine, whereas inhibition of NO synthesis decreased collagen content.

Some conflicting results also exist. Shukla et al. found that inhibition of NOS increased wound collagen content significantly as compared with untreated and SNP (sodium nitroprusside, a NO donor) treated rat transdermal wounds.

![Figure 6](image_url)

**Figure 6.** Inhibition effect of L-NMMA on total protein and collagen synthesis in iNOS gene-transfected human tendon cells. (a) Ad-iNOS 100 pfu/cell: tendon cells transfected with 100 pfu/cell adenovirus containing iNOS gene insert. Ad-iNOS + L-NMMA: tendon cells transfected with 100 pfu/cell adenovirus containing iNOS gene insert with 0.5 mM of L-NMMA. ***: p < 0.001 (Mean ± SD, n = 6). (b) The effects of L-NMMA on collagen synthesis in iNOS gene transfected human tendon cells. Ad-iNOS 50 pfu/cell: human tendon cells transfected with adenovirus 50 pfu/cell containing iNOS gene insert with or without 0.5 mM of L-NMMA. Ad-iNOS 100 pfu/cell: human tendon cells transfected with adenovirus 100 pfu/cell containing iNOS gene insert with or without 0.5 mM of L-NMMA. *: p < 0.05; **: p < 0.01 (Mean ± SD, n = 6).
The mechanisms of how NO affects collagen synthesis still remain unclear. One explanation may be related to transforming growth factor-beta 1 (TGF-β1). TGF-β1 is often associated with stimulation of collagen production and deposition during wound healing.\(^{29,30}\) Vodovotz et al.\(^{31}\) showed that NO can lead to the activation of latent TGF-β1. They found that activated macrophages express iNOS and NADPH oxidase. iNOS produces NO, which in turn can react to form reactive nitrogen species (NO\(_X\)). These NO\(_X\) then bring about a series of reactions in the target cell, which result in the activation of latent TGF-β1. Both in vitro and in vivo, several groups have demonstrated that TGF-β1 can increase production and secretion of collagen and stimulate the expression of extracellular matrix proteins.\(^{32,33}\) Besides TGF-β1, other cytokines, such as basic fibroblast growth factor (bFGF), nuclear transcription factor kappaB (NF-κB), and insulin-like growth factor (IGF) can be induced by NO in wound healing and these cytokines may also play an important role in collagen synthesis.\(^{34–38}\)

In our study, we found dual effects of nitric oxide on tendon cells. High doses of exogenous NO (SNAP) (> or = 800 μM) inhibited the collagen synthesis in cultured human tendon cells, but lower doses of SNAP enhanced \(^3\)H-proline and collagenase sensitive \(^3\)H-proline incorporation into the cells. Our results are consistent with other reports, in the sense that low concentrations of this mediator play homeostatic roles, whereas when NO is overexpressed, it may have damaging effects. It is likely that the timing and level of NO production in the healing wound must be carefully balanced to ensure a beneficial effect.\(^{39–42}\)

This work also demonstrated that human tendon cells have the capacity to synthesize physiologically active NO following transfection of iNOS gene via adenovirus.

For enhancing collagen synthesis, interestingly, the nitrite level (5.0 ± 0.4 μM) from iNOS gene transfection was lower than that (37 ± 5 μM) from SNAP stimulation. One explanation may be: SNAP is an exogenous NO donor; NO is a free radical gas and has a very short half-life of only a few seconds because it readily reacts with other atoms or molecules, whereas as an enzyme, iNOS can consistently act on its substrate in human tendon cells to synthesize a sustained level of NO.\(^{2,43}\)

There are several limitations to consider in this study. Firstly we used the collagenase sensitive \(^3\)H-proline incorporation into the cells as the index of collagen synthesis of the cells. We did not evaluate specific collagen (e.g., type I collagen) protein expression in the cultured tendon cells. Secondly, we investigated NO effects on collagen synthesis in the cells from injured tendon tissue, namely, the wounded tendon cells (WTCs), but did not compare WTCs with normal tendon cells (NTCs). There may be some different characteristics between WTCs and NTCs. Schaffer et al.\(^{10}\) confirmed that wounded fibroblasts are phenotypically altered during the healing process to synthesize NO, which, in turn, regulates their collagen synthetic and contractile activities.

We have performed a series of randomized and double-blinded clinical trials and found that application of topical nitric oxide improved outcomes of patients with extensor tendinosis, chronic Achilles tendinopathy, and chronic supraspinatus tendinosis.\(^{16–18}\) In the clinical trial of extensor tendinosis study, 86 patients with extensor tendinosis were randomized into two equal groups; one group received an active glyceryl trinitrate transdermal patch, and the other group received a placebo patch. The patients in the glyceryl trinitrate group had significantly reduced elbow pain with activity at 2 weeks, reduced epicondylar tenderness at 6 and 12 weeks, and an increase in wrist extensor mean peak force and total work at 24 weeks. At 6 months, 81% of treated patients were asymptomatic during activities of daily living, compared with 60% of patients who had tendon rehabilitation alone. Similar improvements were noted in the glyceryl trinitrate group in the Achilles tendinosis trial,\(^{16}\) and even greater improvements in the supraspinatus tendinosis trial.\(^{18}\)

In summary, our studies show for first time, to our knowledge, that nitric oxide can enhance collagen synthesis in human tendon cells in vitro. These results may explain, in part, the beneficial effects of NO donors in tendonopathies in randomized clinical trials.

ACKNOWLEDGMENTS

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REFERENCES

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The Orthopaedic Research Institute-Ankle Strength Testing System: Inter-rater and Intra-rater Reliability Testing

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ABSTRACT

Achilles tendinosis is a degenerative overuse tendinopathy involving the primary ankle plantarflexors, namely the soleus and gastrocnemius muscles forming the tendon Achilles. The Orthopaedic Research Institute-Ankle Strength Testing System (ORI-ASTS) was designed to record objective measurements of force generated with a resisted ankle plantarflexion test. Testing normal subjects (n=6) was used to establish the reliability of the ORI-ASTS for measuring ankle plantarflexion force. Testing patients with Achilles tendinosis (n=5) over time and comparing Achilles tendon analogue pain scores to ankle plantarflexion force measurements was used to assess the validity of the ORI-ASTS for monitoring progression of Achilles tendinopathy. Inter-rater reliability of the ORI-ASTS with normal subjects was excellent, with single measure intraclass correlation coefficients (ICC) for right mean peak force of 0.92, left mean peak force of 0.96, right mean total force of 0.89, left mean total force of 0.91. The ORI-ASTS also had excellent intra-rater reliability for normal subjects with the following single measure ICC scores: right mean force 0.96, left mean force 0.92, right mean total force 0.97, left mean total force 0.92. The relative technical errors of measurement were calculated from these results, and ranged from 1.0% to 2.7%.

Testing patients with Achilles tendinosis (n=5), and comparing analogue activity pain scores to ORI-ASTS testing demonstrated a strong negative relationship between the two parameters (Spearman Rho -0.87 to -1.0, Kendall tau b -0.82 to -1.0). The relationship was statistically significant at the p=0.01 level for two of the five patients.

The ORI-ASTS shows excellent reliability for testing ankle plantarflexor force, and appears valid for objectively assessing and monitoring patients with Achilles tendinosis.

INTRODUCTION

The Achilles tendon is the largest, strongest tendon in the body and is able to accept weight-bearing forces of up to 7000 N. It is formed in the calf by the combination of the gastrocnemius and soleus muscles, which are the primary ankle plantarflexors. We have developed a resisted ankle plantarflexion testing system that this research aims to show provides reliable, objective measurements of plantarflexion peak force and total force, is a valid measure for monitoring symptomatic patients with Achilles tendinosis, and has low associated error.

The Achilles tendon, formed from the primary ankle plantarflexor muscles, can be placed under longitudinal strain when the soleus and gastrocnemius muscles contract concentrically against resistance during ankle plantarflexion. In diseased Achilles tendons and their musculature, pain, fatigue, and weakness can be produced by repetitive resisted ankle plantarflexion. This serves as a test of functional competence of the Achilles tendon. Thus, weakness in ankle plantarflexion is used in the clinical assessment of Achilles tendinosis, and may occur as a primary manifestation of Achilles tendon dysfunction, atrophy, or inhibition, or as a secondary consequence of pain. An objective measurement of ankle plantarflexion force may illustrate this reduction in strength, and its relationship to patient symptomatology.

Ankle plantarflexion strength tested clinically in the assessment of Achilles tendon disease is, however, by its nature a subjective test. To our knowledge there is no reliable, objective clinical measure to assess the severity or the progress of Achilles tendinosis. We have attempted to address this through the development of the Orthopaedic Research Institute-Ankle Strength Testing System (ORI-ASTS).
MATERIALS AND METHODS

The purpose of the initial stage of testing was to examine the reliability of the ORI-ASTS. In order to test the inter-rater reliability of this system, four examiners were chosen from the staff of the orthopaedics department of St. George hospital and six normal subjects were recruited from the laboratory staff at the Orthopaedic Research Institute (ORI). The examiners consisted of two orthopaedic surgeons, one sports physician registrar, and one biomechanical engineer.

The normal subjects were aged 23 to 42 years, with a median age of 30 and a mean age of 32. There were three female and three male subjects, all were right footed, and none had previous ankle pathology. The testing was conducted over a four week period and was completed on four consecutive Wednesdays.

In order to also assess the intra-rater reliability of the ORI-ASTS, four normal subjects were chosen randomly and were tested by a single examiner once each fortnight for a total of six weeks. The subjects were aged 20 to 40 years, with a median age of 24 and a mean age of 27. There were two female and two male subjects, all were right footed, and none had previous lower limb pathology.

The aim of the second stage of testing was to determine the validity of the testing apparatus for use in patients with noninsertional Achilles tendinosis. Five patients were recruited with a clinical diagnosis of noninsertional Achilles tendinosis. This diagnosis was made on the basis of thickening and tenderness in the Achilles tendon in the region 2 to 6 cm from the tendon insertion, with pain localized to this region on performing repeated standing heel-raises. Ultrasound testing was used to exclude concomitant soft tissue pathology in the retro-Achilles region. These patients were aged 30 through to 60 years, with a median age of 47 years and a mean age of 48 years. There were three female and two male patients, and the affected side was on the left in three patients and on the right in two patients. The subjects were tested with the ORI-ASTS every two weeks for a total of six weeks (Fig. 1), and were asked prior to testing to complete an analogue pain scale to determine the level of Achilles tendon pain experienced with activities.

Prior to testing, ethics approval was obtained from the ethics committee at St. George Hospital. The testing protocol was identical for all testing with the ORI-ASTS, and involved seating the subjects in a chair with the foot securely fastened to the footplate by adjustable straps. The subject's chair was then moved in order to have the knee in 90° of flexion, and the footplate placed in a neutral position (0° of rotation) horizontal to the floor, prior to testing. Apart from reinforcing the testing protocol, no prior training in the use of the ORI-ASTS was provided to any of the examiners.

Each "run" comprised of an effort to forcefully depress the
The axis of rotation was designed to be at the axis of the ankle plantarflexor musculature. It consists of a resisted footplate, against which the foot is secured using adjustable straps, able to rotate about the axis of the ankle into both plantarflexion and dorsiflexion (Fig. 2). The rotation powers a driven belt coupled to a pneumatic piston with a fixed resistance, and is linked in series to a 2000N load cell (RS components, Sydney, Australia). The load cell is connected to a transducer, and rotational force data is recorded and stored directly onto computer hard-drive using National Instruments LabView 5.1 system technology. The data collected consists of readouts of rotational force generated related to time (Table 1).

The axis of rotation was designed to be at the axis of the ankle. In order to establish where to locate the axis, 10 consecutive patients were chosen who were required to have plain weight-bearing X-rays in order to differentiate between the enthesitis of insertional Achilles tendinosis and Haglund’s deformity. The weight-bearing lateral ankle X-rays were taken with the patient standing on a 2-cm thick wooden platform, and the X-ray beam was centered at the midfoot in order to reduce beam refraction.

The films were then examined and the distance from the center of the mortis to the posterior skin surface, and to the inferior skin surface, were measured and recorded. The age of the four male and six female patients ranged from 33 to 75 years (mean 52 years), and there were five left and five right lateral ankle X-rays. The mean supero-inferior distance from mortis to skin was 8.06 cm (range, 7.6 to 8.7 cm), and the mean antero-posterior distance from mortis to skin was 5.5 cm (range, 4.8 to 6.1 cm). As a result of these measurements, the footplate of the ORI-ASTS was constructed with the axis of rotation 5.5 cm from the heelrest, and 8 cm from the base of the footplate.

Once all testing was completed, the Microsoft Excel data files were analyzed. They consisted of force data (in standardized rotational units) related to time (in 0.05 second increments). The average force per unit time for each incremental time period was calculated using the following equation ((F1 + F2)/2)(T2 - T1), where F represents force and T represents time. The total force, in rotational units (U), per 20 second run was calculated by summation of all the individual force per unit time figures. Thus for each subject there was a record of peak force and total force for each run. The right and left mean peak force and mean total force were then calculated by an average of the two runs.

Inter-rater reliability was analyzed to determine the Intraclass Correlation Coefficient (ICC) for measured parameters using a (2,1) model involving two-way randomization and absolute agreement. Intra-rater reliability was also analyzed to determine the ICC (3,1) for measured parameters using a two-way mixed model with absolute agreement using the Microsoft Windows SPSS 10.1 program (Table 1).

The inter-rater data was used to determine the absolute technical error of measurement (TEM) by taking the sum of the squared differences of the individual measurements, dividing by twice the number of subjects and then taking the square root of the product. The relative technical error of measurement (% TEM) was then calculated using the TEM, dividing it by the average mean of the two sets of measurements and multiplying by 100.

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### Table 1: Summarized results of ORI-ASTS intra-rater reliability testing including intraclass correlation coefficients (ICC), and showing 95% confidence intervals. The full scores for the absolute technical error of measurement (TEM, in the units of the test) and relative technical error of measurement (% TEM) are also included. Note that 1 U is equivalent to 17.9 Newtons per meter.

<table>
<thead>
<tr>
<th>Measured Parameter</th>
<th>Single measure ICC</th>
<th>95% Confidence Intervals</th>
<th>TEM</th>
<th>%TEM</th>
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<tr>
<td>Right peak force</td>
<td>0.89</td>
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<td>Right total force</td>
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<td>Left mean total force</td>
<td>0.97</td>
<td>0.65 - 0.99</td>
<td>0.21 U/10s</td>
<td>1.38</td>
</tr>
</tbody>
</table>
RESULTS

The results of inter-rater reliability testing using the ORI-ASTS showed mean measure intraclass correlation coefficients for right mean peak force of 0.92, left mean peak force of 0.96, right mean total force of 0.89, and left mean total force of 0.91.

Intra-rater reliability testing using the ORI-ASTS showed even higher ICC scores on the right side as outlined in Table 1, with mean measure intraclass correlation coefficients for right mean peak force of 0.97, left mean peak force of 0.92, right mean total force of 0.97, and left mean total force of 0.92. The intra-rater testing indicates that greater reliability can be obtained from a single examiner testing multiple subjects using the ORI-ASTS than when multiple different examiners test the same subject group.

The relative technical error of the measurement for the ORI-ASTS with mean measures ranged from 1.0% to 2.7%. These results show that the technical error of the ORI-ASTS is very low.

In patients with Achilles tendinosis there was an excellent negative correlation (inverse relationship) between pain with activity and mean peak force obtained through ankle testing with the ORI-ASTS (Spearman's rho -0.87 to -1.0, and Kendall's tau -0.82 to -1.0). The negative values indicate that the higher patients rated their Achilles tendon pain, the lower they scored on measurement of their ankle plantarflexion force. A similarly strong correlation between Achilles tendon pain with activity and mean total force was also demonstrated (Spearman's rho -0.87 to -1.0, and Kendall's tau -0.82 to -1.0). The results for individual patients are summarized in Table 2.

DISCUSSION

Testing normal subjects with the ORI-ASTS demonstrated excellent inter-rater and intra-rater reliability, and
also showed the total technical error of measurement of the ORI-ASTS to be less than 3\% for mean force measurements.

The ICC is a reliability coefficient used for interval data and is both a bona fide correlation coefficient,\(^1\) and a theoretically sound reliability coefficient.\(^6\) Unfortunately, there are no universally accepted standards for what constitutes poor, good or excellent reliability. One general criteria defines ICC results as: <0.4 (poor), >0.4 to 0.75 (fair to good), and >0.75 (excellent).\(^8\)

The inter-rater and intra-rater reliability scores (ICC) for all measured, and calculated, parameters were of a very high order and indicate that this testing system can be used with great reliability by a single examiner or multiple different examiners. There did not appear to be a significant learning effect as there is no evidence of consistently increasing scores with any measured parameter. This may be because the test is relatively simple, or because subjects were allowed to practice the test prior to the first run. The learning effect may have occurred prior to the commencement of testing.

There is, however, some evidence for a training effect with the inter-rater testing, as the highest individual score measured for all parameters occurs with examiner four in greater than 40\% of cases. The examiners performed their measurements in numerical order, with examiner four testing the subjects last on the penultimate day. The scores, as mentioned previously, did not increase consistently with time, but the fact that the highest individual score was registered on the final day in greater than 40\% of cases seems to indicate that muscular strength had increased in the ankle plantarflexors.

The absolute and relative technical errors of measurement (TEM and %TEM) are indicators of the precision (or rather imprecision) associated with the measure in question,\(^6\) in other words, the error of the method due to both biological and technical factors. The TEM is described in the same units as the variable measured, while the relative TEM is a described as a percentage error.\(^6\) The measures of mean peak force and mean total force obtained by a single examiner showed relative TEMs of between 1.0\% and 2.7\%. These results indicate that the ORI-ASTS has relatively low total error associated with its measurements.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Pain versus mean peak force</th>
<th>Pain versus mean total force</th>
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</thead>
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<td></td>
<td>Spearman’s rho</td>
<td>Kendall’s tau</td>
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<td>-0.82</td>
</tr>
<tr>
<td>2</td>
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<td>-0.82</td>
</tr>
<tr>
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<td>-1.0*</td>
<td>-1.0</td>
</tr>
<tr>
<td>4</td>
<td>-0.82</td>
<td>-0.87</td>
</tr>
<tr>
<td>5</td>
<td>-1.0*</td>
<td>-1.0</td>
</tr>
</tbody>
</table>

*significant at the 0.01 level

Testing conducted on patients with Achilles tendinosis demonstrated that there was a negative correlation between the level of pain experienced when performing daily activities and the amount of peak force, and total force, that could be produced by the ankle plantarflexors using the ORI-ASTS. A negative relationship implies that individuals scoring low on one variable (e.g. pain) tend to score high on a second variable (e.g. mean peak force, or mean total force). The Spearman r correlation coefficient was used to determine this relationship because the analogue pain scale data was ordinal in nature, while the data from the ORI-ASTS was interval data. Spearman r can be thought of as the regular Pearson product moment correlation coefficient in terms of variability accounted for, except that Spearman r is computed from ranks.\(^7\) The Kendall tau b correlation coefficient is similar to Spearman r in regards to underlying assumptions and statistical power, but they imply different interpretations. Spearman r is a measure that expresses the extent to which two variables are related, while Kendall tau represents a probability. That is, it is the difference between the probability that in the observed data the two variables are in the same order versus the probability that the two variables are in different orders.\(^6\)

All five patients demonstrated a strong negative relationship between their Achilles tendon pain with activity, and the ability to produce plantarflexion force when tested with the ORI-ASTS. This is shown by both the Spearman r and Kendall tau b correlation coefficients approaching -1 for all patients. The results for two of the five patients were significant at the 0.01 level (2 tailed) for both the negative relationships of pain versus mean peak force, and pain versus mean total force. As pain with activity is one of the most prominent symptoms in Achilles tendinopathy, it is interesting to note the strong correlation between the patient’s own rating of this symptom and the amount of ankle plantarflexion force.
able to be produced on ORI-ASTS testing. All patients were able to complete the testing. The footplate apparatus is heavily resisted, and over the course of two "runs" (40 seconds total duration) the ankle plantarflexor musculature, potentially atrophied from disuse, might fatigue. Muscle inhibition secondary to pain or pathological changes may also contribute.

There are, of course, accessory plantarflexor muscles such as the tibialis posterior and peroneal longus muscles that may be recruited together to attempt to compensate for any weakness in the Achilles tendon and its musculature. Despite this the correlation between increasing pain and decreasing plantarflexion force persists, demonstrating the importance of the strong primary ankle plantarflexors gastrocnemius and soleus, and the inability of accessory plantarflexors to compensate for Achilles tendon dysfunction.

We have demonstrated that the ORI-ASTS has excellent reliability as a measuring apparatus when used to test ankle plantarflexion force by single or multiple examiners. We have also separately demonstrated that in patients with non-insertional Achilles tendinosis the mean plantarflexion force measurements are inversely related to Achilles tendon pain with activity. Thus the ORI-ASTS could be used to objectively monitor patients with Achilles tendinosis through measurements of force generated by the ankle plantarflexors with this resisted footplate test.

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REFERENCES

Spontaneous recovery of injured Achilles tendon in inducible nitric oxide synthase gene knockout mice

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Abstract. Objective and Design: To determine if inducible nitric oxide synthase (iNOS) gene could affect Achilles tendon healing using iNOS gene knockout mice.

Methods: 21 iNOS knockout (iNOS⁻⁻) mice and 8 of the wild type (iNOS⁺⁺) mice were utilized in this study. Group 1: iNOS⁻⁻ (n = 8), group 2: iNOS⁻⁻ mice (n = 11) and group 3: iNOS⁻⁻ with a NOS inhibitor, (aminoguanidine, 500 mg/kg/day, via an intraperitoneal mini-osmotic pump for 7 days, n = 10). The right Achilles tendons were transected in all mice and harvested on day 7 for cross-sectional area and biomechanical properties. Serum nitrate concentration of the mice was measured by gas chromatography mass spectrometry (GC/MS).

Results: A significant reduction in cross-sectional area of the healing Achilles tendon was observed in group 3 mice compared to group 2 mice (p < 0.01). The serum nitrate concentration in both group 2 and group 3 mice was lower than that in group 1 mice (p < 0.01) iNOS gene deletion and inhibition of NOS did not affect the biomechanical properties of the healing tendons.

Conclusions: iNOS gene is not solely responsible for the beneficial effects of nitric oxide (NO) on tendon healing.

Key words: Nitric oxide synthase - tendon healing - knockout mice - NOS inhibition

Introduction

Tendon healing is a well-ordered and coordinated process involving inflammation, cell proliferation, angiogenesis, matrix deposition, and tissue remodeling [1, 2]. These processes are driven by a complex mixture of growth factors, which are released coordinately into the area of injury. Besides these protein factors and mitogens, evidence is emerging for an important role of small diffusible molecules in wound repair. One of them is nitric oxide (NO), a free radical gas.

NO is produced in higher organisms by the oxidation of one of the terminal guanidinotriazine atoms of L-arginine. This process is catalyzed by the enzyme nitric oxide synthase (NOS). The enzyme NOS exists in three isoforms, neuronal NOS (nNOS, type I), inducible NOS (iNOS, type II), and endothelial NOS (eNOS, type III). The isoforms nNOS and eNOS are constitutively expressed, and their activity is regulated by intracellular calcium concentration. The isoform iNOS is located at chromosome 17 (17cen-q11.2), contains 4.1 kb mRNA encoded a 131 kDa size protein [3–5]. iNOS is inducibly expressed in macrophages and other cells after stimulation by cytokines, lipopolysaccharides, and other immunologically relevant agents. Expression of iNOS is regulated at the transcriptional and posttranscriptional level by signaling pathways that involve agents such as the redox-responsive transcription factor NF-kB or mitogen-activated protein kinases (MAPKs) [6–8].

NO is involved in many physiological and pathological processes, including regulation of blood flow and pressure via endothelium-dependent relaxation of blood vessels, neuronal transmission with activation of guanylylclelase pathways in target neurons, host defense immune response via cytotoxic, antimicrobial and tumoricidal activity, and inflammation [9–13]. There is a growing body of evidence that suggests that NO is important in the healing of soft tissues. For example, NO inhibitors delay the healing of excisional skin wounds while provision of NO via donors accelerates skin wound healing [14, 15]. Mice deficient in iNOS exhibit impaired skin wound healing that is reversible by iNOS gene
Material and methods

Materials

Wizard Genomic DNA Purification Kit (Cat. #A1120) was purchased from the Promega Corporation (Madison, Wisconsin, USA). GeneAmp PCR System 2400 is the product of PERKIN ELMER (Norwalk, CT, 06850 USA). Aminoguanidine (hydrochloride) (Cat. No. 81530) was purchased from Ciytax Chemical (Anto Arbor, MI, 48108, USA).

Mini-osmotic pumps were from ALZET Technical Information Services (DURECT Corporation Cupertino, CA, USA). '*'-labeled potassium nitrate (98% Cambridge Isotope Laboratories, Inc.) was obtained from Novochem Pty Ltd (South Yarra, VIC 3141, Australia), o, m, and p-nitrotoluenes and trifluoroacetic anhydride were obtained from Sigma-Aldrich Pty Ltd. (Castle Hill, NSW 2154, Australia). Hewlett-Packard 5989B mass spectrometer/ Hewlett-Packard 5971A MSD were the products of Hewlett-Packard, Meriden (CT, USA). Reagent grade organic solvents were obtained from commercial suppliers and were used without treatment. Acids, bases, and other chemicals were analytical grade and were obtained from commercial suppliers.

Animals

iNOS gene knockout (iNos-/-) mice and their wild-type (iNos+/+) (C57BL/6) mice were the gifts from Dr Gunasegaran Karupiah, Sydney University of Australia, which was authorized to breed by Cornell University of Australia, which was authorized to breed by Cornell University of USA. The original developers of iNos-/- and iNos+/+ mice embryos. iNos-/- mice were generated as described [25], the gene replacement vector, pNOS-RV, was designed to delete the proximal 585 bases of the iNos promoter, a region required for iNos expression in macrophages, plus exons 1-4, including the ATG translation start site in exon 2. iNos+/- and iNos++/+ littermates (129/SvEv X C57Bl6/J F1) were bred at the specific pathogen-free unit, Biological Resources Centre, University of New South Wales, Australia. Experiments were performed according to each institution’s guidelines for animal use and care.

Wild-type alleles (413-bp amplicands); 5’ primer (5’-GAGCAATGTGACAAAGGCTCTCAGTAG-3’), 3’ primer (5’-GGTGAAGCAGTACGCTCTC-3’).

iNOS PCR Genotyping

iNOS genotype was confirmed by PCR as previously outlined [26]. Briefly, mice tail biopsy DNA was prepared as described by Laird et al [27] using Wizard Genomic DNA Purification Kit (Promega). The 400-600 ng of genomic DNA was analyzed by PCR in a 50-μl reaction volume containing 10𝑛M Tris-HCl (pH 8.3), 1.5 𝑚Μ MgCl2, 200 𝑛M dNTPs, and 2.5 μl of Taq polymerase (Sigma). Primer pairs (200 ng each) were as follows: 5’ primer (5’-ATACGGCTTCTCTGTTCCC-3’), 3’ primer (5’-GCGTTCTCTGTTCCCTC-3’).

Animal model and experimental groups

Twenty-one 8–12 weeks old male iNos-/- mice and eight 8–12 weeks old iNos+/- mice were utilized for this study. Group 1: iNos-/- mice (𝑛=8); group 2: iNos+/- mice (𝑛=11) and group 3: iNos-/- mice treated with a systemic NOS inhibitor, amino-guanidine (AG, 500 mg/kg/day, via a subcutaneous mini-osmotic pump for 7 days (𝑛=10).

All procedures and protocols were approved by the Animal Care and Ethics Committee of the University of New South Wales, Sydney, Australia. The mice were housed at the specific pathogen-free unit, Biological Resources Centre, University of New South Wales, Australia. Anesthesia was achieved by intraperitoneal injection of Nembutal (10 times dilution in 0.9% saline, 40 mg/kg). Surgical division of the right Achilles tendon was performed as previously outlined on rats with some modification [28, 29]. Briefly, a 0.5 cm midline incision was made over the right Achilles tendon, and the Achilles tendon and plantaris were isolated from the surrounding fascia. The Achilles tendon was transected with a scalpel in an axial fashion 0.2–0.5 cm from its calcaneal insertion. The tendinous portion of plantaris was removed to prevent any possible action as an internal split. The skin was then sutured with one or two simple nylon suture of 5-0 ethion monofilament nylon on a PC-1 cutting needle. No operation was performed on the left uninjured hind limb. No cast or dressings were applied and the animals were unrestricted during the healing phase. For group 3, the animals were treated by continuous infusion of AG 500 mg/kg body weight/day using intraperitoneally implanted mini-osmotic pumps (Alzet Osmotic Pumps, Model 2001, DURECT Corporation, Cupertino, CA USA). The laparotomy wounds were closed in two layers with running 4-0 nylon sutures. After operation, daily clinical observation was made. Observation included an evaluation of the behaviour (eating, drinking and moving) of the mice; any sign of infection at the operation site and pain that may need postoperative analgesia.

Cross-sectional area of tendon measurement

A constant gap was created between two glass slides using standard shims placed at each end (0.27 mm for uninjured tendon, 0.53 mm for injured tendon). The tendon was placed between the two slides causing the tissue to flatten. The constrained tendon created a shadow when viewed through a transmission microscope and this was used to determine the tendon width. The cross-sectional area was calculated by multiplying the shim thickness by the tendon width.

Biomechanical testing

Biomechanical assessment was carried out on an in-house mini tensile testing system. The muscle and intramuscular tendinous fibers were compressed between one set of serrated aluminium grips that were attached to the lead screws of the testing system. A block of dry ice was placed against the side of the grips to freeze the compressed tissue. The calcaneus was fixed to a second pairs of grips (set 7.5 mm from the first grips), which were attached to a 50 N load cell. The specimens were...
kept moist throughout the entire testing procedure by constant irrigation with 0.9% saline. Each specimen underwent a constant velocity ramp to failure (2.5 mm/sec). Voltage-time and displacement-time histories for each test were collected using a dedicated computer and software for subsequent data analysis.

Nitrate level of serum measurement via Gas Chromatography-Mass Spectrometry (GC/MS) methods

Nitrate level of serum was measured by gas chromatography-mass spectrometry (GC/MS) following preparation of samples by nitration of toluene as described [30, 31]. Nitration of toluene was done by using trifluoroacetic anhydride (TFAA) as a catalyst. An aliquot of an aqueous solution of [15N] potassium nitrate internal standard (200 µM, 50 µl) was mixed in a screw-top glass vial (13 × 100 mm) with either serum samples (50 µl) or calibrated dilutions of native sodium nitrate for the standard curve. The mixture was then dried under a stream of nitrogen (or SpeedVac rotary evaporator, Savant Instruments, Sayth Scientific & Medical, Clayton VIC Australia 3168). TFAA (200 µl) and toluene (1 ml) were added to the residue and the tubes were capped and heated at 70°C for 60 min. After cooling to room temperature, the toluene solution was washed sequentially with water (1 ml), aqueous sodium bicarbonate (1%, 1 ml), and water (1 ml). The toluene solution was separated, dried over anhydrous sodium sulfate (500 mg), and removed to a gas chromatography (GC) autosampler vial for GC/MS analysis.

Gas chromatography mass spectrometry (GC/MS) was performed on a Hewlett-Packard 5890 gas chromatograph interfaced to either a Hewlett-Packard 5973B mass spectrometer or a Hewlett-Packard 5971A MSD. Chromatographic separations were performed in splitless mode using an HP-5MS capillary column (30 m × 0.25 mm i.d. with 0.25 µm stationary phase film thickness, Hewlett-Packard Australia) with the following temperature program: 70°C constant for 2 min and then 20°C per minute to 250°C. The molecular ions ([M+H]+) for the unlabeled form and [M+2H]2+ for the [15N]-labeled isotope were monitored. The (m/z) peak areas were determined and the area ratios of the ion pairs calculated. Calibration curves were generated for the assay after correction for (a) the contribution to the [15N] nitro-toluene peaks at m/z 138 and m/z 121 due to the natural abundance of 15N present in endogenous or unlabeled nitrotoluene and (b) the contribution to the peaks at m/z 137 and m/z 120 arising due to the small amount of unlabeled nitrate present in the original 15N nitrate used as internal standard. These corrections were made for each assay by the analysis of separate samples containing unlabeled and [15N]-labeled nitrate alone. Nitrate levels were reported as nmol/ml.

Statistical analysis

All data are presented as mean ± SD. Differences among experimental groups were assessed using unpaired two-tailed Student's t-tests and one-way analysis of variance (ANOVA). The level of statistical significance was accepted at p < 0.05.

Results

iNOS PCR genotyping

To confirm the iNOS genotype of the mice, the genomic DNA was extracted from mice tail tips, and analyzed by PCR. One pair of primers, for the wild type allele, was fo-

Cross-sectional area of the Achilles tendon

As in our previous experiments in rats [28], healing mouse Achilles tendons were much thicker than control, un-injured Achilles tendon (Table 1). No significant differences were noted in cross-sectional area of the healing Achilles tendon or un-injured tendon when comparing the iNOS+/− group and the iNOS+ group (Fig. 1/Table 1). A significant reduction, however, in cross-sectional area of the healing Achilles tendon was observed in iNOS−/− mice treated with the nitric oxide synthase inhibitor, AG, 500 mg/kg body weight/day for 7 days. The reduction of the cross-sectional area of healing Achilles tendon in iNOS−/− mice treated with AG was 20% (p < 0.01) compared with the iNOS+/− group mice (Fig. 2). NO synthase inhibition did not alter the cross-sectional area of un-injured tendons at day 7.

Biomechanics

To investigate the biomechanical properties of mouse Achilles tendon, both of un-injured and healing tendons, we designed a mini tensile testing system for mouse Achilles tendon. All load-deflection curves exhibited a typical response for soft tissue structures. The failure site of all un-injured tendons was at the distal tendon-calcaneeus interface. Failure sites of injured Achilles tendons at day 7 were in the mid-substance.
Achilles tendon healing in iNOS knockout mice

Table 1. Effects of iNOS gene deletion and nitric oxide synthase inhibition on cross-sectional area and biomechanical properties of mice Achilles tendon seven days after surgical division of the right Achilles tendon. Mean (SD). **: p < 0.01 when compared with the iNOS+/-(R) group using one-way analysis of variance (ANOVA).

<table>
<thead>
<tr>
<th>Assessments</th>
<th>iNOS+/-(n = 8)</th>
<th>iNOS+/-(n = 11)</th>
<th>iNOS+/+ AG (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-sectional Area (mm²)</td>
<td>0.4 (0.1)</td>
<td>1.6 (0.4)</td>
<td>0.4 (0.1)</td>
</tr>
<tr>
<td>Failure load (Newton)</td>
<td>12 (2.4)</td>
<td>1.7 (0.3)</td>
<td>12 (1.8)</td>
</tr>
<tr>
<td>Stress (Newton/mm²)</td>
<td>29 (6.3)</td>
<td>1.1 (0.2)</td>
<td>33 (10)</td>
</tr>
<tr>
<td>Young modulus (Mpa)</td>
<td>165 (27)</td>
<td>2.0 (0.7)</td>
<td>183 (61)</td>
</tr>
<tr>
<td>Energy (Joules)</td>
<td>5.1 (1.3)</td>
<td>0.7 (0.3)</td>
<td>7.6 (2.6)</td>
</tr>
<tr>
<td>Serum nitrate</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Fig. 2. Effects of aminoguanidine (AG) on the cross-sectional area of Achilles tendon of iNos-/- mice. iNos-/- (uninjured): uninjured Achilles tendon of iNos-/- mice with (n = 10) or without (n = 11) AG, 500 mg/kg/day. iNOS-/- (injured): injured Achilles tendon of iNos-/- mice with (n = 10) or without (n = 11) AG, 500 mg/kg. **: p < 0.01 (Mean ± SD) using unpaired Student's t test.

No significant differences in failure load or stress (failure load/cross-sectional area) of the healing Achilles tendon constructs were found among the three groups. The uninjured Achilles tendon constructs were unaffected by iNOS gene deletion or NOS inhibitor either (Table 1). iNOS gene deletion and inhibition of NO synthase did not affect maximum displacement, stiffness, energy, modulus or maximum stress of the healing tendons of the surgically divided or uninjured Achilles tendon constructs (Table 1).

Serum nitrate

The serum nitrate concentrations of both the iNOS+/+ mice group (n = 11) and the iNOS+/+ mice with AG group (n = 10) were significantly lower than that in iNOS+/- mice group, 62% reduction in iNOS+/+ group and 58% reduction in iNOS+/+ with AG group compared with iNOS+/+ group (p < 0.001). However, there was no significant difference of nitrate concentrations between iNOS+/+ group mice and iNOS+/+ with AG group mice (Fig. 3).

Discussion

To our knowledge, this is the first investigation of the Achilles tendon healing in inducible nitric oxide synthase deficient (iNOS+/+) mice.

Systemic inhibition of nitric oxide synthase by aminoguanidine significantly reduced the cross-sectional area of the healing Achilles tendon in iNOS+/+ mice. However, no significant difference in cross-sectional area or biomechanical properties of the healing Achilles tendon was found between the iNOS+/- group and the iNOS+/+ group.
Systemic NOS inhibition resulted in a reduction in cross-sectional area of the mice healing Achilles tendon, which is consistent with healing Achilles tendons in rats [21–23]. However, deletion of the iNOS gene (iNos−/−) did not affect tendon cross-sectional area or biomechanical properties. This suggests that the iNOS gene may not be the only important contributor to the beneficial effects of NO on tendon healing and other NOSs (eNOS and nNOS) may also play a role in tendon healing. There are some reports that indicate that eNOS may play a significant role in regenerating endothelium, in cell migration, in growth factor-stimulated angiogenesis and in diabetes-impaired healing [32–34].

Aminoguanidine is a competitive NOS inhibitor. In normal mice, aminoguanidine has a partial selectivity for iNOS versus eNOS, and has little selectivity for iNOS versus nNOS [7]. For iNOS gene deficiency mice, however, aminoguanidine is likely to inhibit eNOS and nNOS, as there is no iNOS expression. The serum nitrate levels in iNos−/− mice were 2.5 time of that in iNos−/− mice, with no significant difference between iNos−/− group and iNos−/− + AG group. The explanation for this controversy may be that iNOS is the predominant isoform for NO production after tendon injury, while eNOS and nNOS produce smaller amounts of NO after tendon injury. Our findings are consistent with the results of other researchers: iNOS (inducible NOS) can synthesize prodigious amounts of NO for extended periods of time, whereas both eNOS and nNOS (constitutive NOS) produce NO in relatively low amounts of NO [35–37]. The results in the drinking water, but did not find any effect on cross-sectional area of healing Achilles tendon or serum nitrate (data not shown). Schaffer MR et al. [38] also found that NOS inhibitors given orally in the drinking water or by daily intraperitoneal injection had no effect on wound nitrite/nitrate concentrations or deposition of collagen in mice skin wounds. When given continuously through intraperitoneally placed osmotic pumps, aminoguanidine (500 mg/kg/day) significantly reduced wound fluid nitrite/nitrate concentrations and lower accumulation of collagen in wounds. The diurnal drinking pattern of rodents may, in a fashion similar to intermittent intraperitoneal injection, fail to maintain the plasma or wound fluid concentrations of inhibitor at concentrations effective at inhibiting NOS activity. These findings could reflect their short biological half-life of NOS inhibitors in mice [39, 40].

Taken together, the results of this study indicate that nitric oxide (NO) is beneficial in mouse tendon healing, but the iNOS gene is not solely responsible for the beneficial effects of NO on tendon healing.

Acknowledgements: The authors wish to thank original developers of iNOS−/− and iNOS−/− mice embryos, Dr. John MacMicking and Dr. Carl Nathan (Cornell University, USA), for the authorization to breed, and Dr. Guinsegan Karupiah (Sydney University, Australia) for providing iNOS−/− and iNOS−/− mice embryos; thank Dr. MX Wang, Dr. AD Diwan, Mr. Q Dai, Mr. T Molloy, and Dr. Y Boldik for their kind advice and help; and St. George Hospital/South East Sydney Area Health Service, NSW, Australia for financial support.

References

Vol. 55, 2006 Achilles tendon healing in iNOS knockout mice


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The Orthopaedic Research Institute–Tennis Elbow Testing System: A modified chair pick-up test—Interrater and intrarater reliability testing and validity for monitoring lateral epicondylosis

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Lateral epicondylosis is a degenerative overuse tendinopathy involving the extensor tendons of the forearm, predominantly the extensor carpi radialis brevis, in the region of the lateral epicondyle of the elbow. The Orthopaedic Research Institute–Tennis Elbow Testing System (ORI-TETS) is designed to record objective measurements of force generated with a simulated chair pick-up test. Interrater reliability of the ORI-TETS was excellent, with high intraclass correlation coefficients (ICCs) for right arm mean peak force of 0.93, left arm mean peak force of 0.84, right arm mean total force of 0.93, and left arm mean total force of 0.86. The ORI-TETS also demonstrated excellent intrarater reliability, with ICCs ranging from 0.9 to 0.97. The relative technical error of the ORI-TETS for all measurements ranged from 5.8% to 7.2%. Testing patients with lateral epicondylosis (N = 16) and comparing analog pain scores with ORI-TETS testing demonstrated a strong negative relationship between the two parameters (Spearman ρ = −0.87 to −1.0). Thus, the ORI-TETS is a reliable and reproducible testing system for the forearm extensors. The testing system is inexpensive, takes 5 minutes to perform, and demonstrates good predictive value for objectively assessing patients with lateral epicondylosis. This system could be used for routine clinical monitoring of patients with lateral epicondylosis. (J Shoulder Elbow Surg 2004;13:72-7.)

Lateral epicondylosis, sometimes called epicondylitis, is a common degenerative tendinopathy that causes pain and sensitivity in the lateral elbow. It has an incidence rate of 4 to 7 patients per 1000 per year in general practice, is evenly distributed between the sexes, and occurs in all ages but has a peak in persons aged 35 to 54 years. Despite the colloquial term tennis elbow, only about 5% of cases are caused by playing a racket sport, and it is far more commonly an occupational injury resulting from repetitive arm movements.1

The extensor carpi radialis brevis (ECRB) is the main tendon involved in lateral epicondylosis. Its origin is the anterior slope of the lateral epicondyle of the humerus. Here the tendon lies deep to the origin of the extensor digitorum communis (EDC) and inferior to the origin of the extensor carpi radialis longus (ECRL) and brachioradialis, which partly overlie the ECRB tendon.10

The origins of the extensor tendons of the wrist can be loaded by forceful gripping of an object when the hand is in palmar flexion, the wrist ulnarily deviated, and the forearm pronated, as in the chair pick-up test.6 As the ECRB tendon origin is proximal to the axis of elbow flexion and extension, it is subject to shearing stress in all forearm movements, especially those involving power at the wrist.7 The ECRB muscle crosses both the elbow and wrist joints and contracts eccentrically at both ends during certain maneuvers. In addition, the radial head rotates anteriorly against the ECRB tendon during pronation.3 We are unaware of any objective clinical measure to assess the severity or the progress of lateral epicondylosis.

The chair pick-up test can be used in the clinical assessment of elbow function and is used as a provocative test in patients with lateral epicondylosis, reproducing symptoms at the elbow. The Orthopaedic Research Institute–Tennis Elbow Testing System (ORI-TETS) was designed to simulate the chair pick-up test, and this study was performed to assess the reliability and reproducibility of results obtained through the use of this system. This study also aimed to
determine the validity of the system for monitoring patients with lateral epicondylosis.

To assess reliability of interval data, the intraclass correlation coefficient (ICC) (Pearson $R$) is the gold standard, as it is both a bona fide correlation coefficient$^2$ and a theoretically sound reliability coefficient.$^4$ There are, however, no universally accepted standards for what constitutes poor, good, or excellent reliability. One general criterion defines ICC results as less than 0.4 (poor), 0.4 to 0.75 (good), and greater than 0.75 (excellent).$^5$ We used these criteria to assess the reliability of the ORI-TETS.

MATERIALS AND METHODS

The ORI-TETS is a simulated chair pick-up test and consists of a vertical handboard attached to a horizontal lever arm, which, in turn, is connected to an adjustable vertical tensile cord in series with a 45-N Xtran K4 load cell (RS Components, Sydney, Australia). The load cell is connected to a transducer, and force data are recorded and stored directly on computer hard drive by use of National Instruments LabView 5.1 system technology (Austin, TX) (Figure 1).

The testing protocol involved the subjects standing front-on, behind a line marked on the floor, and grasping the coronally aligned handboard with the thumb on the side of the handboard nearest their body and the other four digits on the side furthest from their body, to ensure a pronated forearm (Figure 2). The elbow was required to be held at the side, with the shoulder and arm in neutral position, and the vertical tensile cord was adjusted so that the elbow was at $90^\circ$ of flexion. Each run comprised an effort to hold and lift the handboard superiorly while maintaining the lever arm horizontal to the floor and was of 10 seconds' duration. Subjects were allowed to practice the testing maneuver for 30 seconds before testing, and this served as the initial warm-up period. The subjects then performed two 10-second runs with both the left and right arms under the instruction of the examiner. The runs alternated from right arm to left and back again.

Before system testing, ethics approval was obtained from the ethics committee of our institution. To test the interrater reliability of this system, 4 examiners were chosen from the staff of the orthopaedics department of St George Hospital (Sydney, Australia), and 6 normal subjects were recruited from the laboratory staff at the Orthopaedic Research Institute (Sydney, Australia). The examiners consisted of 2 orthopaedic surgeons, 1 sports physician registrar, and 1 biomechanical engineer. The subjects were aged 23 to 42 years, with a median age of 30 years and a mean age of 32 years. There were 3 female and 3 male subjects, all were right hand–dominant, and none had previous elbow pathology. The testing was conducted on the same day for 4 consecutive weeks.

In order also to assess the intrarater reliability of the ORI-TETS, 4 subjects were chosen randomly and were tested by a single examiner once each fortnight for a total of 6 weeks. The subjects had a median age of 24 years (range, 20-40 years) and a mean age of 27 years. There were 2 female and 2 male subjects, all were right hand–dominant, and none had previous elbow pathology. The testing protocol was identical to that used for interrater testing.

Once all testing was completed, the data files were analyzed. They were in Excel (Microsoft, Redmond, WA) text format and consisted of force data (in Newtons) related to time (in increments of 0.1 seconds). The average force per unit time for each incremental time period was assessed, and then the total force per 10-second run was calculated by adding the individual measurements. Thus, for each subject, there was a record of peak force and total force for each run. The right arm and left arm mean peak force and mean total force were calculated by an average of the two runs.

Interrater reliability scores were analyzed to determine the ICC with 2-way randomization and 95% confidence intervals with Microsoft SPSS 10.1. Intrarater reliability scores were also analyzed to determine the ICC with a 2-way mixed model and 95% confidence intervals.

The interrater data were used to determine the absolute technical error of measurement (TEM), which is an estimation of the total error of a testing system that takes into account biological and technical factors.$^9$ This was calculated by taking the sum of the squared differences of the individual measurements, dividing by twice the number of subjects, and then taking the square root of the product. The relative TEM (%TEM) was calculated by dividing the TEM by the average mean of the two sets of measurements and multiplying by 100.$^9$

For validity testing, 16 patients were recruited with a clinical and magnetic resonance imaging diagnosis of lateral epicondylosis. These patients were aged 30 to 74 years, with a median age of 47 years and a mean age of 52 years. There were 8 female and 8 male patients, and the affected side was on the left in 4 patients (2 of these patients were left hand–dominant and 2 were right hand–dominant) and on the right in 12 patients (all right hand–dominant). The subjects were tested with the ORI-TETS every 2 weeks for a total of 6 weeks and were asked before testing to complete an analog pain scale to determine the level of
pain experienced with activities. The testing protocol was identical to that described previously.

From these patients with lateral epicondylitis, the arm mean peak force and arm mean total force for each run were calculated in an identical manner to the normal subjects. To ascertain whether there was a correlation between the patient’s rating of elbow pain with activities and their ability to produce force with the affected elbow when tested with the ORI-TETS pain rating scores, mean peak force and mean total force were analyzed with Spearman $\rho$ and Kendall $\tau$ correlation coefficients by use of the Microsoft Windows SPSS 10.1 program.

RESULTS

The results of interrater reliability testing with the ORI-TETS demonstrated ICCs ranging from 0.80 to 0.93 (Table I). ICC scores for all measured parameters in these right hand-dominant subjects were consistently higher on the right hand side than on the left. Intrarater reliability testing with the ORI-TETS demonstrated higher ICC scores as outlined in Table II, with ICCs ranging from 0.9 to 0.97.

The relative technical error of the measurement for
the ORI-TETS was very low, with individual measures ranging from 5.8% to 7.2% and mean measures ranging from 1.9% to 3.3% (Table II).

In patients with lateral epicondylitis, there was a very high correlation between elbow pain with activity and mean peak force obtained through elbow testing with the ORI-TETS (Spearman ρ, 0.78 to −1.0; Kendall τ, −0.71 to −1.0) (Figure 3). A similarly strong correlation between elbow pain with activity and mean total force was also demonstrated (Spearman ρ, −0.78 to −1.0; Kendall τ, −0.71 to −1.0) (Figure 4). The results for individual patients are summarized in Table III.

DISCUSSION

The intrarater reliability scores (ICCs) for all measured and calculated parameters were also of a very high order and were higher than reliability scores from interrater testing. The intrarater testing indicates that greater reliability can be obtained from a single examiner testing multiple subjects with the ORI-TETS than when several different examiners test the same subject group. This indicates that the ORI-TETS is both reliable and reproducible for use by a single examiner in subjects tested on repeated occasions over time. Once again, there did not appear to be a significant learning effect, as scores did not increase sequentially, and there was no evidence for a training effect, as the highest scores were distributed evenly across the three testing periods.

The absolute and relative technical errors of measurement (TEM and %TEM) are indicators of the total error associated with the measure in question. The TEM is described in the same units as the measured variable, whereas the relative TEM is described as a percentage error. The measures of peak force and peak total force obtained by an individual examiner showed relative TEMs of between 5.8% and 7.2%, whereas the measures of mean peak force and mean total force showed relative TEMs of between 1.9% and 3.3%. The individual measures show that the ORI-TETS has a relatively low total error associated with its measurements, and this error appears to be lowered further by using the mean values of the two runs. This is consistent with the values obtained for ICCs.

Testing conducted on patients with lateral epicondylitis demonstrated that there was a strong negative correlation between the level of pain experienced when performing daily activities and the amount of peak force and total force that could be produced by the forearm extensors with use of the ORI-TETS. A negative relationship implies that individuals scoring low on one variable (eg, pain) tend to score high on a second variable (eg, mean peak force or mean total force). The Spearman ρ and Kendall τ correlation coefficients were used to determine this relationship because the analog pain scale data were ordinal in nature, whereas the data from the ORI-TETS were interval data.

All 16 patients demonstrated a strong negative relationship between their elbow pain with activity...
and the ability to produce force when tested with the ORI-TETS. This is shown by both the Spearman $\rho$ and Kendall $\tau$ correlation coefficients approaching $-1$ for all patients. The results for 3 of the 16 patients were significant at the 0.01 level, and the results for 4 of the 16 patients were significant at the 0.05 level for both the negative relationships of pain versus mean peak force and pain versus mean total force. The ORI-TETS is an inexpensive testing system, with the only significant cost being the computer software.

### Table I  Summarized results of ORI-TETS interrater reliability testing

<table>
<thead>
<tr>
<th>ORI-TETS parameter</th>
<th>ICC</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
</tr>
<tr>
<td>Right arm peak force</td>
<td>0.91</td>
<td>0.78</td>
</tr>
<tr>
<td>Left arm peak force</td>
<td>0.8</td>
<td>0.59</td>
</tr>
<tr>
<td>Right arm mean peak force</td>
<td>0.89</td>
<td>0.75</td>
</tr>
<tr>
<td>Left arm mean total force</td>
<td>0.81</td>
<td>0.62</td>
</tr>
<tr>
<td>Right arm mean peak force</td>
<td>0.93</td>
<td>0.77</td>
</tr>
<tr>
<td>Left arm mean peak force</td>
<td>0.84</td>
<td>0.58</td>
</tr>
<tr>
<td>Right arm mean total force</td>
<td>0.93</td>
<td>0.76</td>
</tr>
<tr>
<td>Left arm mean total force</td>
<td>0.86</td>
<td>0.62</td>
</tr>
</tbody>
</table>

### Table II  Summarized results of ORI-TETS intrarater reliability testing

<table>
<thead>
<tr>
<th>ORI-TETS parameter</th>
<th>ICC</th>
<th>95% Confidence interval</th>
<th>TEM</th>
<th>%TEM</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
<td>TEM</td>
</tr>
<tr>
<td>Right arm peak force</td>
<td>0.90</td>
<td>0.73</td>
<td>0.98</td>
<td>0.8 N</td>
</tr>
<tr>
<td>Left arm peak force</td>
<td>0.90</td>
<td>0.68</td>
<td>0.98</td>
<td>0.9 N</td>
</tr>
<tr>
<td>Right arm mean peak force</td>
<td>0.93</td>
<td>0.70</td>
<td>1.0</td>
<td>0.3 N</td>
</tr>
<tr>
<td>Left arm mean peak force</td>
<td>0.93</td>
<td>0.66</td>
<td>1.0</td>
<td>0.4 N</td>
</tr>
<tr>
<td>Right arm total force</td>
<td>0.90</td>
<td>0.69</td>
<td>0.98</td>
<td>8.4 N/10 s</td>
</tr>
<tr>
<td>Left arm total force</td>
<td>0.93</td>
<td>0.78</td>
<td>0.98</td>
<td>7.6 N/10 s</td>
</tr>
<tr>
<td>Right arm mean total force</td>
<td>0.94</td>
<td>0.69</td>
<td>1.0</td>
<td>2.3 N/10 s</td>
</tr>
<tr>
<td>Left arm mean total force</td>
<td>0.97</td>
<td>0.83</td>
<td>1.0</td>
<td>2.7 N/10 s</td>
</tr>
</tbody>
</table>

### Table III  Results of testing in subjects with lateral epicondylitis ($N = 16$) demonstrating relationship between elbow pain with activity and functional force testing with ORI-TETS

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Pain vs mean peak force</th>
<th>Pain vs mean total force</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spearman $\rho$</td>
<td>Kendall $\tau$</td>
</tr>
<tr>
<td>1</td>
<td>-0.87</td>
<td>-0.82</td>
</tr>
<tr>
<td>2</td>
<td>-1.0**</td>
<td>-1.0**</td>
</tr>
<tr>
<td>3</td>
<td>-1.0**</td>
<td>-1.0**</td>
</tr>
<tr>
<td>4</td>
<td>-0.87</td>
<td>-0.82</td>
</tr>
<tr>
<td>5</td>
<td>-1.0**</td>
<td>-1.0**</td>
</tr>
<tr>
<td>6</td>
<td>-0.95*</td>
<td>-0.91*</td>
</tr>
<tr>
<td>7</td>
<td>-0.82</td>
<td>-0.78</td>
</tr>
<tr>
<td>8</td>
<td>-0.95*</td>
<td>-0.91*</td>
</tr>
<tr>
<td>9</td>
<td>-0.78</td>
<td>-0.71</td>
</tr>
<tr>
<td>10</td>
<td>-0.95*</td>
<td>-0.91*</td>
</tr>
<tr>
<td>11</td>
<td>-0.95*</td>
<td>-0.91*</td>
</tr>
<tr>
<td>12</td>
<td>-0.78</td>
<td>-0.71</td>
</tr>
<tr>
<td>13</td>
<td>-0.78</td>
<td>-0.71</td>
</tr>
<tr>
<td>14</td>
<td>-0.78</td>
<td>-0.71</td>
</tr>
<tr>
<td>15</td>
<td>-0.78</td>
<td>-0.71</td>
</tr>
<tr>
<td>16</td>
<td>-0.95*</td>
<td>-0.91*</td>
</tr>
</tbody>
</table>

*Significant at $P < .05$.  
**Significant at $P < .01$.  

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January/February 2004
Testing a single patient involves four 10-second efforts, and the testing process takes 2 minutes to perform; subsequent data analysis is uncomplicated and takes only a few minutes. The whole process is completed in approximately 5 minutes and can feasibly be performed in a clinic situation.

The ICCs for ORI-TETS of both intrarater and interrater reliability testing demonstrated excellent reliability and reproducibility of force measurements of the wrist extensors with this modified chair pick-up test. For an individual examiner, the TEM by use of the ORI-TETS was on the order of 5.8% to 7.2%. In patients with lateral epicondylitis, there was a strong negative correlation between patient-rated activity pain and measured mean peak force and mean total force. Given its low cost, simplicity of use, excellent reliability, and low associated error, the ORI-TETS could be used by single or multiple examiners to monitor patients with lateral epicondylitis objectively.

REFERENCES

Topical Nitric Oxide Application in the Treatment of Chronic Extensor Tendinosis at the Elbow

A Randomized, Double-Blinded, Placebo-Controlled Clinical Trial

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Background: Extensor tendinosis ("tennis elbow") is a degenerative overuse tendinopathy of the wrist extensors at their attachment to the lateral humeral epicondyle. No treatment has been universally successful. Topical application of nitric oxide has been used effectively to treat fractures and cutaneous wounds in animal models, presumably by stimulation of collagen synthesis in fibroblasts.

Purpose: To determine whether topical nitric oxide can improve outcome of patients with extensor tendinosis.

Study Design: Prospective, randomized, double-blinded clinical trial.

Methods: Eighty-six patients with extensor tendinosis were randomized into two equal groups; both were instructed to perform a standard tendon rehabilitation program. One group received an active glyceryl trinitrate transdermal patch, and the other group received a placebo patch.

Results: Patients in the glyceryl trinitrate group had significantly reduced elbow pain with activity at 2 weeks, reduced epicondylar tenderness at 6 and 12 weeks, and an increase in wrist extensor mean peak force and total work at 24 weeks. At 6 months, 81% of treated patients were asymptomatic during activities of daily living, compared with 60% of patients who had tendon rehabilitation alone.

Conclusions: Application of topical nitric oxide improved early pain with activity, late functional measures, and outcomes of patients with extensor tendinosis.

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Tennis elbow, or lateral epicondylitis, is a common cause of chronic elbow pain and wrist extensor dysfunction in adults, especially those between the ages of 35 and 54 years.¹⁰ No treatment is universally successful in managing this condition, which causes considerable time lost to work and recreation. The current best practice management of tendon rehabilitation involves relative rest, forearm bracing, and a graduated stretching and strengthening exercise program.²

Inhibition of nitric oxide has been shown to reduce collagen content, contraction, and synthesis by wound fibroblasts in vitro.¹⁴ In animal studies, nitric oxide synthase inhibition resulted in a significant reduction in the cross-sectional area and load to failure of healing tendon,⁸ suggesting that nitric oxide stimulates collagen synthesis by wound fibroblasts. This hypothesis is a proposed mechanism for nitric oxide modulation of healing in tendons, which rely on fibroblastic production of collagen for repair. The goal of our study was to assess whether application of continuous topical nitric oxide can alter outcome measures in patients with chronic tennis elbow when compared with standard tendon rehabilitation treatment alone.

Topical application of glyceryl trinitrate has been used for over 100 years as a therapy for angina pectoris.⁹ It is

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now accepted that the mechanism of action of the organic nitrates is through production of nitric oxide, the endothelium-derived relaxing factor. Thus, organic nitrates such as glyceryl trinitrate may be viewed as prodrugs of endogenous nitric oxide. In our clinical trial we chose to deliver nitric oxide via transdermal glyceryl trinitrate patches because of the ease of dosing and of application.

The current best practice management for lateral epicondylitis involves a graduated tendon rehabilitation program that begins with relative rest of the injured tendon through avoidance of aggravating activities. Although this treatment has not been scientifically validated, it is anecdotally important, empirically effective, and logically sound. The early use of counterforce braces has been reported, through biomechanical and EMG analysis, to reduce angular acceleration at the tendon origin and to reduce muscle duration responses above the braced area.

Stretching of the tendons of the elbow and wrist is thought to be important for maintaining normal range of motion at these joints, particularly during a period of relative rest, and assists in regaining flexibility. Stretching may also aid tendon healing through gentle longitudinal traction along normal lines of stress.

Muscle strengthening is instituted as soon as pain permits and is thought to assist in healing through tensile loading and improved tendon collagen fiber orientation, as well as by gradually restoring lost strength in forearm musculature. Initially, isometric exercises are commenced, and then, as each exercise stage can be performed without pain, increased resistance can be added. Further exercises are gradually instituted, including concentric and eccentric types.

In five randomized and controlled clinical trials, corticosteroids have been shown to provide some improvement in elbow pain between 2 and 6 weeks, with no improvement beyond 2 months. For this reason, we decided not to use corticosteroid injections in this clinical trial and to exclude patients who had received this therapy in the preceding 3 months. There is no type II evidence to support ultrasound, laser, and electrogalvanic stimulation in the treatment of lateral epicondylitis, and, for this reason, these measures were neither actively included nor excluded in this trial.

MATERIALS AND METHODS

The study was designed to meet CONSORT guidelines and was approved by the South Eastern Sydney Area Health Service Ethics Committee. A power analysis determined that, to have a 90% probability of finding a 40% difference between groups, it was necessary to recruit 80 patients for the trial. All subjects were required to be more than 18 years of age. Subjects were excluded if they had extensor tendinosis of less than 3 months' duration, current pregnancy, previous surgery or dislocation of the affected elbow or wrist, distal neurologic signs, or a local corticosteroid injection in the previous 3 months.

The 86 recruited patients were allocated to one of the two clinical trial groups through coded randomization, with 43 patients in each group. This randomization process was controlled by the senior pharmacist in our institution (JN), who also supervised the packaging of transdermal patches and their distribution to patients. Patients in one group received the active transdermal patch (one-quarter of a 5-mg/24-hour Nitro-dur patch, Schering-Plough, Sydney, Australia), and the other group received a placebo transdermal patch (one-quarter of a Nitro-dur demonstration patch, Schering-Plough).

The patches were distributed to the patients by the pharmacist along with a supply of 24 500-mg paracetamol tablets to use for possible headache. The transdermal patches were intact when distributed, and patients were required to cut the patches into quarters before application. Both the patients and the clinical examiner were blinded as to group assignment.

Patients were instructed in the application of the patches at their initial visit. They were informed that the dosing regimen was one-quarter of a transdermal patch to be applied daily to the affected elbow. The patches were to be left in place for 24 hours and then replaced with a new quarter patch. The site of application was demonstrated as immediately distal to the lateral epicondyle of the humerus, and patients were instructed to rotate the patch application site around this point with each new patch application.

At the initial clinical assessment, all patients were instructed in the performance of a tendon rehabilitation program encompassing the current best practice management for extensor tendinosis. The program involved 1) rest from aggravating activities in the early stages (particularly repetitive wrist flexion and extension, repetitive forearm pronation and supination movements, and forceful gripping), 2) the continuous use of a forearm counterforce brace until completion of the first phase of the muscle-strengthening program, 3) regular stretching of the extensor carpi radialis brevis muscle and tendon, and 4) a muscle-strengthening program involving gradually increasing resistance.

At the initial visit and at all subsequent visits, the patient was also required to complete an extensor tendinosis symptom assessment form with use of verbal descriptor scales to rate the severity (0 to 4) of their elbow pain with activity, at rest, and at night. This assessment form was also used to exclude mechanical symptoms in the elbow and to indicate changes in elbow symptoms, either improvement or deterioration.

A single examiner (JAP) assessed each patient and recorded the following: 1) the level of local epicondylar and proximal common extensor tendon tenderness by using a four-point scale (none, mild, moderate, or severe tenderness), 2) handheld dynamometer measurement of resisted third finger metacarpophalangeal extension with a fully extended elbow (Maudsley’s test), 3) wrist extensor tendon mean peak force by using a modified chair pick-up test (the Orthopaedic Research Institute Tennis Elbow Testing System, ORI-TETS), and 4) total work as measured by the ORI-TETS. This modified chair pick-up test was performed with the elbow flexed to 90° and with a vertically oriented hand board gripped palm downward and pulled.
superiorly. The hand board was linked to a load cell, and the readings were stored directly onto a computer’s hard drive.

All clinical assessments were repeated at 0, 2, 6, 12, and 24 weeks with an identical format. Records of headaches, paracetamol use, and compliance with patch application and the tendon rehabilitation program were also made at these scheduled visits.

The symptoms and signs used as outcome measures for assessing response to treatment in this clinical trial were patient-rated elbow pain at rest (0 to 4), patient-rated elbow pain with activity (0 to 4), patient-rated elbow pain at night (0 to 4), local epicondylar and tendon tenderness (0 to 3), dynamometer-measured strength with Maudsley’s test (measured in newtons), and wrist extensor mean peak force and mean total work as measured by the ORI-TETS (calculated with LabView 5.1 biomechanical software, National Instruments, Austin, Texas).

Outcome measures were analyzed with Sigmastat 2.0 statistical software (Jandel Scientific, San Rafael, California) with use of the Mann-Whitney rank sum test to compare differences between groups and the Wilcoxon signed-rank test to compare differences within the groups. The level of significance was defined at $P = 0.05$. A chi-square analysis of patient-reported symptom outcomes at week 24 was performed. Effect size estimates were calculated by dividing the mean z-score at week 24 by the square root of the sample size.$^{13}$

RESULTS

Eighty-six patients (95 elbows) with a clinical diagnosis of extensor tendinosis were recruited through newspaper advertisements and private consulting rooms. There were 42 men and 44 women, with a median age of 46 years (range, 30 to 74). Bilateral disease was present in nine patients, all of whom were right-hand dominant. In total, 81 of the patients were right-hand dominant; in 16 of these patients, the nondonimant side was affected alone. Five patients were left-hand dominant, and four of them had the dominant side affected. The median duration of symptoms was 17 months (range, 3 to 232).

Of the 86 patients originally recruited for the clinical trial, 5 patients in the glyceryl trinitrate patch group had treatment discontinued during the course of the study because of side effects (2 patients had severe and persistent headaches, 2 patients had dermatitis rash, and 1 patient experienced transient facial flushing and angiodysplasia). Of the remaining 38 patients in the glyceryl trinitrate patch group, 2 patients dropped out at the 2-week stage, with no reason given, and 1 patient dropped out at the 3-week stage because of an occupational back injury that required hospitalization. Thus, there were 35 patients (39 elbows) in the glyceryl trinitrate patch group who completed the 6-month follow-up.

In the placebo patch group there were four dropouts. Three of these dropped out within the first 3 weeks of the trial, with no reason given, and the fourth dropped out at the 14-week stage when this patient relocated to another state. Thus, there were 39 patients (41 elbows) in the placebo patch group who completed the 6-month follow-up period. There were no significant differences in the patient characteristics or the drop-out rate between the two treatment groups, and the statistical analysis was made on the basis of intention to treat.

Analysis of the clinical trial outcome measures determined that the data were not normally distributed. A Mann-Whitney rank sum test comparison of the glyceryl trinitrate group compared with the placebo group revealed a significant decrease in elbow pain with activity at week 2 ($P = 0.01$) (Fig. 1). It was noted that patients in the glyceryl trinitrate group had less elbow pain with activity once treatment began, and this difference continued to the conclusion of the trial at week 24. There was, however, a greater decrease in elbow pain with activity in the glyceryl trinitrate group at the week-2 stage than at any other time period.

Mann-Whitney rank sum tests comparing lateral epicondylar and tendon tenderness between groups showed a significant decrease in clinically assessed lateral epicondylar and tendon tenderness at week 6 ($P = 0.02$) and at week 12 ($P = 0.02$). There appeared to be a trend toward diminishing elbow tenderness over the course of the clinical trial in the glyceryl trinitrate group, especially when compared with the placebo group (Fig. 2).

Mann-Whitney rank sum tests comparing ORI-TETS measurements between groups showed a significant increase in mean peak force at week 24 ($P = 0.03$) and a significant increase in mean total work at week 24 ($P = 0.03$). Both the peak force and total work produced by the affected elbow increased over time, with the glyceryl trinitrate group increasing more than the placebo group (Fig. 3).

Within the glyceryl trinitrate group the data were not normally distributed, and analysis with the Wilcoxon signed-rank test showed that all measures at all testing periods were significantly improved compared with the
baseline measures at week 0. Patient-reported outcomes at week 24 showed that 81% of patients (35 of 43) in the glyceryl trinitrate group had excellent improvement (asymptomatic with activities of daily living) over the course of the trial; 16% of patients (7 of 43) were unchanged (equivalent to less than 10% worsening or improvement) over the course of the trial; and 2% of patients (1 of 43) rated their elbow as poor (equivalent to worsening by more than 10%) after completion of the clinical trial. This result compared with 60% of patients in the placebo group rating their condition as excellent (26 of 43), 23% as unchanged (10 of 43), and 16% as poor (7 of 43). A chi-square analysis comparing outcomes between the two groups revealed that the glyceryl trinitrate group had a significantly increased \( P = 0.005 \) chance of being asymptomatic with activities of daily living at 24 weeks.

The mean estimated effect size at week 24 was 0.12 (95% confidence interval, 0.06 to 0.19). This measure was derived from the mean \( P \) value of all outcome measures at the week-24 stage and was equivalent to a binomial effect size display, or change in patient outcome success rates, of 12%. In other words, from an estimation of the size of the mean effect on outcome measures at week 24 in the glyceryl trinitrate group, it would be expected that the between-groups difference in successful outcome (asymptomatic at week 24) would be 12% of patients.

The reported and observed side effects in the glyceryl trinitrate group included headache in 63% (27 of 43 patients), a dermatitis rash in 21% (9 of 43 patients), facial flushing and cutaneous angiodyplasia in 2% (1 of 43 patients), ipsilateral axillary sweating in 2% (1 of 43 patients), and a feeling of uneasiness/apprehension in 2% (1 of 43 patients). Only 35% (15 of 43 patients) experienced no side effects during the 6 months of the clinical trial.

In the placebo patch group, the reported side effects were headache in 58% (25 of 43 patients), and a dermatitis rash in 9% (4 of 43 patients). Between the two groups, there was no significant difference in the number of days affected by headache or of the amount of paracetamol required for headache during the course of the trial.

The glyceryl trinitrate group reported a total of 136 days with at least one headache, which was equal to an average of 5 days (2% of clinical trial days for those with headache) per person of those patients who experienced this side effect. The median was 3 days of headache, with a range of 0 to 16 days. The patients who had headaches required a total of 214 paracetamol tablets (500 mg) during the course of the clinical trial. The average number of paracetamol tablets required for headache over the 6-month period was 8 tablets, with a median of 4 tablets and a range of 0 to 38 tablets.

The placebo patch group reported a total of 166 days with at least one headache, which was equal to an average of 6.5 days (2% of clinical trial days for those with headache) per person of those patients who experienced this side effect. The median was 1 day of headache, with a range of 0 to 34 days. A total of 250 paracetamol tablets (500 mg) were used by patients who had a headache during the course of the clinical trial. The average number of paracetamol tablets required for headache over the 6-month period was 10 tablets, with a median of 0 tablets and a range of 0 to 90 tablets.

DISCUSSION

The group of patients with extensor tendinosis at the elbow who had continuous topical glyceryl trinitrate therapy demonstrated significantly reduced elbow pain with activity at 2 weeks, reduced lateral epicondylar and tendon tenderness at 6 and at 12 weeks, and improved elbow peak force and total work (as tested by ORI-TETS) at 24 weeks. These effects of topical nitric oxide were in addition to the significant improvement of all measures at all test-
The results of this clinical trial demonstrated that there were significant improvements in symptoms, clinical signs, provocative functional tests, and patient outcomes in patients with extensor tendinosis treated with topical nitric oxide therapy (glyceryl trinitrate application) when compared with tendon rehabilitation alone. This is the first clinical trial of this therapy that we are aware of that
shows patient- and examiner-determined subjective and objective improvements in outcome for tennis elbow.

Clearly, more research needs to be done in this area to define the exact mechanism of action of nitric oxide in tendon healing, to confirm the validity and reproducibility of the results, and to delineate the most effective dosage regimen to maximize effect and limit side effects. It is also important to determine the reason for treatment failure in the 19% of patients who did not improve and to fully elucidate the role and effects of nitric oxide in tendon healing and rehabilitation. However, because this is a well-tested medication in humans and there are no irreversible side effects in healthy subjects, there does appear to be a role for use of continuous topical nitric oxide therapy in the treatment of patients with chronic extensor tendinosis in combination with a comprehensive tendon rehabilitation program.

ACKNOWLEDGMENTS

This research was supported in part by St. George Hospital - South Eastern Sydney Area Health Service. The support of Schering-Plough Australia by their donation of the glyceryl trinitrate and placebo patches used in the clinical trial was greatly appreciated.

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Reliability of five methods for assessing shoulder range of motion

Kimberley Hayes, Judie R Walton, Zoltan L Szomor and George AC Murrell
University of New South Wales

In symptomatic subjects, the reliability of tests for shoulder joint range of motion has yet to be determined. For this reason, inter-rater and intra-rater agreement trials were undertaken to ascertain the reliability of visual estimation, goniometry, still photography, "stand and reach" and hand behind back reach for six different shoulder movements. Intra-class correlation coefficients (Rho) were derived by using a random effects model. For flexion, abduction and external rotation fair to good reliability was demonstrated for both trials using visual estimation (inter-rater Rho = 0.57-0.70; intra-rater Rho = 0.59-0.67), goniometry (inter-rater Rho = 0.64-0.69; intra-rater Rho = 0.53-0.65) and still photography (inter-rater Rho = 0.62-0.73; intra-rater Rho = 0.56-0.61). The tests had standard errors of measurement of between 14 and 25 degrees (inter-rater trial) and 11 and 23 degrees (intra-rater trial).

Key words: Clinical Protocols; Range of Motion, Articular; Reproducibility of Results; Shoulder

Introduction

Of all the joints, the shoulder has the greatest range of motion. The assessment of shoulder range of motion is important in the diagnosis of disorders of the shoulder and for the evaluation of the strategies that may alter shoulder function.

There have been a number of tools designed to measure joint range of motion varying from simple visual estimation to high speed cinematography (Clapper and Wolf 1988, Fish and Wingate 1985, Hellebrandt et al 1949, Low 1976, Moore 1949, Moore 1949, Youdas et al 1994). Several of these have been trialled for measurement reliability (Boone et al 1978, Clapper and Wolf 1988, Fish and Wingate 1985, Hellebrandt et al 1949, Low 1976, Mayerson and Milano 1984, Riddle et al 1987, Williams and Callaghan 1990, Youdas et al 1994). However, notably lacking is information specific to the shoulder joint, in symptomatic subjects, under conditions that reflect how measurements are taken in the clinical environment. Given that range of motion reliability varies from one patient population to the next (Ashton et al 1978, Bartlett et al 1985, Harris et al 1985), from one joint to the next (Boone et al 1978, Clapper and Wolf 1988, Hellebrandt et al 1949, Low 1976) and from one joint movement to the next (Hellebrandt et al 1949, Riddle et al 1987), there is a clear need to establish patient specific, joint specific and movement specific reliability indices for clinical practice. For this purpose, we determined the inter-rater and intra-rater reliability, and the standard error of the measurement for five easily applied methods for assessing active and passive shoulder range of motion for six movements, in patients with a spectrum of shoulder dysfunctions (rotator cuff repair, adhesive capsulitis and scapulothoracic fusion).

Method

Subjects Two groups of subjects gave informed consent for trials which investigated the inter-rater and intra-rater reliability of five joint range of motion assessment tests. All patients with shoulder pathology that warranted consultation with the participating orthopaedic surgeon between January 1996 and October 1998 were considered for inclusion to these trials. From this patient list, subjects were randomly selected and contacted by telephone for the purpose of recruitment. This process was repeated until the desired sample size had been obtained.

Inter-rater reliability trial The inter-rater reliability trial consisted of eight volunteers, three males and five females, ranging in age between 57 and 72 years (mean age = 66 years, SD = 5.7). All subjects had a current shoulder complaint. Six patients had undergone rotator cuff repair surgery within the past 24 months, one patient was 17 months post scapulothoracic fusion, and one patient had adhesive capsulitis.

Intra-rater reliability trial The intra-rater reliability trial consisted of nine volunteers, five males and four females, ranging in age between 29 and 74 years (mean age = 64 years, SD = 14.7). One of these subjects had two symptomatic shoulders. Of the 10 shoulders, eight were symptomatic, being within 36 months of rotator cuff repair.
surgery. The remaining two shoulders were asymptomatic at the time of testing.

Raters

Inter-rater reliability trial Four raters, identified here as A to D were used for the inter-rater reliability trial. Rater A was an orthopaedic surgeon, Rater B was a sports physician trainee and Raters C and D were qualified physiotherapists. All raters tested all eight subjects with the five range of motion assessment tests. All raters were blinded to the results of each other’s assessments.

Intra-rater reliability trial Only Rater A was used for the intra-rater reliability trial. This rater tested all nine subjects (including the one subject being tested bilaterally) on three separate occasions within a 48 hour period, using the same five range of motion assessment tests. The operating/treating surgeon was not involved in assessments for the inter-rater or intra-rater reliability trials.

Measurement tests

The five tests used for measuring range of motion for both the inter-rater and intra-rater trials were:

1. Visual estimation of passive range of motion (measured in degrees) for the movements of flexion, abduction, external rotation and hand behind back (measured to the hand behind back position, the rater moved the affected extremity upwards and towards the midline to the highest vertebral level reached by the tip of the subject’s extended thumb.

2. Goniometry (measured in degrees), using a plastic, 41 cm universal goniometer, for the active movements of flexion, abduction and external rotation. The subject was positioned as for visual estimation tests. The subject moved the affected extremity (thumb pointing upwards) to the end of active range of shoulder flexion, abduction and external rotation (0 degrees glenohumeral joint abduction, 90 degrees elbow flexion, neutral supination/pronation position). The flexion angle was formed by joining the acromion process, and the end of rib 12. The abduction and external rotation angle was formed by aligning the goniometer with the lateral epicondyle of the humerus; the middle of the posterior glenohumeral joint line, and a vertical line in the transverse plane.

3. Still photography, using a standard polaroid camera, for the active movements of flexion, abduction and external rotation. Each rater labelled eight reference points on each subject prior to the commencement of photographic testing using the following bony landmarks: T1 spinous process, T7 spinous process, postero-lateral acromion process of the scapula, lateral epicondyle of the humerus, a point 6 cm inferior to the postero-lateral aspect of the acromion process of the scapula, end of rib 12, olecranon process of the ulna, and ulna styloid process.

For flexion and abduction, the subject stood with feet shoulder width apart. For external rotation, the subject was supine and the affected extremity was supported on the treatment table (0 degrees glenohumeral joint abduction, 90 degrees elbow flexion, neutral supination/pronation position). The subject moved the affected extremity (thumb pointing upwards) to the end of active range of shoulder flexion, abduction and external rotation. The rater took a polaroid photograph of each end of range position from a perspective aligned with the axis of joint motion. Using a plastic 15 cm goniometer and the developed photograph, the rater manually calculated the resultant joint angle in degrees. The flexion angle was formed by joining the lateral epicondyle, the point 6 cm below the postero-lateral acromion process, and the end of rib 12. The abduction angle was formed by the intersection of a line that ran through the lateral epicondyle and the postero-lateral acromion process and another line that ran through T1 and T7 spinous processes.

4. Stand and reach test of maximum overhead reach (measured in centimetres), using a metric vertical wall scale. The subject faced the wall and reached the affected extremity to a maximum overhead position. The stand and reach value was recorded as the position of maximum reach minus the patient’s standing head height.

5. Tape measured distance of hand behind back reach in centimetres. The subject was standing with feet shoulder width apart. With thumb extended, the subject reached the affected extremity upwards and towards the midline to a maximum hand behind back position. The rater used a tape measure to record the distance in centimetres from the thumb tip to T1 spinous process.

Procedures For the inter-rater trial, all raters were briefed on the study protocol and a five hour training session was conducted to ensure familiarisation and standardisation of the five range of motion tests. The intra-rater trial was conducted several weeks after the inter-rater trial and involved a different group of subjects. Both trials followed the procedures described.

For each test, raters were asked to correct for compensatory
Table 1. Inter-rater reliability and standard error of the measurement for five methods of assessing shoulder range of motion.

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD, (range)</th>
<th>ICC (Rho)</th>
<th>95% CI of ICC</th>
<th>SEM</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Visual estimation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flexion</td>
<td>142±34, (45 - 180)°</td>
<td>0.70</td>
<td>0.42 - 0.92</td>
<td>19°</td>
<td>±38°</td>
</tr>
<tr>
<td>Abduction</td>
<td>121±33, (45 - 180)°</td>
<td>0.66</td>
<td>0.37 - 0.90</td>
<td>19°</td>
<td>±38°</td>
</tr>
<tr>
<td>External rotation</td>
<td>47±21, (13 - 80)°</td>
<td>0.57</td>
<td>0.26 - 0.87</td>
<td>14°</td>
<td>±26°</td>
</tr>
<tr>
<td><strong>Goniometry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flexion</td>
<td>132±45, (0 - 170)°</td>
<td>0.69</td>
<td>0.40 - 0.91</td>
<td>25°</td>
<td>±50°</td>
</tr>
<tr>
<td>Abduction</td>
<td>118±39, (30 - 170)°</td>
<td>0.69</td>
<td>0.37 - 0.92</td>
<td>21°</td>
<td>±42°</td>
</tr>
<tr>
<td>External rotation</td>
<td>46±23, (-15 - 80)°</td>
<td>0.64</td>
<td>0.31 - 0.91</td>
<td>14°</td>
<td>±26°</td>
</tr>
<tr>
<td><strong>Still photography</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flexion</td>
<td>120±45, (5 - 170)°</td>
<td>0.73</td>
<td>0.46 - 0.93</td>
<td>23°</td>
<td>±46°</td>
</tr>
<tr>
<td>Abduction</td>
<td>118±44, (15 - 160)°</td>
<td>0.73</td>
<td>0.45 - 0.93</td>
<td>23°</td>
<td>±46°</td>
</tr>
<tr>
<td>External rotation</td>
<td>50±25, (-10 - 90)°</td>
<td>0.62</td>
<td>0.32 - 0.89</td>
<td>15°</td>
<td>±30°</td>
</tr>
<tr>
<td>Stand and reach</td>
<td>42±7, (-64 - 54)cm</td>
<td>0.74</td>
<td>0.45 - 0.94</td>
<td>3cm</td>
<td>±6cm</td>
</tr>
<tr>
<td>Hand behind back</td>
<td>T10±3, (T7 - S4)</td>
<td>0.26</td>
<td>-0.01 - 0.69</td>
<td>2</td>
<td>±4</td>
</tr>
<tr>
<td>(vertebral level) (cm)</td>
<td>26±8, (16 - 48)cm</td>
<td>0.39</td>
<td>0.09 - 0.77</td>
<td>6cm</td>
<td>±12cm</td>
</tr>
</tbody>
</table>

Inter-rater reliability and standard error of the measurement for five methods of assessing shoulder range of motion. Each test was performed on eight subjects by four raters. Intra-class correlation coefficients (Rho) were calculated using a two-way mixed effect model. Mean, standard deviation and range of scores were calculated from the raw data. (SD = standard deviation, ICC = intra-class correlation coefficient, CI = confidence interval, SEM = standard error of the measurement).

Statistical analysis

For the inter-rater and intra-rater reliability trials, two-way random effect intra-class correlation coefficients (2,1) (Rho), together with their confidence intervals, were calculated with SPSS statistical software (Yaffee 1998). In accordance with the suggestions of Fleiss (1986), a Rho value of < 0.4 was deemed as representing poor reliability; 0.4-0.75 as fair to good reliability; and > 0.75 as excellent reliability.

In addition, the standard error of the measurement (SEM) and 95% confidence intervals (+2 SEM) were calculated for each range of motion test and expressed in units of the measure. The standard error of the measurement was calculated according to the equation: SEM = SD x \(\sqrt{\text{1 - ICC}}\), where SD is the standard deviation and ICC is the Rho score (Portney and Watkins 1993).

Results

**Intra-class correlation coefficient** The intra-class correlation coefficient (Rho score) is an index of reliability for measurements of the same material. Two types of measurement reliability can be distinguished according to whether each of several raters independently measures the same material (inter-rater reliability), or whether one rater makes two or more measurements of the same material (intra-rater reliability). An instrument with a Rho score of 1 will produce the same measurement each time it is used. In contrast, an instrument with a Rho score of 0 will produce markedly different measurements. The confidence interval of the intra-class correlation coefficient represents a range of possible Rho scores, on the basis of the sample data, for the population score (95% probability).

**Inter-rater reliability trial** The inter-rater intra-class correlation coefficients (Rho) for each movement tested with each of the range of motion tests are displayed in Table 1.
Table 2. Intra-rater reliability and standard error of the measurement for five methods of assessing shoulder range of motion.

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean±SD, (range)</th>
<th>ICC (Rho)</th>
<th>95% CI of ICC</th>
<th>SEM</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Visual estimation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flexion</td>
<td>143±21, (100-170)</td>
<td>0.59</td>
<td>0.28 - 0.85</td>
<td>13</td>
<td>±26</td>
</tr>
<tr>
<td>Abduction</td>
<td>135±33, (70-170)</td>
<td>0.60</td>
<td>0.30 - 0.86</td>
<td>21</td>
<td>±42</td>
</tr>
<tr>
<td>External rotation</td>
<td>47±19, (10-80)</td>
<td>0.67</td>
<td>0.38 - 0.89</td>
<td>11</td>
<td>±22</td>
</tr>
<tr>
<td><strong>Goniometry</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flexion</td>
<td>135±26, (77-165)</td>
<td>0.53</td>
<td>0.21 - 0.82</td>
<td>17</td>
<td>±34</td>
</tr>
<tr>
<td>Abduction</td>
<td>129±35, (56-170)</td>
<td>0.58</td>
<td>0.27 - 0.85</td>
<td>23</td>
<td>±46</td>
</tr>
<tr>
<td>External rotation</td>
<td>41±23, (0-80)</td>
<td>0.65</td>
<td>0.36 - 0.86</td>
<td>14</td>
<td>±28</td>
</tr>
<tr>
<td><strong>Still photography</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flexion</td>
<td>128±28, (63-168)</td>
<td>0.56</td>
<td>0.24 - 0.84</td>
<td>19</td>
<td>±38</td>
</tr>
<tr>
<td>Abduction</td>
<td>131±35, (57-170)</td>
<td>0.61</td>
<td>0.31 - 0.86</td>
<td>22</td>
<td>±44</td>
</tr>
<tr>
<td>External rotation</td>
<td>48±21, (7-79)</td>
<td>0.60</td>
<td>0.30 - 0.86</td>
<td>13</td>
<td>±26</td>
</tr>
<tr>
<td>Stand and reach</td>
<td>39±19, (-22-59) cm</td>
<td>0.49</td>
<td>0.18 - 0.80</td>
<td>13cm</td>
<td>±26cm</td>
</tr>
<tr>
<td>Hand behind back</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(vertebral level)</td>
<td>9±2, (T6-S1)</td>
<td>0.14</td>
<td>-0.11 - 0.55</td>
<td>2</td>
<td>±4</td>
</tr>
<tr>
<td></td>
<td>25±7, (15-39) cm</td>
<td>0.39</td>
<td>0.08 - 0.75</td>
<td>6cm</td>
<td>±12cm</td>
</tr>
</tbody>
</table>

Intra-rater reliability and standard error of the measurement for five methods of assessing shoulder range of motion. Each test was performed on nine subjects, on three occasions by a single trained rater. Intra-class correlation coefficients (Rho) were calculated using a two-way mixed effect model. Mean, standard deviation and range of scores were calculated from the raw data. (SD = standard deviation, ICC = intra-class correlation coefficient, CI = confidence interval, SEM = standard error of the measurement).
**Intra-rater reliability trial** For the movements of flexion, abduction and external rotation, the standard error of the measurement ranged from 11-21 degrees for visual estimation, 14-23 degrees for goniometry, and 13-22 degrees for still photography. For the stand and reach test, the standard error of the measurement was 13 centimetres. For the movement of hand behind back, measured passively to the vertebral level, and as an active reach, the standard error of measurement was two vertebral levels, and six centimetres, respectively.

**Discussion**

The results of this study demonstrated fair-good reliability for visual estimation, goniometry, still photography and the stand and reach test for the movements of shoulder flexion, abduction, external rotation and overhead reach in patients with a variety of orthopaedic shoulder disorders (rotator cuff repair, adhesive capsulitis and scapulothoracic fusion) when assessed by either an individual trained measurer or by a number of trained measurers. Hand behind back, whether tested passively or as an active reach, was found to be the least reliable movement of this study.

Williams and Callaghan (1990) have shown that in the normal shoulder, visual estimation when employed by an experienced clinician permits measurements that are as reliable as those obtained with a mechanical goniometer. Using one asymptomatic subject, Low (1976) showed that examiners from various clinical backgrounds were more consistent in measuring full elbow flexion and full wrist extension positions with a goniometer than by estimating the same angles by eye. While there is some evidence to suggest that the goniometer can be used reliably for measuring certain shoulder joint movements (Boone et al. 1978), studies of the shoulder in symptomatic patients are limited. In a comprehensive evaluation of passive shoulder range of motion in symptomatic subjects, Riddle (1987) reported variable results (Rho = 0.26-0.90 inter-rater; Rho = 0.87-0.99 intra-rater). The results of this study showed comparable reliability for visual estimation and goniometry for both the inter-rater (visual estimation Rho = 0.57-0.70; goniometry Rho = 0.64-0.69) and the intra-rater (visual estimation Rho = 0.59-0.67; goniometry Rho = 0.53-0.65) trials.

Photographic procedures have been shown by Fish and Wingate (1985) to be more reliable than goniometry for measurements of elbow joint position in an asymptomatic subject. In the present study, all movements measured by still photography (active range of motion) demonstrated fair-good reliability for both the inter-rater trial (flexion Rho = 0.73, abduction Rho = 0.73, external rotation Rho = 0.62) and the intra-rater trial (flexion Rho = 0.56, abduction Rho = 0.61, external rotation Rho = 0.60). In general, comparable reliability and measurement error was demonstrated for visual estimation, goniometry, and still photography. Of note, large confidence intervals were associated with these same tests for the inter-rater trial (visual estimation = ±28-38 degrees; goniometry = ± 28-50 degrees; still photography = ± 30-6 degrees) and the intra-rater trial (visual estimation = ±22-42 degrees; goniometry = ± 28-46 degrees; still photography = ± 26-44 degrees). On the basis of this data, large differences in repeated scores are therefore prerequisite when using these tests to assess actual change in clinical status.

We evaluated the stand and reach test as it is a functional movement of practical importance. The results of this study demonstrated fair to good reliability for the stand and reach test (inter-rater Rho = 0.74; intra-rater Rho = 0.49). Interestingly, this test was associated with a smaller standard measurement error for the inter-rater trial (3cm) than for the intra-rater trial (13cm).

Poor reliability was demonstrated for both hand behind back tests for both the inter-rater trial (Rho = 0.26 for passive hand behind back; Rho = 0.39 for active reach) and the intra-rater trial (Rho = 0.14 for passive hand behind back; Rho = 0.39 for active reach). These findings may be a reflection of the complexity of the movement itself. To effect the manoeuvre, contributions to range of motion are required from the shoulder complex, elbow, forearm, wrist and hand. Alternatively, the lower reliability associated with this movement may reflect an attenuated range of available scores. In the presence of certain shoulder joint pathologies, hand behind back range of motion is frequently diminished (Murrell and Walton 2001). There was a standard measurement error of two vertebral levels for passive hand behind back and six centimetres for active hand behind back reach for both the inter-rater and intra-rater trials. Hand behind back range of motion is of functional importance and one that warrants further reliability testing.

The intention of this study was not to compare the reliability of active and passive range of motion tests for the shoulder joint, in symptomatic subjects. Rather, this study established the reliability and measurement error of five range of motion tests currently performed in clinical practice.

Both the inter-rater and intra-rater trials were conducted over a relatively short timeframe. The reliability of these methods for longer reassessment timeframes requires that the raters do not change their technique. This study did not establish the reliability of shoulder range of motion assessments over time.

Estimates of measurement reliability are applicable to the range of test scores and to the conditions of assessment. The mean values obtained for flexion, abduction and external rotation, for the inter-rater and intra-rater trials (Table 1, 2) show that most subjects obtained scores for these movements in the upper ranges of motion. We did not determine the reliability of these tests for scores measured primarily in the lower ranges of motion.

In summary, fair to good reliability was demonstrated for three shoulder movements assessed by visual estimation,
Reliability of 3 methods for assessing shoulder strength

Kimberley Hayes, BApSc(Physiotherapy),a,b Judie R. Walton, PhD,1 Zoltan L. Szomor, MD,a and George A. C. Murrell, MBBS, DPhil,a Sydney, Australia

The reliability of tests for isometric strength of the shoulder joint in symptomatic subjects has yet to be established. For this purpose, interrater and intrarater agreement trials were undertaken to ascertain the reliability of manual muscle tests, a handheld dynamometer, and a spring-scale dynamometer for 5 different shoulder movements in symptomatic subjects. Intraclass correlation coefficients were calculated from a random-effects model. All movements tested with the handheld dynamometer demonstrated excellent reliability for the interrater trial ($p = 0.79-0.92$). Excellent reliability was also demonstrated for elevation, external rotation, and internal rotation for the intrarater trial ($p = 0.79-0.96$). For the interrater trial, measurement of the lift-off maneuver with the handheld dynamometer was significantly more reliable than with manual muscle tests ($p = .002$). In summary, the handheld dynamometer was the most reliable and discriminatory means for assessing strength of the rotator cuff in symptomatic subjects. ([Shoulder Elbow Surg 2002;11:33-9.]

INTRODUCTION

Disorders of the rotator cuff are the most common cause of shoulder pain and disability.2,3,4 Despite the prevalence of disorders of the rotator cuff and the many methods of managing them, little has been done to establish the reliability of an isometric strength assessment in patients with rotator cuff dysfunction.

Reliability is defined as the extent to which a measurement is repeatable20 and can be estimated from measurements made by a single rater or multiple raters on the same material (agreement). Two different types of agreement can be distinguished according to whether one rater makes two or more measurements of the same material (intrarater agreement) or each of several raters independently measures the same material (interrater agreement).

There have been many studies on the reliability of strength assessment tests* and the variables associated with measurement error.† However, the majority have been conducted on either subjects with normal shoulders3,5,6,8,11,17,32 or neurologically impaired patients,1,3,4,9,16,18,29,33 A limited number of studies have included patients with various orthopaedic conditions9,21,33; however, in these cases the exact pathology has been poorly specified. To our knowledge, there are no published studies that have investigated the reliability of isometric strength assessment for the shoulder in patients with disorders of the shoulder.

The aim of this study was to determine the interrater and intrarater reliability of 3 tests for assessing isometric shoulder muscle strength in patients with a range of shoulder dysfunctions.

MATERIALS AND METHODS

Two separate groups of subjects gave informed consent for trials that investigated the interrater and intrarater reliability of 3 muscle strength assessment tests.

The interrater reliability trial consisted of 8 volunteers, 3 men and 5 women, ranging in age from 57 to 72 years (mean, 66 years). All subjects had a current shoulder complaint. Six patients had undergone rotator cuff repair surgery within the past 24 months, 1 patient was 17 months post scapulothoracic fusion, and 1 patient had adhesive capsulitis.

The intrarater reliability trial consisted of 9 volunteers, 5 men and 4 women, ranging in age from 29 to 74 years (mean, 64 years). One of these subjects had 2 symptomatic shoulders. Of the 10 shoulders, 8 were symptomatic, being within 36 months of rotator cuff repair surgery. The remaining 2 shoulders were asymptomatic at the time of testing.

Four raters, identified here as A to D, were used for the intrarater reliability trial. Rater A was an orthopaedic surgeon; rater B was a sports physiotherapy trainee, and raters C and D were qualified physiotherapists. All raters tested all 8 subjects with the 3 muscle strength assessment tests. Each rater was blinded to the results of the other raters' assessments.

Only rater A was used for the interrater reliability trial. This rater tested all 9 subjects (including the one subject being tested bilaterally) on 3 separate occasions within a

*References 1, 3-6, 8, 9, 11, 16-18, 21, 27, 29, 32, 33.
†References 10, 12, 13, 22, 23, 25, 26, 28, 30.
48-hour period, using the same 3 muscle strength assessment tests.

The 3 tests used for measuring isometric muscle strength for both the interrater and intrarater trials comprised manual muscle tests, testing with a handheld dynamometer, and testing with a spring-scale dynamometer. Manual muscle tests were used for the movements of elevation, external rotation, internal rotation, and hand behind back lift-off. We graded muscle strength as follows: grade 1, trace or absent muscle contraction as determined by observation and palpation of the tendons and muscle bulk; grade 2, subject was able to move the affected extremity through its range of motion with gravity eliminated; grade 3, subject had antigravity power and could hold a mid-range position against a low load resistance applied by the examiner (submaximal resistance for that individual); grade 4.5, subject had antigravity power and could hold a mid-range position against a greater resistance applied by the examiner (greater resistance than for grade 4 and lesser resistance than for grade 5); and grade 5, subject had antigravity power and could hold a mid-range position against a resistance that the examiner considered to be maximal for that individual.

A handheld dynamometer (PowerTrack MMT; JTech Medical Industries, Alpine, Utah, and Muscletester; Hoggan Health Industries, South Draper, Utah) was used for the movements of elevation, external rotation, internal rotation, and hand behind back lift-off. This device had a force-measuring capacity of 4.4 to 445 N, in 4.4-N increments (Figure 1).

A spring-scale dynamometer (Manley 2012 spring scale; Manley Tool and Machine, Independence, Mo) (Figure 2) was used for the movements of elevation, external rotation, internal rotation, and adduction. The lift-off maneuver was not measured with this device because it was not possible to perform this test with the spring-scale dynamometer. This device had a force-measuring capacity of 0 to 25 kg, in 0.25-kg increments. Velcro cuff loops were secured to either end of the spring scale to allow both subject and rater attachment. This device was tested as a possible cost-effective alternative to the handheld dynamometer.

For the interrater trial, all raters were briefed on the study protocol and a 5-hour training session was conducted to ensure familiarization and standardization of the 3 muscle strength tests. The intrarater trial was conducted several weeks after the interrater trial and involved a different group of subjects. Both trials followed the procedures outlined below.

For each test, raters were asked to ensure that resistance was applied perpendicular to the limb segment at the appropriate joint angle and that adequate manual stabilization was provided to prevent compensatory muscular action. A "make" test procedure6,7 was used for all 3 tests to determine the isometric strength for each movement. The subject was asked to build to a maximum contraction over a 1- to 2-second period and to hold the maximum effort against applied resistance for a further 4 to 5 seconds. The recorded measure reflected the maximum isometric value.
achieved by the individual. Each rater performed all 3 tests on a given subject before starting the assessment of the next subject. This procedure was repeated until all 9 subjects had been measured by all 4 raters. With the exception of manual muscle tests, which were always performed first, the order of the other 2 tests was random. This was done to prevent inadvertent biasing of the manual muscle grade with objective data such as those provided by the handheld dynamometer or spring-scale dynamometer. The following assessment protocol for the 3 muscle strength tests was used.

(1) Manual muscle tests

(a) Elevation. The subject was seated upright on the edge of the treatment table with the feet supported on a footstool. The rater stood in front of the subject and placed the affected extremity into a position of 90° of elevation 30° in front of the coronal plane. The palm of the hand faced downward for this test. The subject was asked to hold this position as the rater applied a downward resistance through the distal end of the dorsal forearm (Figure 3).

(b) External rotation. The subject was positioned as for position 1a, and the rater stood to the subject's affected side. The subject's affected extremity was placed midline with his or her side with 90° elbow flexion and neutral forearm pronation/supination. He or she was asked to hold this position as the rater applied a medially directed resistance through the dorsal aspect of the distal forearm. The rater provided stabilization to the distal end of the humerus with the non-testing hand to prevent unwanted humeral movement.

(c) Internal rotation. The subject, the rater, and the affected extremity were positioned as for position 1b. The subject was asked to hold this position as the rater applied a laterally directed resistance through the volar aspect of the distal forearm. The rater provided stabilization to the distal end of the humerus with the non-testing hand to prevent unwanted humeral movement.

(d) Hand behind back lift-off maneuver. This maneuver was first described by Gerber and Krushell15 as a method with which to test subscapularis strength. The subject was positioned as for position 1a, and the rater stood behind him or her. The subject's affected extremity was placed midline behind the back to a reach that was governed by the individual's upper limb flexibility. The dorsal forearm was clear of skin contact. The subject was asked to hold this test position as the rater provided an anteriorly directed force through the volar aspect of the distal forearm.

(2) Handheld dynamometer

(a) Elevation. The subject, the rater, and the affected extremity were positioned as for position 1a. The dynamometer was centered on the dorsal aspect of the distal forearm and was kept parallel to the ground throughout the testing procedure. The subject was asked to hold this position as the rater applied a downward force through the dynamometer.

(b) External rotation. The subject was in the supine position, and the affected extremity was supported on the treatment table in 90° abduction, 90° elbow flexion, and a mid-forearm position. The rater stood to the affected side of the subject and stabilized the medial aspect of the distal humerus with the non-testing hand. The dynamometer was centered on the dorsal aspect of the distal forearm and was kept perpendicular to the ground throughout the testing procedure. The subject was asked to hold this position as the rater applied a caudally directed force through the dynamometer.

(c) Internal rotation. The subject, the rater, and the affected extremity were positioned as for position 2b. The rater stabilized the lateral aspect of the distal humerus with the non-testing hand. The dynamometer was centered on the volar aspect of the distal forearm and was kept perpendicular to the ground throughout the testing procedure. The subject was asked to hold this position as the rater applied a rostrally directed force through the dynamometer.

(d) Hand behind back lift-off maneuver. The subject, the rater, and the affected extremity were positioned as for position 1a. The dynamometer was centered on the volar aspect of the distal forearm and was kept perpendicular to the long axis of the forearm throughout the testing procedure. The subject was asked to hold the test position as the rater applied an anteriorly directed force through the dynamometer.

(3) Spring-scale dynamometer

(a) Elevation. The subject, the rater, and the affected extremity were positioned as for position 1a. The superior cuff of the spring-scale dynamometer was looped around the distal aspect of the subject's elevated forearm, and the subject was asked to hold this test position as the rater applied a downward force through the inferior cuff attachment. The body of the spring-scale dynamometer was kept perpendicular to the ground throughout the testing procedure.

(b) External rotation. The subject and the affected extremity were positioned as for position 1b. The rater stood in front of the subject. The superior cuff of the spring-scale dynamometer was looped around the distal aspect of the subject's affected forearm, and the subject was asked to hold this position as the rater applied a medially direct-
ed force through the inferior cuff attachment. The body of the spring-scale dynamometer was kept parallel to the ground throughout the testing procedure.

(c) Internal rotation. The subject, the rater, and the affected extremity were positioned as for position 1b. The superior cuff of the spring-scale dynamometer was looped around the distal aspect of the subject’s affected forearm, and the subject was asked to hold this position as the rater applied a laterally directed force through the inferior cuff attachment. The body of the spring-scale dynamometer was kept parallel to the ground throughout the testing procedure.

(d) Adduction. The subject stood, and the rater faced the affected side of the subject. The subject’s affected extremity was abducted 30° from the neutral position. The superior cuff of the spring-scale dynamometer was looped around the distal aspect of the subject’s affected extremity, and the subject was asked to hold this position as the rater applied a laterally directed force through the inferior cuff attachment.

For the interrater and intrarater reliability trials, 2-way random-effects intraclass correlation coefficients (p), together with confidence intervals, were calculated with SPSS (SPSS, Inc, Chicago, Ill) statistical software. In accordance with the suggestions of Fleiss, a p value of less than 0.4 was deemed as representing poor reliability; 0.4 to 0.75 as fair to good reliability; and greater than 0.75 as excellent reliability.

In addition to determining the 2-way random-effects intraclass correlation coefficient, we calculated the Pearson correlation coefficient (r) for movements assessed with the handheld dynamometer and the spring-scale dynamometer. This correlation coefficient (r) was used as a measure of association for the 2 objective strength tests.

### RESULTS

The interrater intraclass correlation coefficients (p) for each movement tested with each of the strength tests are displayed in Table I. For all tests performed with the handheld dynamometer (elevation, external rotation, internal rotation, and lift-off) and for most tests performed with the spring-scale dynamometer (elevation, internal rotation, and adduction), agreement between the 4 raters was excellent (p = 0.79-0.92 and p = 0.75-0.96, respectively). Agreement for external rotation, as assessed by the spring-scale dynamometer, was fair to good (p = 0.75).

Correlation between the 2 types of dynamometer measurements was high, with Pearson correlation coefficients (r) of 0.88 for elevation, 0.77 for external rotation, and 0.82 for internal rotation (p < .0001).

For the 4 movements assessed by manual muscle tests (elevation, external rotation, internal rotation, and lift-off), agreement between the 4 raters ranged from poor to fair to good (p = 0.36-0.72). The lift-off maneuver, as assessed by manual muscle tests, was the least reproducible movement of the trial (p = 0.36). The same movement assessed by the handheld dynamometer was significantly more reliable than the manually tested lift-off maneuver (p = 0.79, p < .002).

Figure 4, A and B graphically represent the distribution of raw data for the lift-off maneuver as assessed by manual muscle tests and the handheld dynamometer, respectively. Interestingly, subjects 1 and 2 were assigned strength grades for the lift-off maneuver that ranged from grade 3 (no force-generating capacity against resistance) to grade 5 (maximal force-generating capacity) by raters using manual muscle tests (Fig-
coefficients that were as low as

can ordinal grade to the test muscle group, the examin-

was fair to good

with what he or she considers to be normal for age,

was high, with Pearson correlation coefficients (r) of 0.99 for elevation, 0.85 for external rotation, and 0.86 for internal rotation (P < .00001).

For the 4 movements assessed by manual muscle tests, agreement between the 3 measurement occasions was excellent for elevation (p = 0.79), external rotation (p = 0.86), and internal rotation (p = 1.00), but poor for the lift-off maneuver (p = 0.29).

Data for internal rotation measured by the spring-scale dynamometer was converted from kilograms to Newtons of force, to allow for direct comparison with the handheld dynamometer. For this movement, all subjects were assigned grade 5 muscle strength, by means of manual muscle tests. In contrast, the same subjects effected strength outcomes that ranged from 50 to 175 N with the handheld dynamometer and 49 to 196 N with the spring-scale dynamometer.

DISCUSSION

The results of this study show that the handheld dynamometer and the spring-scale dynamometer are reliable methods for assessing isometric shoulder muscle strength in subjects with shoulder joint disease. The reliability of manual muscle tests was less consistent than that of the handheld dynamometer and the spring-scale dynamometer, as demonstrated by correlation coefficients that were as low as 0.29.

Manual muscle tests are the most widely used method of physical examination for clinical evaluation of muscle strength. However, manual muscle tests are also recognized as being a subjective measurement of strength, dependent on the experience, strength, and judgment of the examiner. In assigning an ordinal grade to the test muscle group, the examiner relies on an internal basis for comparing test results with what he or she considers to be normal for age, sex, and weight of the individual. The results of our study support the argument of individual examiner bias in the use of manual muscle tests. This was well demonstrated in the interrater trial (Figure 4, A) for the lift-off maneuver, in which raters judged subjects 1 and 2 as having lift-off strength that ranged from no force-gener-

Figure 4 Variations in lift-off strength measurements made by 4 raters in 8 subjects for manual muscle tests (A) and handheld dynamometer (B).

ating capacity against manual resistance (grade 3) to maximal force-generating capacity (grade 5). Further, manual muscle tests generally had better intrarater reliability than interrater reliability. These results suggest that an examiner is reliable in reproducing a muscle grade in accordance with his or her own grading system, but less able to reproduce a second examiner's outcome under similar measurement conditions.

A further criticism of manual muscle tests has been their inability to detect small differences in muscle strength, particularly in the good to normal strength categories. This study confirmed the lack of discrimination between various strength outcomes when assessed by manual muscle tests. The ability of manual muscle tests to discriminate between internal rotation power was particularly poor. For the intrarater trial,
internal rotation strength values varied from 50 to 175 N with the handheld dynamometer and from 49 to 196 N with the spring-scale dynamometer. However, the same subjects were all deemed to have grade 5 strength when assessed by manual muscle tests. Thus the information obtained from manual muscle tests appears to be relatively imprecise and of less value in identifying changes in strength status.

In 1991 Gerber and Krushell described a lift-off test with which to evaluate the integrity of the subscapularis musculotendinous unit. In that study, patients with a subscapularis rupture were unable to effect the maneuver. More recently, electromyographic analysis of selected shoulder girdle muscles was conducted for 4 versions of the lift-off test to establish a precise starting position for the upper limb that would best isolate the internal rotation function of the subscapularis muscle from other synergists to this movement. For most versions of this test, the subscapularis muscle was shown to be highly active. However, a position of maximum internal rotation whereby the dorsum of the hand was placed in the midline of the posterior thorax at the level of the inferior aspect of the scapulae was shown to be the position best able to isolate the internal rotation function of subscapularis. For patients with supraspinatus tears, it may be particularly difficult to achieve this position, as this patient group has reduced range of active internal rotation. To our knowledge, the reliability of this test has not been determined.

Raters were poor at manually assessing strength in this position in these patients. When the same movement was measured with the handheld dynamometer, reproducibility was considerably improved (P = .002 in the interrater trial). By using a measurement test that provides a direct recording of force output, clinicians may be able to assess the integrity of the subscapularis complex with greater accuracy than that afforded by a subjective grading system.

The results of this study are in keeping with those of previous trials that have shown the handheld dynamometer to be a reliable measurement tool, from both an interrater and intrarater perspective, for the measurement of shoulder muscle strength in patients with normal shoulders and neurologically impaired patients. Intrarater reliability coefficients for various shoulder muscle groups have been stated to be as high as 0.95 to 0.994, 0.98,32 and 0.95 to 0.97,8 to a somewhat lower value of 0.69.33 Interrater reliability of the handheld dynamometer at the shoulder joint has not been examined as extensively. A value of 0.88 has been reported for shoulder external rotation,9 but to our knowledge, interrater reliability for other shoulder movements has not been reported.

Although its reliability has not been investigated as extensively as that of other strength assessment tests, there is evidence to suggest that reproducible measurements can be taken with a spring-scale dynamometer. The results of this study demonstrated excellent reliability for most movements assessed with the spring-scale dynamometer. In addition, high correlation was demonstrated for measurements taken with the spring-scale dynamometer and the handheld dynamometer. To its advantage, the spring-scale dynamometer is portable, is easy to operate, and is a cost-effective measurement tool (assembly cost, approximately $200). However, assessment of the lift-off maneuver was biomechanically impossible with the spring-scale dynamometer, because the instrument measured distraction force rather than compression force, which limits its usefulness in the assessment of subscapularis muscle strength.

In summary, reliable isometric strength measurement of the shoulder musculature in symptomatic subjects was shown with the handheld dynamometer and the spring-scale dynamometer. The reliability of manual muscle tests was found to be less consistent than that of the handheld dynamometer and the spring-scale dynamometer. Lift-off strength was unreliable when assessed manually. Thus, for a strength examination that requires information specific to all components of the rotator cuff, the handheld dynamometer is the strength assessment test of choice.

We wish to thank the patients and clinicians (Larry Bryant, MBBS, BSc, Kim Wade, BAppSc(Physiotherapy), and Sophia Short, BAppSc(Physiotherapy) who assisted in this study. We also thank Robert Crouch (Precision Fitness Industries, Hindmarsh, Adelaide, South Australia) and Karen Ginn (Senior Lecturer, Department of Biological Sciences, Health Sciences Faculty, University of Sydney) and acknowledge the support of the St George Private Hospital/Health Care of Australia and the Sutherland Hospital, South Eastern Sydney Area Health Service.

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The glenohumeral joint relies on muscular control for dynamic stability, and the rotator cuff muscles are the main muscular stabilizers of this joint. Supraspinatus tendinosis, histologically a noninflammatory degenerative tendinopathy, leads to lack of humeral head control and often subsequent symptoms and signs of subacromial impingement. No treatment is universally successful in managing this condition, which causes considerable time lost at work and recreation.

Topical glyceryl trinitrate (GTN) has been used for more than 100 years as a therapy for angina pectoris, and it is now accepted that the mechanism of action of the organic nitrates is through production of nitric oxide (NO), the endothelium-derived relaxing factor. Thus, organic nitrates such as GTN may be viewed as prodrugs of endogenous NO, and in this clinical trial, we chose to deliver NO via transdermal GTN patches because of their...
ease of dosing and of application. Topical GTN has previously been demonstrated to improve immediate short-term pain scores in patients with acute supraspinatus tendinopathy.\(^5\)

Inhibition of NO has been shown to reduce collagen content, contraction, and synthesis by wound fibroblasts in vitro,\(^24\) and in animal studies, NO synthase inhibition has resulted in a significant reduction in healing tendon cross-sectional area and load to failure,\(^16\) suggesting that NO resulted in a significant reduction in healing tendon cross-section. We aimed to assess if continuous topical GTN treatment altered outcome measures in patients with supraspinatus tendinopathy. A proposed mechanism for NO modulation of healing in tendons, which rely on fibroblastic production of collagen for repair. We aimed to assess if continuous topical GTN treatment altered outcome measures in patients with chronic supraspinatus tendinopathy when compared with tendon rehabilitation alone.

MATERIALS AND METHODS

Power analysis determined that to have a 90% chance of detecting a 40% alteration in pain, the study would require 25 subjects per group. The study was approved by our institutional ethics committee. All subjects were required to be older than 18 years, and patients were excluded if they had supraspinatus tendinopathy of less than 3 months’ duration, current pregnancy, previous surgery or dislocation of the affected shoulder or arm, distal neurologic signs, ischemic heart disease, or a local corticosteroid injection in the previous 3 months.

The diagnosis of supraspinatus tendinopathy was made only if subjects met 3 criteria: (1) clinical signs of impingement (positive Hawkins sign in internal rotation or impingement in 90° of forward flexion with forced external rotation), (2) pain with supraspinatus muscle testing in the “empty can” position, and (3) MRI evidence of high signal intensity without a frank tear in the supraspinatus tendon.

Fifty-three recruited patients (57 shoulders) were randomized into 2 groups (26 patients in the GTN group, 27 patients in the placebo group), and this process was controlled by the senior pharmacist at our institution, who also supervised the packaging of transdermal patches and their distribution to patients. Patients were allocated to either of the 2 clinical trial groups through coded randomization. One group received the active transdermal patch (one quarter of a 5 mg/24-h GTN patch [Nitro-Dur, Schering-Plough, New South Wales, Australia]), and the other group received a placebo transdermal patch (one quarter of a demonstration patch [placebo patch, Schering-Plough]). The patches were indistinguishable from each other.

The patches were distributed to the patients by the pharmacist (J.N.), along with a supply of 24 acetaminophen (paracetamol) tablets (500 mg) for use with possible headache. The transdermal patches were intact when distributed, and patients were required to cut the patches into quarters before application. Both the patients and the clinical examiner were blinded as to which group the patients were in.

Patients were instructed in the application of the patches at their initial visits. They were informed that the dosing regimen was one quarter of a transdermal patch to be applied daily to the affected shoulder. The patches were to be left in situ for 24 hours and then replaced with a new quarter patch. The site of application was demonstrated as immediately distal to the anterolateral edge of the acromion, and patients were instructed to rotate the patch application site around this point with each new patch application. Subjects used the patches until full symptom resolution, when the patches were discontinued and not reinstituted, or until the conclusion of the clinical trial.

At the initial clinical assessment, all patients were instructed in the performance of a tendon rehabilitation program. The aim of this program was to encompass the current best practice management for supraspinatus tendinopathy and involved (1) rest from aggravating activities in the early stages (particularly repetitive rotatory movements of the shoulder and overhead or behind-the-back activities that may cause impingement), (2) daily range of motion exercises, (3) daily stretching of the posterior shoulder capsule and pectoral muscles, and (4) a muscle-strengthening program initially involving scapular retraction exercises and closed kinetic chain isometric exercises and gradually progressing to dynamic open kinetic chain isotonic resistance exercises.\(^12,14\)

Despite the popularity of nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroid injections, the biologic basis of their effect in histologically noninflammatory conditions such as tendinosis is unknown, and systematic evidence of anything more than a small, short-lived effect is lacking.\(^6,18,24\) Prospective, randomized, controlled clinical trials investigating anti-inflammatory therapies—NSAIDs and subacromial corticosteroid injections—have demonstrated superior efficacy to placebo in improving shoulder abduction range of motion.\(^5,7,19\) These treatments have equivocal efficacy in improving shoulder pain, but there is no evidence of improvements in muscular strength or functional measures.\(^6\) For these reasons, we decided not to use NSAIDs or corticosteroid injections in this clinical trial, and it is why we excluded patients who had received corticosteroid injections within the preceding 3 months.

There is no type II evidence (randomized, controlled clinical trial) to support electrotherapeutic modalities (ultrasound, laser, and electrogalvanic stimulation) in the treatment of noncalcified human tendon.\(^20,25\) For this reason, these measures were not included in this trial.

Also at the initial visit and at all subsequent visits, the patients were required to complete a supraspinatus tendinopathy symptom assessment sheet using verbal descriptor scales to rate the severity (0-4: no pain, mild pain, moderate pain, severe pain, very severe pain) of their shoulder pain with activity, at rest, and at night. This sheet was also used to indicate changes in shoulder symptoms (eg, improvement or deterioration).

A single examiner (J.A.P.) assessed the patient and recorded the following: (1) the level of subacromial tenderness using a 4-point scale (0-3): no tenderness, mild tenderness, moderate tenderness, severe tenderness (based
on viewing patient response to palpation; (2) visual assessment of passive shoulder range of motion in abduction, forward flexion, external rotation, and internal rotation (hand behind back); (3) handheld dynamometer measurement of muscle force in “empty can” position (90° of abduction in the scapular plane with thumb pointing inferiorly),
adduction, external rotation, internal rotation, and subscapularis pushoff; and (4) impingement tests in internal rotation and external rotation.

All clinical assessments were repeated at weeks 0, 2, 6, 12, and 24 with an identical format. Records of headaches and acetaminophen (paracetamol) use were assessed from a headache diary that the patient was provided with at the start of the study, and compliance with patch application and the tendon rehabilitation program was also made at these scheduled visits.

The symptoms and signs used as outcome measures for assessing response to treatment in this clinical trial were patient-rated shoulder pain at rest (0-4); patient-rated shoulder pain with activity (0-4); patient-rated shoulder pain at night (0-4); subacromial tenderness (0-3); passive shoulder range of motion in abduction, forward flexion, external rotation (degrees), and hand behind back (centimeters from vertebra prominens); dynamometer-measured muscle force in “empty can” position, adduction, external rotation, internal rotation, and subscapularis pushoff (in newtons); and impingement tests in internal rotation and external rotation (0-1: negative or positive).

Outcome measures were analyzed with SigmaStat 2.0 statistical software (Jandel Scientific, San Rafael, Calif), using Mann-Whitney tests to compare differences between groups and using the Wilcoxon signed rank test to compare differences within the groups. The level of significance was defined at \( P = .05 \). A \( \chi^2 \) analysis of patient-reported symptom outcomes at week 24 was performed. Effect size estimates were calculated by dividing the mean \( z \) score at week 24 by the square root of the sample size.

RESULTS

Group Demographics

Fifty-three patients (57 shoulders) with a clinical and MRI diagnosis of supraspinatus tendinopathy were recruited through newspaper advertisements and private consulting rooms. There were 26 patients (28 shoulders) in the GTN group and 27 patients (29 shoulders) in the placebo group (Table 1). There were 24 men and 29 women, with a median age of 52 years (range, 25-79 years). The mean age in the placebo group was 49 years; the mean age in the GTN group was 53 years. In total, 50 patients were righthand dominant, with 30 of these patients having the dominant side affected, 18 having the nondominant side affected, and 2 patients with bilateral disease. Three patients were left-hand dominant, with 1 patient having the dominant side affected and 2 patients with bilateral disease. The median duration of symptoms was 14 months (range, 4-96 months). There were no differences between groups with respect to age, sex, affected side, or duration of symptoms.

Of the 53 patients originally recruited for the clinical trial, 2 patients discontinued use of the patch during the course of the study because of side effects (2 patients with severe and persistent headaches). Both of these patients were in the GTN patch group. No subjects were excluded because of poor compliance.

From the remaining 24 patients in the GTN patch group, there was 1 patient who dropped out of the clinical trial. This dropout was at the 12-week stage of the trial, and he dropped out because of an ipsilateral radius fracture sustained from a fall. Thus, there were 23 patients (25 shoulders) in the GTN patch group who completed the 6-month follow-up period.

In the placebo patch group, there were 2 dropouts. Both of these dropouts were within the first 4 weeks of the trial, and no reason was given. Thus, there were 25 patients (27 shoulders) in the placebo patch group who completed the 6-month follow-up period. There were no significant differences in the patient demographics or in the drop-out rates between the 2 treatment groups, and the statistical analysis was made on the basis of intention to treat. Analysis of the clinical trial outcome measures determined that the data were not normally distributed.

OUTCOME MEASURES

There were no significant between-group differences with forward flexion range of motion (\( P = .36 \)), external rotation range of motion (\( P = .17 \)), subacromial tenderness (\( P = .53 \)), or impingement in external rotation (\( P = .24 \)).

Week 2

The GTN group demonstrated a significant increase in subscapularis force (\( P = .01 \)) compared with the placebo group.

Week 6

The GTN group showed a significant increase in supraspinatus force (\( P = .01 \)) (see Figure 1A).
Week 12

These results are summarized in Figures 1A, 1B, and 2A. The GTN group showed significant decreases in shoulder pain at night \((P = .03)\) and shoulder pain at rest \((P = .04)\). There were significant increases in supraspinatus force \((P = .001)\), external rotation force \((P = .01)\), internal rotation force \((P = .01)\), subscapularis force \((P = .02)\), and adduction force \((P = .003)\).

Week 24

These results are summarized in Figures 1A, 1B, 2A, 3A, 3B, and 4. The GTN group had significant decreases in shoulder pain with activity \((P = .01)\) (see Figure 2B), shoulder pain at night \((P = .01)\), and shoulder pain at rest \((P = .03)\). There were significant increases in supraspinatus force \((P = .001)\), external rotation force \((P = .01)\), internal rotation force \((P = .01)\), subscapularis force \((P = .02)\), and adduction force \((P = .003)\). Range of motion was significantly increased in abduction \((P = .02)\) and internal rotation \((P = .04)\), and impingement in internal rotation (Hawkins sign) was significantly decreased \((P = .02)\). Patient-reported outcomes at week 24 showed that 46% of shoulders treated (13/28 shoulders) in the GTN group had excellent improvement (specifically defined as asymptomatic with activities of daily living) during the course of the trial. Forty-six percent of patients (13/28) rated their shoulders as moderately improved, and 7% of patients (2/28) rated their shoulders as poor after completion of the clinical trial. These results compared with 24% of patients who rated their shoulders as excellent (7/29), 66% of patients who rated their shoulders as moderately improved (19/29), and 10% of patients who rated their shoulders as poor (3/29) in the placebo group. A \(\chi^2\) analysis comparing outcomes between the 2 groups revealed that the GTN group had a significantly increased \((P = .007)\) chance of the shoulder being asymptomatic with activities of daily living at 24 weeks.

The mean estimated effect size at week 24 was 0.26 (95% confidence interval, 0.19-0.32). This measure was derived from the mean \(P\) value of all outcome measures at the week-24 stage, and it is equivalent to a binomial effect size display, or change in patient outcome success rates, of 26%. In other words, from an estimation of the size of the mean effect on outcome measures at week 24 in the GTN group, the GTN group had a significantly increased chance of the shoulder being asymptomatic with activities of daily living at 24 weeks.
group, it would be expected that the between-groups difference in successful outcomes (eg, asymptomatic at week 24) would be 26% of patients.

Post hoc analysis of group mean outcome measures demonstrates a general improvement in all outcome measures in the GTN group relative to the placebo group (see Figure 5). An analysis of the between-group means at week 0 compared with week 24 demonstrates that in the GTN group, patient-rated pain scores (with activity, at night, and at rest) decreased by a mean of 65% (range, 64%-67%), whereas the placebo group scores decreased by a mean of 30% (range, 27%-33%) (see Figure 5).

Passive range of motion measurements in the GTN group demonstrated range of motion increases by a mean of 24% (range, 17%-31%), whereas the placebo group increased by a mean of 8% (range, 4%-13%). Mean increases for the GTN group were 29° for abduction, 22° for forward flexion, 14° for external rotation, and 9 cm closer to the vertebra prominens for internal rotation (see Figure 5).

Dynamometer-measured shoulder force in the GTN group increased by a mean of 29% (range, 10%-61%), whereas the force in the placebo group increased by a mean of 12% (range, 5%-32%). Mean increases for the GTN group were 15.2 N for supraspinatus testing, 11.8 N for external rotation, 13.7 N for internal rotation, 15.4 N for subscapularis pushoff, and 6.3 N for adduction testing. These results compare with the placebo group mean increases of 4.1 N for supraspinatus testing, 2.2 N for external rotation, 2.9 N for internal rotation, 7 N for subscapularis pushoff, and 3.6 N for adduction testing (see Figure 5). The adduction force was included in the trial testing to act as a control measurement because this measurement should not be affected by supraspinatus function, and the movement is not controlled by muscles of the rotator cuff. It was interesting to see that in the placebo group, adduction force did increase by 10%, but in the GTN group, the increases in rotator cuff force, and in particular...
the increase in supraspinatus tendon force, were at least
twice the magnitude.

Impingement signs in the GTN group decreased by a
mean of 76% (range, 74%-78%), whereas the signs in the
placebo group decreased by a mean of 43% (range, 40%-47%) (see Figure 5).

SIDE EFFECTS

The side effects are summarized in Table 2. In the GTN
group, there was a significant increase in the number of
days affected by headache (P = .001) and in the amount of
acetaminophen (paracetamol) required for headache (P = .001) during the course of the trial. In the GTN group, 70%
of headaches were experienced within the first 2 weeks,
and only 2% of headaches occurred after week 12; these
results are in comparison with the placebo patch group, in
which 62% of headaches were experienced within the first
2 weeks, and 9% of headaches occurred after week 12.

With regard to headaches, the GTN group had a total of
127 days with at least 1 headache, a mean of 8.5 days per
person of those patients who experienced this side effect,
and a median of 3.5 days (range, 0-32 days). These patients
with headaches required a total of 138 acetaminophen
(paracetamol) tablets (500 mg) during the clinical trial, a
mean of 9 tablets over the 6-month period, and a median
of 2 tablets (range, 0-25 tablets).

The placebo patch group reported a total of 37 days with
at least 1 headache, a mean of 4.1 days per person of those
patients who experienced this side effect, and a median of
0 days (range, 0-9 days). These patients with headaches
required a total of 69 acetaminophen tablets during the
course of the clinical trial, a mean of 8 tablets over the 6-
month period, and a median of 0 tablets (range, 0-15
tables).

DISCUSSION

The use of continuous topical GTN therapy for supraspinatus
tendinopathy resulted in significantly improved shoulder
pain with activity, at rest, and at night; significantly
increased shoulder range of motion in abduction, forward
flexion, and internal rotation; significantly increased
dynamometer-measured force with supraspinatus testing,
external rotation, internal rotation, subscapularis pushoff,
and adduction; and significantly decreased shoulder
impingement in internal rotation.

Forty-six percent of patients in the GTN group reported
their shoulders as asymptomatic with activities of daily
living at the completion of the 6-month clinical trial. This
result compares with 24% of patients in the placebo group
who were asymptomatic with activities of daily living at
week 24.

The calculated mean estimated effect size at week 24
was 0.26, which is equivalent to a binomial effect size display,
or change in patient success rate, of 26%. 21 This figure
is comparable with the 22% improvement with topical
NO therapy noted in patient-rated outcomes.

<table>
<thead>
<tr>
<th>Side Effect</th>
<th>Glyceryl Trinitrate Group (n = 26)</th>
<th>Placebo Group (n = 27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Headache</td>
<td>15 58***</td>
<td>9 33</td>
</tr>
<tr>
<td>Rash</td>
<td>3 12</td>
<td>1 4</td>
</tr>
<tr>
<td>No adverse effects</td>
<td>9 35</td>
<td>18 67</td>
</tr>
</tbody>
</table>

*All adverse effects were reversible, and some patients experienced
more than one side effect. There was a significant increase in the number of patients with headaches (***P < .001) and the level of
paracetamol use (P = .001) in the glyceryl trinitrate group.

These results are consistent with the hypothesis that
topical GTN therapy demonstrates significant improve-
ments in symptomatic and functional outcome measures
at the 6-month stage beyond that achieved through tendon
rehabilitation alone.

The mechanism of action of topical GTN (presumed NO
donation) on tendon is currently unknown. Any effect may
be mediated by either local or systemic action. It is known
that healing tendon, like most human soft tissues, relies
on fibroblastic production of collagen for repair. 15,22
Perhaps providing exogenous topical NO to the degenerate
tendon can substitute for any reduced levels of NO, or NO
synthase, and this NO donation may stimulate wound
fibroblasts to increase collagen synthesis and remodeling.
The site of action may also be the supraspinatus bursa,
interrupting the cycle of impingement. Other potential
mechanisms for this demonstrated improvement include
increased blood supply to the region due to local vasodila-
tion or increased clearance of local cellular mediators or
bioactive proteins such as substance P. There is also a pos-
sibility that NO may have an effect on the apoptotic
process 1,27 or have an effect on neural structures at a local
level that may modulate pain.

The tendon rehabilitation program that was used in this
clinical trial can be expected to provide shoulder improve-
ment such that 24% of patients are asymptomatic with
activities of daily living at 6 months. It should be noted
that this rehabilitation program was home based and not
strictly controlled except through verbal checks of compli-
ance at each examination, but it still afforded fair results
and led to decreased tendon pain and impingement and
increased shoulder range of motion and force production.

Comparing the outcomes of our placebo group (rehabili-
tation only) with other studies of nonoperative treatment
of supraspinatus tendinopathy is interesting. Blair et al, 3
in their prospective study on corticosteroid injections for
subacromial impingement syndrome, a clinical and radio-
logic diagnosis, had a control group of 21 subjects who
underwent a similar rehabilitation program involving pas-

TABLE 2

Numerical Breakdown of Side Effects
and Paracetamol Use in the
Glyceryl Trinitrate Group and the Placebo Group

<table>
<thead>
<tr>
<th>Side Effect</th>
<th>Glyceryl Trinitrate Group (n = 26)</th>
<th>Placebo Group (n = 27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Headache</td>
<td>15 58***</td>
<td>9 33</td>
</tr>
<tr>
<td>Rash</td>
<td>3 12</td>
<td>1 4</td>
</tr>
<tr>
<td>No adverse</td>
<td>9 35</td>
<td>18 67</td>
</tr>
</tbody>
</table>

*All adverse effects were reversible, and some patients experienced
more than one side effect. There was a significant increase in the number of patients with headaches (***P < .001) and the level of
paracetamol use (P = .001) in the glyceryl trinitrate group.
sive assisted range of motion exercises and isometric rotator cuff strengthening that then progressed to isotonic strengthening. This group had a mean duration of symptoms of 8 months and were followed up for 28 weeks, and the results showed 19% of subjects had no impingement signs, there was a 17% reduction in pain, and 41% of patients had increased range of motion in forward flexion (mean, 10°) and in external rotation (mean, 5°), but there was no increase in functional status. Morrison et al\textsuperscript{15} performed a retrospective study on nonoperative management of subacromial impingement syndrome, a clinical diagnosis, in 616 subjects with a mean duration of symptoms of 16 months and a mean follow-up period of 27 months. They used NSAIDs and an exclusively isotonic exercise regimen, and there was a 64% reduction in impingement signs, 9% of patients were asymptomatic at 6 months, 30% of patients had an excellent outcome, and 37% had a good outcome.

Considering that patients in our study had chronic supraspinatus tendinopathy, with a median duration of symptoms of 14 months, the results of tendon rehabilitation alone compared favorably with previously published rehabilitation outcomes. This finding indicates that a structured, evidence-based tendon rehabilitation program can be useful in treating supraspinatus tendinopathy, even if home based and unsupervised. Patient compliance was excellent, with no patient excluded because of lack of compliance, and this result may be because of the thorough explanation of the rehabilitation program, both oral and written; the regular examinations and checks of compliance; or the personalities of patients enrolling in clinical trials.

The major side effects of this medication are headache, weakness, or dizziness (symptoms of hypotension) and local skin irritation or rash at the application site. It is wise to exercise caution in patients with marked anemia, those who are currently pregnant, those with known hypersensitivity to organic nitrates with prior episodes of hypotension, sufferers of migraine headaches, or those on diuretic therapy. A relative contraindication to GTN therapy is severe ischemic heart disease, especially if the patient requires other nitrate vasodilators for angina pectoris. It is important for patients to maintain adequate hydration, and hence blood volume, during nitrate therapy.\textsuperscript{25}

It was necessary to discontinue 8% of patients (2/26 patients) in the GTN group owing to the severity of headache. This side effect occurred despite a continuous dosing regimen aimed at achieving a tolerance to the vascular effects of NO. The incidence of headache and acetaminophen (paracetamol) usage for this side effect was significantly higher in the GTN group. In patients receiving topical NO therapy who experienced headaches, the mean was 8.5 days of the 6-month period, or 1.5 days of headache per month. The incidence of headaches lessened with time.

All topical therapies may cause cutaneous lesions in patients with contact allergies or sensitive skin, and this effect is something that cannot be easily avoided. It is known that there is variable transdermal GTN absorption across a population, with 5- to 10-fold individual variabil-

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\textbf{REFERENCES}