Histological Changes in the Proximal and Distal Tendon Stumps Following Transection of Achilles Tendon in the Rabbits

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ABSTRACT
Objective: To determine tendon stump changes following unrepaired Achilles tendon lacerations in an animal model.
Study Design: An experimental study.
Place and Duration of Study: King Saud University, Riyadh, Saudi Arabia, from October 2013 to January 2014.
Methodology: A rabbit model was developed and studied tendon retraction and histological changes in the proximal and distal stumps following transection of the Achilles tendon.
Results: Over a period of 12 weeks, retraction of the distal tendon stump was minimal (2 - 3 mm). In contrast, retraction of the proximal tendon stump peaked to reach 6 mm at 4 weeks post-injury and plateaued to reach 7 - 8 mm at the 12-week interval.
Conclusion: Following complete transection of the Achilles tendon, tendon retraction correlated with the density of myofibroblast expression within the tendon stump. Further research is needed to investigate the pathophysiology of these findings.


INTRODUCTION
Achilles tendon injuries are common and may result from either lacerations or spontaneous ruptures.1-3 Surgical exploration of neglected Achilles tendon injuries reveals a gap at the injury site which is attributed to a combination of muscle and tendon retraction.4 Muscle retraction occurs only proximally and is responsible for most of the gap seen between the proximal and distal ends of the tendon. Tendon retraction occurs proximally and distally at the tendon stumps.5
To overcome the resulting gap in the Achilles tendon, several innovative techniques have been proposed such as the use of allografts,6 tendon transfers,7,8 and free autogenous grafts.9 No previous studies were found in the literature investigating tendon stump changes following unrepaired Achilles tendon lacerations. This experimental study was designed to investigate the histological changes in the proximal and distal tendon stumps following complete transection of the Achilles tendon.

METHODOLOGY
This animal study was approved by the Institutional Review Board of our Institution.

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The experimental model utilized female New Zealand rabbits (4 - 6 months-old weighting 2.5 - 3 kg). Ketamine was used for anesthesia. A longitudinal incision was made in the skin of the left hind limb over the Achilles tendon. A transverse complete transection of the Achilles tendon was made 2 cm proximal to its bony insertion. The length of the proximal tendon stump was measured from the musculo-tendinous junction while the length of the distal stump was measured from the point of bony insertion. Measurements were done with a ruler in mm by the same person in all rabbits. Histology of the cut ends of the tendons were studied using Hematoxylin and Eosin (H&E), Masson's trichrome (which stains collagen green), and α-smooth muscle actin (α-SMA, used for the detection of myofibroblasts) stains. A total of 18 rabbits were used and were divided into 6 groups (3 rabbits in each group) to study tendon measurements and histology at 7 intervals following tendon transection: day 3, week 1, week 2, week 4, week 5, and week 12. In each rabbit, tendon stump lengths were measured immediately following tendon transection and at each time interval. Tendon stump shortening was calculated by subtracting the latter length from the former. Besides the 18 experimental rabbits, one extra rabbit was used to study the normal histology of the Achilles tendon.

The specimens of both ends of the tendons were preserved in 4% paraformaldehyde then fixed in 10% neutral buffered formalin in 0.1 M phosphate buffer (pH 7.4) for 24 hours. The specimens were processed and embedded in paraffin blocks to prepare 5-µm-thick paraffin sections. After de-paraffinization in xylene and hydration in descending grades of ethanol, these sections were stained with H&E for routine histological
examination and Masson’s trichrome stain for demonstration of the vasculature and collagen fibers.

For detection and localization of myofibroblasts by detection of \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA) in the cytoplasm of these cells, paraffin sections were fixed on charged slides, then deparaffinized, rehydrated and washed in 0.05 M tris-buffered saline (TBS) pH 7.6. Immunostaining was performed using a mouse monoclonal fixed in paraformaldehyde 4% containing 0.2% glutaraldehyde. After fixation, specimens were embedded in paraffin, and 3 mm thick sections were cut and fixed on glass slides. Representative sections from each specimen were stained with H&E and Masson’s trichrome stains for light microscopy and immunohistochemical studies were carried out using a mouse monoclonal antibody recognizing \( \alpha \)-SMA (clone 1A4, ab7817). The stained sections were evaluated and photographed using Olympus BX51 bright field microscope equipped with an Olympus DP72 camera (Olympus Corporation, Japan).

**RESULTS**

All rabbits tolerated the surgical procedure well. There were no skin wound complications. Tendon stump shortening values in the proximal and distal stumps are shown in Table I. Distally, tendon shortening over the 12-week study period was minimal (2 - 3 mm). In contrast, initial shortening of the proximal tendon stump was 2 - 3 mm, and then became more pronounced (6 mm) at 4-week post-injury, and finally reached 7 - 8 mm at 12 weeks.

Histology of the normal Achilles tendon (Figure 1) at the area of transection showed thick parallel collagen bundles (Figure 1B) with absence of \( \alpha \)-SMA immune-positive cells (Figure 1C). The normal native spindle-shaped fibroblasts were best seen in the H&E stain (Figure 1A).

In experimental tendons, the histological changes during the first 5 weeks at the proximal and distal cut ends were similar when viewed using the H&E and Masson trichrome stains. At 3 days, a hemorrhagic clot was seen at the cut end of the tendon. This was replaced by granulation tissue with extensive extravasation of red blood cells at 1 week. By 2 weeks, the hemorrhage has resolved. Cellular infiltrate appeared within the collagen bundles at 4 weeks and this infiltrate became more pronounced at 5 weeks. At 12 weeks, the final histological appearance of the proximal tendon stump was different from the appearance of the distal stump. Proximally, the tendon stump appeared atrophic with loss of normal thick collagen bundle arrangement. In contrast, the distal end maintained a more normal

**Table I:** Tendon stump shortening following complete transection of the Achilles tendon in the rabbit model.

<table>
<thead>
<tr>
<th>Time after Achilles tendon transection</th>
<th>Number of tendons studied</th>
<th>Tendon stump shortening in each proximal stump</th>
<th>Tendon stump shortening in each distal stump</th>
</tr>
</thead>
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<td>3 days</td>
<td>3</td>
<td>2 mm, 2 mm, 3 mm</td>
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<tr>
<td>1 week</td>
<td>3</td>
<td>2 mm, 3 mm, 3 mm</td>
<td>2 mm, 2 mm, 2 mm</td>
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<tr>
<td>2 weeks</td>
<td>3</td>
<td>3 mm, 3 mm, 3 mm</td>
<td>2 mm, 2 mm, 2 mm</td>
</tr>
<tr>
<td>4 weeks</td>
<td>3</td>
<td>6 mm, 6 mm, 6 mm</td>
<td>2 mm, 2 mm, 2 mm</td>
</tr>
<tr>
<td>5 weeks</td>
<td>3</td>
<td>7 mm, 7 mm, 6 mm</td>
<td>3 mm, 3 mm, 2 mm</td>
</tr>
<tr>
<td>12 weeks</td>
<td>3</td>
<td>8 mm, 7 mm, 7 mm</td>
<td>3 mm, 3 mm, 2 mm</td>
</tr>
</tbody>
</table>

![Figure 1](image1.png)

Figure 1: Histology of normal Achilles tendon (scale bar = 100 um). (A) H&E stain showing the normal spindle shaped tendon fibroblasts (arrow) between the collagen bundles. (B) Masson trichrome stain showing thick parallel collagen bundles (arrow). (C) \( \alpha \)-SMA immune-stain showing absence of positive cells.

![Figure 2](image2.png)

Figure 2: \( \alpha \) SMA staining of the proximal tendon stump (scale bar = 100 um) (A,B) 3 days and 1 week: few myofibroblasts (arrows) at cut edge of tendon (C) 2 weeks: few myofibroblasts (arrows) are now seen between collagen bundles. (D) 4 weeks: the most pronounced expression of myofibroblasts (arrow). (E) 5 weeks: only few myofibroblasts are seen (arrow) (F) 12 weeks: negative staining.
architecture and collagen bundle arrangement. Furthermore, increased vascularity was evident in the distal stump and not in the proximal stump.

The cellular infiltrate within the collagen bundles seen in the proximal and distal tendon stumps arose from tendon epitendon cells, which were best seen using the H&E stain. Epitenon cells initially migrated from the sides of the tendon to its cut edge at 2 weeks post-injury, and then cells migrated between the collagen bundles at 4 weeks.

Histologically, normal tendon fibroblasts had spindle-shaped nuclei (longitudinally arranged along the tendon axis between the collagen bundles). The nuclei of migrating epitendon cells appeared more round in shape and stained darker than native tendon fibroblasts.

Findings with α-SMA staining of the proximal stump were different from those of the distal stump at all experimental intervals. In the proximal stump, positive myofibroblasts were few and limited to the cut tendon edge at 3 days post-injury (Figure 2A). Myofibroblasts gradually increased in number at the edge (Figure 2B) and then infiltrated the collagen bundles (Figure 2C) at 1 week and 2 weeks, respectively. At 4 weeks (corresponding with the sudden increase in proximal stump tendon retraction), myofibroblast staining was most pronounced (Figure 2D). Minimal staining was seen at 5 weeks (Figure 2E) and staining was negative at 12 weeks (Figure 2F).

Figure 3: α-SMA staining of the distal tendon stump (scale bar = 100 um) (A) 3 days: Myofibroblasts (arrow) are most pronounced (B, C, D, E) at 1, 2, 3, and 5 weeks; only very few myofibroblasts are seen (arrows) (F) at 12 weeks. No myofibroblasts are seen. The positive staining is seen in the new blood vessels (arrow).

DISCUSSION

This study delineates the histological changes and the degree of tendon retraction at the proximal and distal tendon stumps following Achilles tendon transection. There are no similar studies in the literature, and hence we are unable to compare our findings with others. The study showed that tendon retraction is more pronounced at the proximal compared to the distal stump.

Differences between the healing of the medical collateral ligament (MCL, an extra-synovial ligament) of the knee and the healing of the anterior cruciate ligament (ACL, an intra-synovial ligament) were extensively studied. The partially injured MCL has a relatively efficient healing capacity; and unrepaired completely transected MCL retract. Healing and retraction of the MCL is closely related to a high density expression of myofibroblasts. In contrast, injured ACL shows a limited healing capacity and retraction and this is also associated with a low-density expression of myofibroblasts. In this study, the degree of tendon retraction was also associated with a high density expression of fibroblasts. For example, expression of myofibroblasts in the proximal tendon stump peaked at the 4-week interval after injury (Figure 2D) and this correlated with a sudden increase in tendon retraction (Table I). Myofibroblasts are characterized by the expression of α-smooth muscle actin (α-SMA) which mediates the contractile activity of myofibroblasts. This contractility is translated into contraction of the extra-cellular collagen via specialized focal adhesions of the cell membrane of myofibroblasts. These focal adhesions are formed by complexes of hundreds of molecules including integrins, talin, vinculin and kinases; and they provide direct communications between the contractile intra-cellular actin and the extra-cellular fibronectin/collagen fibers. The origin of myofibroblasts in connective tissue remains a controversial issue. Most agree that the main origin is from residing fibroblasts (i.e. native tendon fibroblasts in case of tendons or dermal fibroblasts in case of skin). Transformation of fibroblasts into myofibroblasts is a complex process and occurs under the influence of many factors including transforming growth factor-beta (TGF-β). Other sources of myofibroblasts include pericytes (perivascular cells) and fibrocytes (circulating blood cells originating from the bone marrow).
sheath is main source of fibroblasts for healing of repaired and immobilized flexor tendons (extrinsic healing). In contrast, repaired and mobilized flexor tendons heal intrinsically from epitelen cells. These epitelen cells migrate from the sides of the tendon to its cut edge and then they migrate into the tendon to form a new fibroblast population mediating the repair process. In this rabbit model, the Achilles tendon was not repaired, and the limb was not immobilized. A similar migration of epitelen cells was seen at both the proximal and distal tendon stumps.

As with any new experimental research, several questions arise. In this model, perhaps the most important question is that why did the proximal tendon stump retract more than the distal stump? One possible explanation is the fact that the proximal stump is attached to the Achilles muscle belly, which is still contracting since the experimental rabbits were not immobilized. Hence, tension and mobility of the proximal segment is expected to be more than the distal segment. It is well known that the degree of differentiation of myofibroblasts is affected by many factors including the degree of extra-cellular matrix rigidity and the degree of mobilization. In fact, myofibroblasts can feel the stress and this stress perception results in an increase in the degree of TGF-β release from the latent TGF-β binding protein, and will induce further differentiation of myofibroblasts. This may explain the higher degree of myofibroblast expression (and in turn, the higher degree of retraction) in the proximal tendon stump.

CONCLUSION

Following complete transection of the Achilles tendon, tendon retraction correlated with the density of myofibroblast expression within the tendon stump. Further research is needed to investigate the pathophysiology of these findings.

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REFERENCES