Low-level mechanical stimulation is sufficient to improve tendon healing in rats

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Andersson T, Eliasson P, Hammerman M, Sandberg O, Aspenberg P. Low-level mechanical stimulation is sufficient to improve tendon healing in rats. J Appl Physiol 113: 1398–1402, 2012. First published August 30, 2012; doi:10.1152/japplphysiol.00491.2012.—Treatment of tendon injuries often involves immobilization. However, immobilization might not prevent mild involuntary isometric muscle contraction. The effect of weak forces on tendon healing is therefore of clinical interest. Studies of tendon healing with various methods for load reduction in rat Achilles tendon models show a consistent reduction in tendon strength by at least half, compared with voluntary cage activity. Unloading was not complete in any of these models, and the healing tendon was therefore still exposed to mild mechanical stimulation. By reducing the forces acting on the tendon even further, we now studied the effects of this mild stimulation. Rat Achilles tendons were transected and allowed to heal spontaneously under four different loading conditions: 1) normal cage activity; 2) calf muscle paralysis induced by botulinum toxin A (Botox); 3) tail suspension; 4) Botox and tail suspension, combined, to eliminate even mild stimulation. Healing was evaluated by mechanical testing after 8 days. Botox alone and suspension alone both reduced tendon callus size (transverse area), thereby impairing its strength compared with normal cage activity. The combination of Botox and suspension did not further reduce tendon callus size but drastically impaired the material properties of the tendon callus compared with each treatment alone. The peak force was only a fifth of that in the normal cage activity group. The results indicate that also the mild loading that occurs with either Botox or suspension alone stimulates tendon healing. This stimulation appears to affect mainly tissue quality, whereas stronger stimulation also increases callus size.

Achilles tendon; mechanical stress; wound healing; hindlimb unloading; immobilization

ALTHOUGH MECHANICAL STIMULATION is important for tendon healing, the consequences of complete absence of such stimulation are unclear. Studies of mechanical stimulation of tendon healing depend on the ability to create in vivo models where loading can be removed. Such models include Achilles tendon healing with fore-foot amputation (5), tail suspension (1), muscle paralysis with Botox (4), nerve transection (5), or external immobilization, e.g., in a plaster (11). In rat models, all these methods have reduced the strength of the healing tendon compared with voluntarily loaded controls, mainly by reducing callus size, and with minimal effects on the mechanical properties of the callus tissue per se. Because all these models have yielded a reduction of tendon strength between one-half and two-thirds, we believed that this corresponded to the full extent of the effect of mechanical stimulation. Moreover, the results in all the above-mentioned models suggested that reduced loading in vivo mainly decreases callus size (i.e., reduces proliferation and matrix synthesis) but has little effect on tissue differentiation and matrix organization.

Previous studies also show that short daily episodes of loading can improve the strength of an otherwise unloaded healing tendon (1). Loading can stimulate healing both when applied during the early inflammatory phase of healing as well as during the later, more proliferative phase (2). Recently, we have shown that only one single episode of loading is sufficient to improve the strength of the unloaded healing tissue (3).

The used methods for “unloading” may still allow a small amount of mechanical stimulation. Even with complete Botox-induced paralysis of the calf muscles (or with nerve transection), the remaining stiffness of the muscle will cause traction forces in the tendon with passive motion of the joint. With fore-foot amputation, hindlimb unloading by tail suspension, or immobilization in a plaster, the animals can still load the tendon by isometric contraction and with tail suspension also by scratching themselves.

To challenge the perception that the above models all had reduced loading below a threshold under which no stimulation occurred, we studied tendon healing when mechanical loading had been reduced further. This was achieved by combining tail suspension with Botox injection so that neither ground reaction forces nor isometric contraction or scratching could produce traction forces.

MATERIAL AND METHODS

Experimental Design

We used 40 female Sprague-Dawley rats, mean weight 203 g (SD 11, no differences between groups). The right Achilles tendon was transected and allowed to heal spontaneously under four different loading conditions: 1) normal cage activity; 2) calf muscle paralysis induced by Botox; 3) tail suspension; 4) Botox and suspension combined (10 rats in each group). The healing tendons were evaluated by mechanical testing and histological analysis 8 days after surgery (Fig. 1). This time point corresponds to the proliferative and matrix-forming phase of tendon healing in this model. The study was approved by the Regional Ethics Committee for Animal Experiments, and institutional guidelines for care and treatment of laboratory animals were adhered to. The rats were kept one per cage and were housed in rooms with temperature maintained at 21°C (normal cage rats) or 24°C (tail-suspended rats) and 12-h light/dark cycle. Rats were given food and water ad libitum.

Surgery

All rats were anesthetized with isoflurane gas (Forene, Abbot Scandinavia, Solna, Sweden), 5% in a chamber and then with 3.5% isoflurane on a mask. Antibiotics (25 mg/kg, Oxytetracycline, Enge mycin; Intervet, Boxmeer, Holland) were given preoperatively, and
analgesics (0.045 mg/kg, Buprenorphine, Temgesic; Schering-Plough, Brussels, Belgium) were given both pre- and postoperatively. The surgery was performed under aseptic conditions. The skin on the right hindlimb was shaved and washed with chlorhexidine ethanol. A transverse skin incision was made lateral to the right Achilles tendon, and the tendon complex was exposed. The plantaris tendon was removed to simplify the mechanical evaluation at the end of the experiment. Thereafter, the Achilles tendon was cut transversely, and a 3-mm full-thickness segment was removed. The tendon was left to heal spontaneously without any sutures, and the skin was closed by two stitches.

Unloading

**Botox.** Twenty rats were given intramuscular Botox injections to induce calf muscle paralysis (12) 3 days before tendon transection (Botox, Allergan, Irvine, CA). The rats were anesthetized with iso-flurane gas, and the right hindlimb was shaved. Botox was injected into the gastrocnemius lateralis and medianus and the soleus muscles. Flurane gas, and the right hindlimb was shaved. Botox was injected (Botox, Allergan, Irvine, CA). The rats were anesthetized with iso-

Mechanical Testing

Eight days after surgery, all 40 rats were euthanized with carbon dioxide. Eight rats from each group were used for mechanical testing. The right Achilles tendon together with the calcaneal bone and the gastrocnemius and soleus muscle complex were dissected free and harvested. They were kept moist, and the mechanical testing was performed within 1 h. Sagittal and transverse diameters of the mid-part of the callus tissue were measured with a slide caliper, and transverse area was calculated by assuming an elliptical geometry. The old tendon stumps were visualized by transillumination of the entire callus, and the distance between them was measured and referred to as the gap distance. The weight of the gastrocnemius and soleus muscle complex was recorded. For mechanical testing, the muscles were carefully scraped off to produce a fan of tendon fibers, and fine sand paper was used to fix the fibers in a metal clamp. The distance between the metal clamp and the calcaneal bone was used as an approximation of the tendon length. The calcaneal bone was fixated in a custom-made clamp in 30° dorsiflexion relative to the direction of traction. The tendon was mounted vertically via the metal clamp in the materials testing machine (100R, DDL, Eden Prairie). The machine pulled at a constant speed (0.1 mm/s) until failure. Peak force, stiffness, and energy uptake were calculated by the software of the testing machine. The investigator marked a linear portion of the elastic phase of the curve for stiffness calculation. Energy uptake was defined as the area under the curve up to the peak force. Peak stress and an estimate for elastic modulus were calculated afterward. Modulus was calculated as if the specimens were mechanically homogenous. Because parts of the specimen consist of the tendon stumps, we use the expression “estimate for e-modulus” since it at least should reflect the modulus of the callus component. All measurements and calculations were carried out by an investigator blinded for treatment allocation of the specimens.

Histological Analysis

Two rats from each group were used for histological analysis. The right Achilles tendon callus tissue was dissected free, harvested, and fixated in 4% phosphate-buffered formaldehyde. The specimens were dehydrated and then embedded in paraffin and sectioned parallel to the longitudinal axis of the tendon (5-μm sections). One slide per specimen, covering the full length of the tendon callus, was stained with Ehrlich hematoxylin and eosin. The slides were analyzed in a light microscope to look for obvious differences between slides from different groups. Considering the small numbers, the evaluation should be seen as a check that no dramatic differences occurred.

| Table 1. Weight of the calf muscle complex and mechanical properties of tendon callus 8 days after surgery |
|----------------|----------------|----------------|----------------|
|               | Normal Cage Activity (n = 8) | Botox (n = 8) | Suspension (n = 8) | Botox and Suspension (n = 8) |
| Muscle weight, g | 2.6 (5.0–2.4) | 1.9 (2.6–1.3) | 2.5 (2.8–2.1) | 1.8 (1.9–1.5) |
| Gap distance, mm | 9.9 (11.4–6.9) | 6.0 (6.3–4.4) | 8.8 (10.3–6.3) | 5.4 (7.1–4.8) |
| Transverse area, mm² | 8.6 (9.9–6.1) | 4.2 (5.4–3.6) | 4.4 (5.4–3.3) | 4.1 (5.3–2.9) |
| Peak force, N | 25.8 (28.9–17.2) | 8.6 (10.3–5.5) | 8.5 (11.8–7.1) | 4.8 (8.2–2.4) |
| Stiffness, N/mm² | 6.9 (8.5–5.2) | 4.5 (5.8–1.9) | 4.3 (4.8–3.1) | 2.1 (3.0–1.2) |
| Peak stress, N/mm² | 3.0 (3.9–2.1) | 1.9 (2.5–1.2) | 2.2 (3.1–1.6) | 1.2 (1.6–0.5) |
| Elastic modulus, MPa | 10.9 (11.6–8.0) | 10.3 (17.2–4.4) | 12.0 (16.7–8.2) | 4.7 (5.9–3.2) |
| Energy, Nmm | 63.3 (78.5–35.7) | 11.0 (14.3–5.7) | 11.6 (19.1–9.6) | 7.7 (19.3–4.0) |

Values are medians (ranges).
Table 2. P values for all measured variables

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To correct for multiple comparisons, only P values of <0.01 were considered significant.

Statistical Analysis

Because the variance appeared not to be similar between groups for some variables (according to Levene's test), nonparametric methods were chosen. The primary variable was peak force, which is the result of transverse area and peak stress. The two latter variables were therefore considered more interesting than other secondary variables. The goal of the study was to compare the three groups with different methods for load reduction, with the hypothesis that Botox and tail suspension together would show different results compared with each load reduction method alone.

First, all variables of Table 1 were analyzed with Kruskall-Wallis test, confirming heterogeneity between the groups for all variables. The data for the combination of Botox and tail suspension was then compared with the data from the other groups, using Mann-Whitney U-test. Also, data from Botox treatment was compared with data from tail suspension. To correct for multiple comparisons, only P values of <0.01 were considered significant.

RESULTS

General Observations

A clear effect of the Botox treatment could be seen on gait, since the rats were loading their entire paw and not only the fore paw as they normally do. The tail-suspended rats mainly held their ankle joint at ~90°, but they sometimes used the hind paw for scratching themselves. However, this behavior was not systematically analyzed. Apart from porphyria (red tears) during the first days, the rats appeared to tolerate tail suspension well.

Mechanical Testing

All tendons ruptured in the callus tissue. No slipping in the clamps was observed. In general, the combined regimen did not reduce callus transverse area but still reduced peak force, stiffness, peak stress, and estimate for e-modulus by about one-half compared with Botox or tail suspension alone (Tables 1–3).

The groups with suspension alone or Botox alone showed no statistically significant difference between them except for gap distance, which was lower in the Botox group. Transverse area, peak force, stiffness, peak stress, estimate for e-modulus, energy uptake, and muscle weight were all similar.

The group with both Botox and tail suspension combined had a transverse area similar to each unloading regime alone, but peak force, stiffness, peak stress, and estimate for e-modulus were all further reduced compared with Botox alone (P value for each was <0.006; Table 2). The same variables (together with muscle weight and gap distance) were also reduced by combined treatment compared with suspension alone (P value for each was <0.002; Table 2).

Histological Analysis

The reduction in callus size for unloaded tendons compared with loaded ones could be seen also in the histological specimens. No further differences, explaining the differences in the material properties, could be demonstrated.

DISCUSSION

The results indicate that light mechanical loading also stimulates healing. In its absence, the tendon callus was only able to withstand one-fifth of the force that a healing tendon with voluntary loading could hold. It also appears that there are different thresholds for mechanical stimulation of tissue quality vs. volume of the callus (Fig. 2). With almost complete absence of mechanical forces, a callus with poor mechanical tissue prop-

Table 3. Confidence intervals (95%) for the effect of different loading regimes during tendon healing
Material properties.

Fig. 2. Suggested theoretical model of mechanical stimulation of tendon healing. Increasing the loading first does not increase callus size (transverse area), whereas a further increase does. In contrast, material properties such as modulus and peak stress improve also with little loading. In reality, there might be a continuum rather than the illustrated thresholds.

properties is formed. With modest forces, these properties improve, with little change in callus dimensions. Only with strong forces do callus dimensions increase. The results correlate with a study on intact patellar tendons showing that the effects of unloading on the mechanical properties are dependent on the degree of unloading (7).

Patients with Achilles tendon ruptures have an intriguingly large variation in the stiffness of the healing tissue, both early and late during the healing process (9, 10). This variation reflects important clinical differences, because there is a correlation between early mechanical properties and late functional results (9, 10). These data are derived from cases with immobilization in a cast. Possibly, some patients with an immobilized ankle unknowingly perform mild isometric training in the cast, whereas others are completely relaxed (6). If also weak mechanical forces have strong effects on tendon healing, rather moderate differences in spontaneous patient behavior could explain the surprisingly large variation in stiffness and functional results. Moreover, our present results agree with clinical observations of healing human Achilles tendons during the period after plaster removal, when full loading has started. Between 7 and 19 wk after Achilles tendon rupture, the healing tendon increases dramatically in transverse area, without any improvement in elastic modulus (9).

Little is known about the molecular events behind mechanical stimulation of healing. The observation that weak forces improve mechanical qualities and stronger forces mainly increase tissue volume suggests that different transcription factors and possibly different mechanotransduction mechanisms might be involved.

The difference between Botox alone and suspension alone regarding gap distance agrees with previous data (unpublished observations) but is intriguing. Possibly, the explanation might lie in the resting position of the leg. With Botox, the animals are able to position the ankle joint passively in plantar flexion so that the tendon stumps are brought closer together, allowing the callus to contract more. With suspension, it seems the animals cannot passively plantar flex the joint, which is mainly kept in a neutral position, with ~90° of flexion.

This study has several weaknesses. Only one time point in the healing process was studied, and it is possible that the effects of different degrees of loading are different during different phases of healing. We have not measured the forces acting on the tendon in the different models. Although the results suggest different “thresholds” for mechanical stimulation of quality vs. volume, this could in reality reflect a continuum. The lack of significant differences in transverse area among the three groups with reduced loading could be due to a type 2 error. However, the relative variation was not larger than for other variables, and there was a statistically significant difference between these groups and the one with voluntary loading. The histological part of the study did not allow conclusions regarding tissue quality and was made only to provide general information about the appearance of the tissue. Quantification of collagen alignment and cell density with sufficient statistical strength would require a larger number of specimens than we were able to achieve.

In conclusion, our results suggest that even mild loading stimulates tendon healing and that this stimulation appears to affect mainly tissue quality, whereas stronger stimulation also increases callus size. The observation that stimulation occurs also with weak forces indicates that mechanical stimulation is even more important than suggested by previous data.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


