

# Decreasing Inflammatory Response of Injured Patellar Tendons Results in Increased Collagen Fibril Diameters

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Tissue inflammation is essential in the healing process, but its effect on the quality of the healing tissue is not clear. This study determines the effect of decreasing early inflammation during wound healing in genetic deficient mice on collagen fibril diameter. Two strains of mice were used: three C3H/HeJ mice and three C3H/HeN mice for each of two time points (7 and 14 days postinjury). C3H/HeJ mice have a genetic deficiency in the production of tumor necrosis factor by macrophages and other cytokines in response to endotoxin, and C3H/HeN mice have no genetic deficiency. The right patellar tendon of both mouse strains was transversely transected, whereas the left patellar tendon was left intact for control. After 7 and 14 days, both right and left patellar tendons were harvested, and tendon samples were examined with transmission electron microscopy. We found that at 7 days, transected tendons of C3H/HeJ mice exhibited on average 1.6 times larger collagen fibril diameters than transected C3H/HeN tendons, whereas at 14 days, collagen fibril diameters of the C3H/HeJ mice were 1.3 times that of C3H/HeN mice. Also, at both 7 days and 14 days, collagen fibrils in C3H/HeJ mice appeared more organized than C3H/HeN mice. In addition, control tendons in both mouse strains showed no significant differences in collagen fibril diameter and organization. Therefore, these results suggest that decreasing the inflammatory response in the early stages of tendon wound healing enhances the quality of the healing tendon through increased collagen fiber diameter and better organization.

**Keywords** Collagen Fibrils, Inflammation, Tendon

## INTRODUCTION

Connective tissue wound healing starts immediately after injury [16] and continues for months or even years [9, 10, 13]. The first phase of this healing process is inflammation, which overlaps with the proliferation phase. The inflammation phase

is noted for inflammatory cell recruitment, the release of inflammatory factors (*e.g.*, tumor necrosis factor [TNF]- $\alpha$ ), and increased tissue construction. In addition, there is vascular ingrowth and a local build-up of new tissue [3, 9–11]. Both the inflammatory and proliferation phases are essential for wound healing; but an *in vivo* study has shown that skin wound healing in TNF-receptor deficient mice (Rp55 deficient mice) was accelerated compared with wild type mice. This suggests that inflammation caused by TNF- $\alpha$  at the wound site negatively affects wound healing by reducing collagen accumulation as well as angiogenesis [15]. Furthermore, a previous study using genetically deficient mice (C3H/HeJ) to determine the production of TNF by macrophages and other cytokines suggested that inflammatory cytokines present in the early phases of skin wound healing impair the quality of the healing tissue by delaying the increase of tensile strength [2].

Based on these previous studies, we hypothesized that decreasing the early inflammatory response to injury would improve the quality of the healing tendon. To test this hypothesis, injuries were created by transecting the right patellar tendons of C3H/HeJ and C3H/HeN mice, and collagen fibrils of injured and noninjured tendons for control from both mice were examined at 7 and 14 days postinjury. The results of this study show that the healing patellar tendons of C3H/HeJ mice exhibited markedly larger collagen fibril diameter than those of C3H/HeN mice.

## MATERIALS AND METHODS

C3H/HeJ mice (Charles River, Wilmington, MA, USA) and C3H/HeN mice (Jackson Labs, Bar Harbor, ME, USA) were used as animal models in this study. C3H/HeJ mice have a genetic deficiency that results in the reduced production of TNF by macrophages and other cytokines in response to endotoxin and other stimulatory factors [2, 5, 22]. C3H/HeN mice were used as control [22]. Three mice of each strain for 7 and 14 days postinjury, respectively, were used to create tendon injuries according to the following procedures.

Mice were anesthetized using a solution of ketamine/xylazine containing 50 mg/kg and 5 mg/kg, respectively. Anesthetized

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mice were weighed before their operation. The hair on the skin of the right knee was removed with a hair remover. Mice were immobilized in a supine position using tape. The skin surrounding the operative field was treated with Povidine solution and prepared using sterilization techniques. A medial linear parapatellar incision of 10 mm was produced at the right knee of each mouse. The patellar tendon was recognized, and its dimensions were measured. The patellar tendon was transected in the middle of the length, with a #15 surgical blade. The patellar ends were reattached using two nylon nonabsorbable 5-0 sutures.

Knots were made on each side of the transected tendon with one suture placed on the proximal tendon segment and the other one placed on the distal segment. Both were made parallel to the cut line and in the intact tendon substance, to not interfere with the wound tissue (Figure 1). The skin wound was closed with nylon nonabsorbable 5-0 subcutaneous and nylon nonabsorbable 4-0 sutures with the knot made outside the skin. After surgery, all mice that underwent surgeries were allowed free activity in cages.

Then, 7 or 14 days after tendon injuries with transection, tendon samples were harvested according to the following method. Skin sutures were removed and the distal border of the healing tendon was cut through the skin incision with a # 15 surgical blade, whereas the posterior border was removed from the anterior joint capsule with a # 11 surgical blade. As a normal control group, untransected patellar tendons were harvested using the same technique.

For transmission electron microscopy, tendon samples were fixed for at least 1 hr in phosphate buffered saline (PBS) solution containing 2% glutaraldehyde. Secondary fixation was in PBS containing 1% osmium tetroxide. The sample was washed in distilled water and dehydrated in ethanol. Propylene oxide was used as a transitional solvent. The tendons were embedded in Spurr's resin, and the midsubstance of each sample was sectioned ( $0.1 \mu\text{m}$ ) perpendicular to the tendon's longitudinal

axis using a diamond knife. Thin sections were stained with 1% uranyl acetate and Reynold's lead citrate. Tendon sections were viewed on a transmission electron microscope, and images of collagen fibrils from tendon samples were obtained. Note that a total of 6 fields for each tendon sample was imaged, which yielded 6 images per sample.

A software (NIH Image J) was used to analyze the images, with a negatively stained catalase crystal used as a standard to estimate collagen fibril diameters. A total of 261 to 1939 collagen fibrils were used to construct frequency distributions of collagen fibril diameters. Because collagen fibril diameters remained relatively constant, the selected sample size of collagen fibrils did not significantly affect their distributions (Figure 2). For statistical analysis, the Kolmogorov-Smirnov test [12] was used to compare collagen fibril distributions between C3H/HeJ and C3H/HeN mice for both injured and noninjured tendons of these mice. An unpaired Student's *t*-test also was used to compare the means of collagen fibril diameter from the C3H/HeJ and C3H/HeN mice. A difference was considered to be significant if the *p* value was less than 0.05.

## RESULTS

We found that the collagen fibrils of the healing tendons in the C3H/HeJ mice were consistently larger than those of the C3H/HeN mice, either at 7 days (Figures 2A and 2B) or 14 days postinjury (Figures 2E and 2F). In addition, the space between collagen fibrils of healing tendons in C3H/HeJ mice appeared more uniform than collagen fibrils of C3H/HeN mice. The collagen fibrils of noninjured tendons, however, had a similar size and organization either at 7 days (Figures 2C and 2D) or 14 days postinjury (Figure 2G and 2H).

Furthermore, the distribution of collagen fibrils from the injured tendons of C3H/HeJ mice appeared markedly different from that of C3H/HeN mice either at 7 days (Figure 3A) or 14 days postinjury (Figure 3B), and the two collagen fibril distributions were significantly different from each other ( $p < .0001$ ). However, the collagen fibril distributions from noninjured tendons of C3H/HeJ and C3H/HeN for both 7-day and 14-day groups appeared similar and found not to be significantly different ( $p < .05$ ). At 7 days, the collagen fibril diameter of injured tendons in C3H/HeJ mice was  $46 \pm 11$  compared with  $29 \pm 7$  (nm) in C3H/HeN mice (Figure 4A), where the difference was significantly different ( $p < .01$ ). At 14 days, the collagen fibril diameter of injured tendons in C3H/HeJ mice was  $43 \pm 7$  (nm), whereas the collagen fibril diameter was  $33 \pm 6$  (nm) in C3H/HeN mice (Figure 4B). At 7 days, the collagen fibril diameters of noninjured tendons in C3H/HeJ and C3H/HeN mice were  $101 \pm 38$  and  $96 \pm 43$  (nm), respectively, whereas at 14 days, they were  $114 \pm 38$  (nm) for C3H/HeJ mice and  $111 \pm 33$  (nm) for C3H/HeN mice. The difference in collagen fibril diameters of the noninjured tendons between C3H/HeJ and C3H/HeN mice at either 7 days or 14 days was not significantly different ( $p > .05$ ).

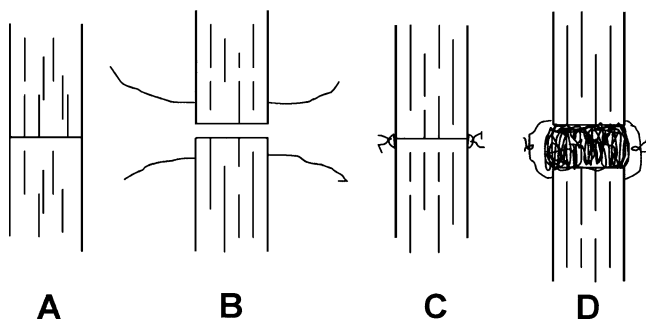


FIG. 1. An illustration of creating injured patellar tendons of C3H/HeJ and C3H/HeN mice. (A) the tendon was transected in the middle substance, perpendicular to the long axis of the tendon. (B) One 5-0 nylon suture was passed through each cut end. (C) The two sutures were tightened to each other by making a knot on each side. In this way, the tendon's transected ends were re-attached without leaving sutures passed through the gap. (D) At 7 or 14 days postinjury, a reproducible gap ( $\sim 2$  mm) filled with healing tissue was observed.

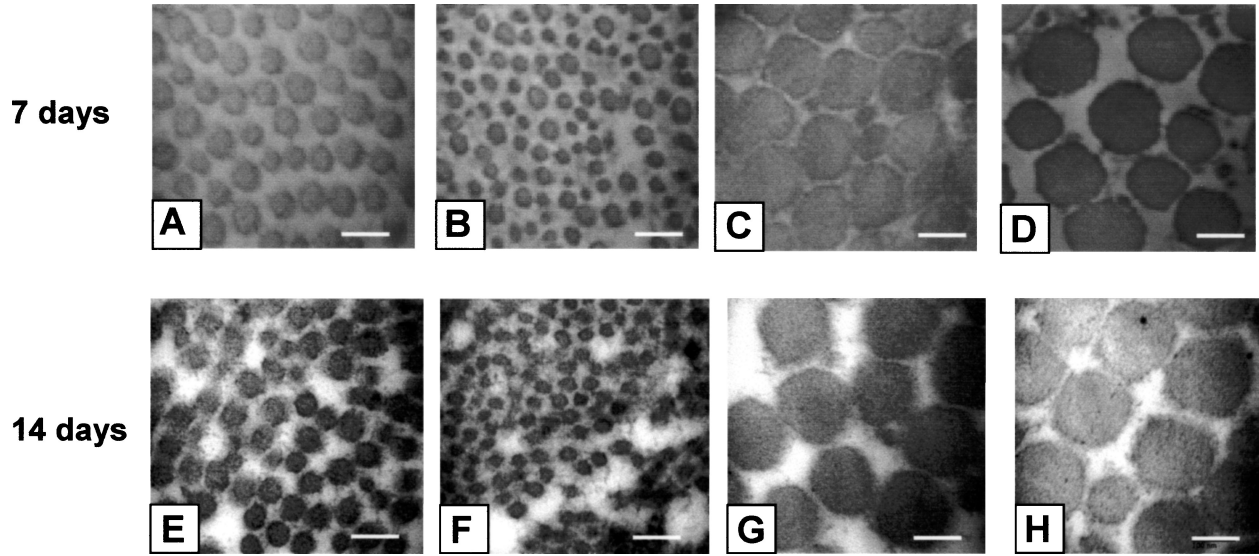


FIG. 2. TEM photographs showing collagen fibrils of injured and noninjured patellar tendons of C3H/HeJ and C3H/HeN mice at 7 days (A–D) and 14 days (E–H) postinjury. Injured tendon from a C3H/HeJ mouse (A, 7 days; E, 14 days). Injured tendon from C3H/HeN mice (B, 7 days; F, 14 days), noninjured tendon from a C3H/HeJ mouse (C, 7 days; G, 14 days), and noninjured tendon from a C3H/HeN mouse (E, 7 days; H, 14 days). It is evident that at both 7 and 14 days postinjury, collagen fibril diameters of the injured tendon in C3H/HeJ mice are larger than those in C3H/HeN mice. However, they are much smaller than those of noninjured tendons. Bars: 100 nm.

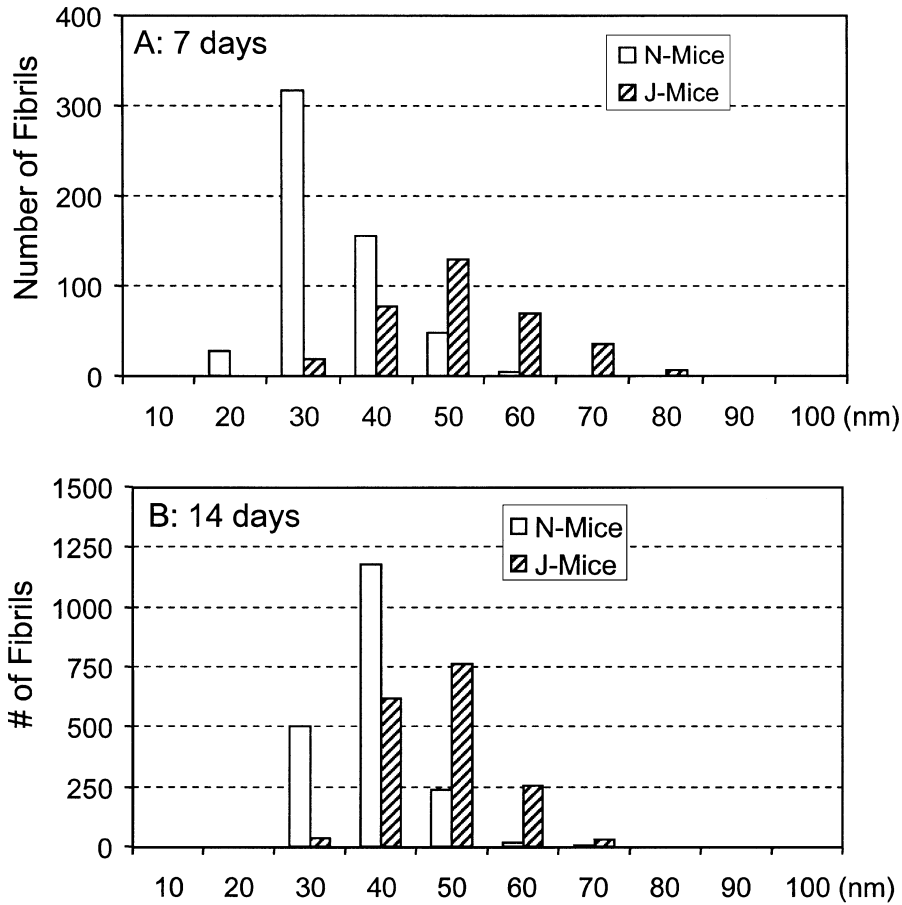


FIG. 3. The distributions of collagen fibril diameters of injured tendons for both C3H/HeJ and C3H/HeN mice are shown here (A, 7 days; B, 14 days). Overall, the collagen fibrils of injured tendons from C3H/HeJ mice at either 7 or 14 days had markedly different distribution than those of C3H/HeN mice ( $p < .01$ ).

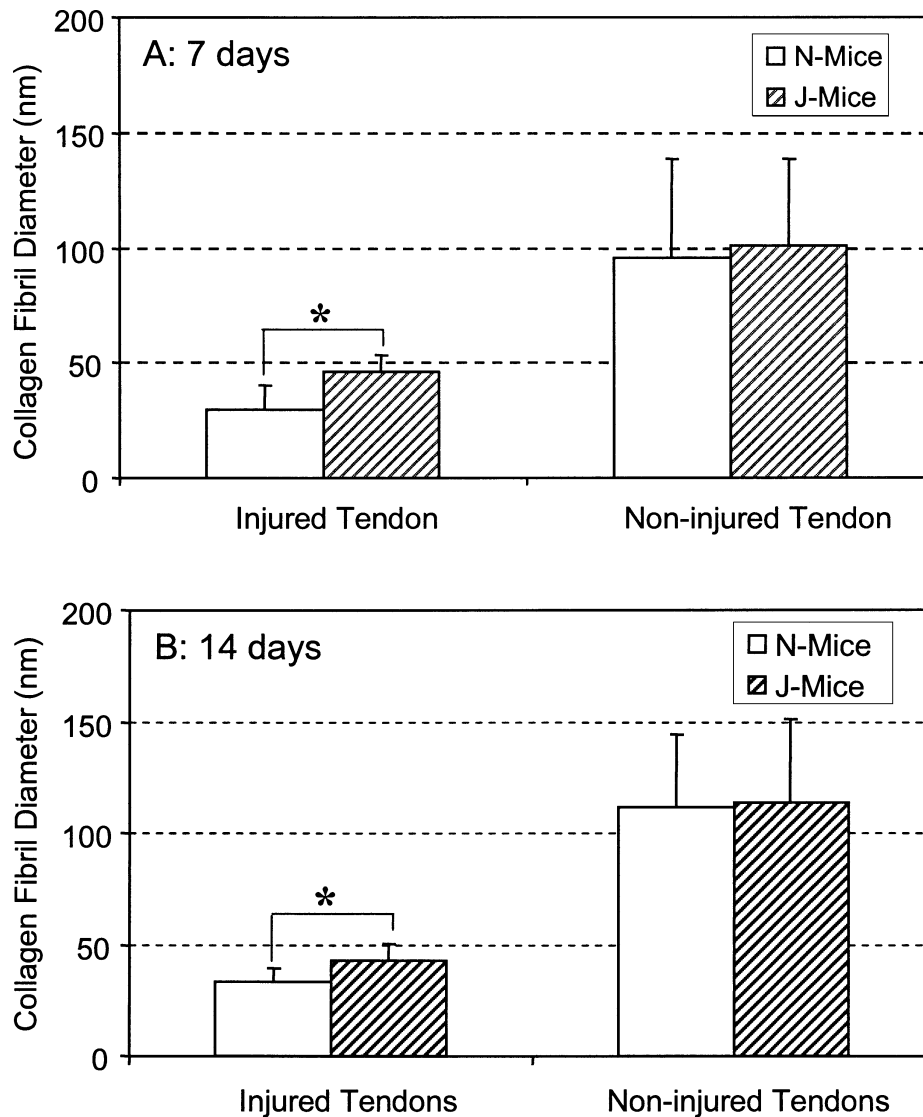


FIG. 4. The comparison of collagen fibril diameters from injured tendons of C3H/HeJ and C3H/HeN mice. The collagen fibril diameter of the injured tendon of C3H/HeJ mice was significantly larger than that of C3H/HeN mice at either 7 days (A) or 14 days (B) postinjury ( $p < .01$ ). However, the collagen fibril distributions of noninjured tendons from C3H/HeJ and C3H/HeN mice were not significantly different ( $p > .05$ ).

## DISCUSSION

When tendons are injured, they undergo inflammation, which is the initial phase of the tissue healing response. Our working hypothesis in this study was that this early inflammatory response affects the quality of the healing tissue. Using genetically deficient mice, C3H/HeJ, this study showed that reducing the inflammatory response increased the diameter of collagen fibrils and improved the organization of collagen fibrils of healing patellar tendons compared with control mice, C3H/HeN [2, 5, 22]. These findings are important for two reasons: the collagen fibril diameter and organization of the healing tissue remain inferior to those of normal tissue after weeks or several months [20], and collagen fibril diameter is associated with the mechanical strength of the tendon tissue [7]. Therefore, to enhance

the quality of injured tendons in the early stages of healing, it may be desirable to reduce tissue inflammation of injured tendons.

To our knowledge, this is the first study that used C3H/HeJ mice to study the influence of the early stages of inflammation on the healing of injured patellar tendons. C3H/HeJ mice exhibit a decreased inflammatory response to stimulatory agents due to decreased macrophage activation and production of cytokines including  $\text{TNF-}\alpha$  [22], where cytokines produced by macrophages and platelets are present early in the wounded tissue.  $\text{TNF-}\alpha$ , an inflammatory cytokine, influences tissue wound healing by regulating macrophage and differentiation [23], increasing the synthesis of collagenase [21], and downregulating the production of collagen [19].  $\text{TNF-}\alpha$  also decreases the rate

of collagen synthesis in human granulation tissue fibroblast cultures [18], also inflammatory cytokines in the wound healing process regulate chemotaxis, migration, local cell proliferation, and synthesis of tissue structures and can control in part the production and release of other cytokines [8]. The multiple roles of the inflammatory cytokines affect the presence of fibroblasts, their activation, and their function in the tendon wound healing tissue and eventually influence the formation and organization of collagen fibrils in the extracellular matrix. All these factors may explain why decreasing inflammation leads to the increase in the collagen fibril diameter observed in this study.

Previous studies have shown that inflammatory cytokines of injured tissues influence the healing quality of injured tissues. For example, in TNF-Rp55 deficient mice that had reduced leukocyte infiltration, skin wound healing was enhanced [15]. Also, using knocked-out mice, researchers found that TNF- $\alpha$  affects connective tissue breakdown by promoting acute smoke-induced inflammation [4]. With genetically deficient mice, C3H/HeJ, inflammatory cytokines at early healing times impair healing and delay improved tensile strength of skin wounds. Thus, our study was consistent with previous studies in that injured tissue inflammation, while necessary to wound healing, reduces the quality of the healing tissue. Further, persistent inflammation has long been associated with chronic nonhealing skin wounds [1, 24].

Many studies have used nonsteroidal anti-inflammatory drugs (NSAIDs) to evaluate the effect of inflammation on tissue healing. NSAIDs are anti-inflammatory drugs, which decrease inflammation in injured tissues by inhibiting the production of prostaglandins. Piroxicam, an NSAID, increased the early tensile strength of healing medial collateral ligament (MCL) in rats [6]. Diclofenac and indomethacin, two more NSAIDs, were found to increase the strength of skin wounds in rats at 10 days after injury [17]. Nevertheless, the inconsistent results from using NSAIDs also were reported. For example, oral administration of ibuprofen for 14 days after rabbit MCL injuries did not result in significant changes in the mechanical properties of the healing MCL in ibuprofentreated rabbits compared with rabbits treated with placebo [14]. These studies suggest that the effect of NSAIDs on the healing process of injured tissues may be different from each other, and they may have different "side effects" on the healing process of an injured tissue. Other factors that possibly influence the outcome of NSAIDs treatment of injured tissues may include the subtle differences in healing responses between different tissues and the healing time when an NSAID is administered. Finally, NSAIDs mainly reduce the production of prostaglandins. Therefore, their effects would be different from those of inflammatory cytokines (e.g., TNF- $\alpha$  and interleukin-1), which were addressed in this study using the C3H/HeJ mouse model.

This study has a few limitations. First, although changes in inflammatory responses in C3H/HeJ mice have been shown in previous studies, they were not determined in this study. Therefore, the changes in collagen fibril diameter could be attributed

to other differences between the C3H/HeJ mice and control mice (i.e., C3H/HeN mice). Second, this study only investigated the diameter of collagen fibrils and their overall organization in the healing tendon using transmission electron microscopy technique. Future studies should evaluate the effect of decreased inflammation on the mechanical properties (e.g., stiffness, ultimate tensile strength), although mechanical testing of small tendon specimens in this study was a challenging task. Also, biochemical properties (e.g., collagen type and cross-links) of the healing tendon also should be evaluated. All these assays will provide a more complete picture of the influences of early inflammation on tendon healing, so that new strategies can enhance the histological, biochemical, and biomechanical properties of injured tendons. Third, the molecular mechanisms responsible for increased collagen fibril diameters due to reduced inflammation are not clear from this study. However, the mechanisms may involve TNF- $\alpha$  that can downregulate collagen deposition during graduation [18].

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