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A new model for partial immobilization of rat hind limb after Achilles tendon excision/reinterposition

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Abstract: There have been several attempts to repair the Achilles tendon by surgery or replacement with graft materials. Because tissue repair is a complex process, tendon healing occurs slowly. In rats, immobilization of the limb is essential during recovery. There are immobilization methods such as plaster or fiber casting. According to prolonged recovery, most of the procedures cause skin ulceration, decrease in weight, restrictions in checking the surgery area, and slipping out of the cast. Our aim was to apply a suture around the defect for immobilization. Tendons of 30 male Wistar rats (250 ± 50 g and aged 8 ± 2 weeks) were dissected and the cut ends were immediately sutured proximally-distally with an 8-0 nonabsorbable suture. For partial immobilization a needle with a 4-0 suture was passed 3 mm proximally and 3 mm distally between the gastrocnemius and calcaneus. A knot was applied close to the calcaneus. The skin was closed with a continuous, Ford interlocking 6-0 suture pattern. The animals were sacrificed after 2 and 4 weeks. No difference was observed in means of muscle atrophy when compared to contralateral intact muscle. No ulcer, sore, or swelling was observed. Tendons were intact and showed superior histological characteristics. Recent techniques seem to be effective in applying strength to protect the defect area from muscle tensile stress of the gastrocnemius during the recovery period.

Key words: Achilles tendon, immobilization, tendon healing, surgery

1. Introduction

The Achilles tendon is the major connective tissue with parallel arranged collagen fibers, which transmits the tensile force generated by the gastrocnemius and soleus muscle complex to the calcaneal bone. Structurally, the tendon is composed of tenoblasts and tenocytes lying longitudinally in a network of collagen molecules. Tenoblasts transform into tenocytes as they age with decreased metabolic activity (1). The extracellular components of the tendon are mainly collagen type I, III, and V; proteoglycans; fibronectin; and elastic fibrils (2,3). The arrangement of these molecules provides the tendon with a unique strength against strain.

Partial or total rupture of the Achilles tendon and failure in its biomechanical properties are a common form of large muscle-tendon injury, occurring mostly in athletes (4,5). The regeneration of the tendon is a complex process that includes several histopathological events taking more than 12 weeks with a remodeling phase (6,7). It is well known that during the repair process the damaged tissue area is replaced by newly formed extracellular matrix components (8). Healing of a ruptured tendon occurs in 3 phases: the cellular phase, the fibrous protein synthesis phase, and the remodeling phase (9). Because of low mitotic activity and the lack of vascularization, tendon remodeling occurs very slowly when compared to other connective tissues (10–12). For this reason, there have been several attempts to repair the damaged zone either by surgical techniques or by replacing the tissue with graft materials (13,14). However, the optimal repair method of a ruptured tendon is under considerable debate. The most frequently applied treatment method is conservative, surgical, and percutaneous repair (15). There are several tissue engineering approaches that have aimed to produce either biological or synthetic polymers for the repair of damaged tissue (16,17).

Over the past few years clinicians have become interested in accelerating the process of tendon healing. For this purpose, there have been several attempts with laboratory animals, including rats and rabbits. During recovery, immobilization of the hind limb is frequently used to reduce the amount of force acting on the damaged zone, which is also possible by self-limitation due to pain

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reaction (18). Nevertheless, it is still a matter of controversy whether postoperative mobilization causes any successful changes in the phases of tendon healing or if mobilization accelerates the restoration of tendon matrix when compared with postoperative immobilization (18,19). Tendon repair starts with hematoma formation and ends with remodeling. It has been previously reported by Virchenko and Aspenberg that mechanical loading during recovery accelerates tendon remodeling (20). However, mechanical stimulation is reported to be seldom used because of the possibility of overloading the fibrogenic callus and distracting the newly forming tendon (20).

On the other hand, there have been several studies in which postoperative immobilization was preferred, including different types of casting methods (15,21–23), tail suspension (24), external fixation (25), denervation (26), extremity suspension (27), and botulinum toxin A injection (20). However, according to prolonged recovery, most of these casting procedures may cause skin ulceration due to the retention of urine by the cast, decrease in weight, restrictions in checking the surgery area, and slipping out the casts by the animal, especially in rodent studies (23). It was shown that immobilization has a detrimental effect on tendon healing. The immobilization of limbs results in atrophy of the muscles, which are fixed in a resting position. This situation is described by a decrease in the contractile protein synthesis rate during the first 6 h of immobilization, which plays a role in initiating muscular atrophy (28,29). The models described in the literature also report harmful effects such as muscular fiber hypotrophy, increased connective tissue, loss of muscular extensibility, and limitation of joint movement (30).

The model in this study was to apply a suture around the defect area for partial immobilization. The objective of the present study was to propose partial immobilization to the defected area without restriction of limb movement, which would be functional for the extremity by keeping only the area of surgery immobilized.

2. Materials and methods

The entire surgical procedure was approved by the Institutional Animal Health and Care Committee of Hacettepe University (approval number: 2009/8). Thirty male Wistar albino rats were used in this study. The age was 9 weeks with an average weight of 275 g (range: 250 ± 50 g). The animals were randomly divided into 2 groups, each containing 15 animals. The experimental study was conducted on the right Achilles tendons of the rats; the left Achilles tendon in each animal served as the control. All rats received tap water and were fed ad libitum. Rats were housed in plastic cages in an air-conditioned room at 22 ± 2 °C, in relative humidity of 45%, and with a 12-h light/dark cycle. The gross appearance and body weight were evaluated at the beginning of the surgical procedure and at the end of study.

2.1. Surgical technique

The rats were anesthetized with an intraperitoneal injection of 2 mg/kg xylazine HCl (Alfasan; Woerden, the Netherlands) and 15 mg/kg ketamine HCl (Richter Pharma; Wels, Austria). The fur on both hind limbs was shaved with a disposable razor following soap-and-water cleaning. The surgical area was disinfected with a povidone 10% solution (Diagnokim, İstanbul, Turkey) and covered with a sterilized cloth. Under sterile conditions, a longitudinal incision was made on the skin from the distal end of the gastrocnemius muscle to the calcaneal bone. Achilles tendon was isolated and a mid-3/1 section (3 mm long) was sharply excised with a scalpel blade (Figure 1A). An immediate interposition of the excised tendon part was sutured proximally and distally using 8-0 absorbable suture material (SMI, Hünningen, Belgium) (Figure 1B). For partial immobilization of the Achilles tendon, 4-0 nonabsorbable suture material (Ethicon, Woluwe, Belgium) was used. The needle was passed 3 mm proximally and distally around the excised tendon through the gastrocnemius muscle and close to the calcaneal bone (Figure 1C). A knot was applied close to the calcaneal bone (Figure 1D). Skin was closed with a continuous, Ford interlocking suture pattern using 6-0 nonabsorbable suture material (SMI) (Figure 1E).

2.2. Postoperative management

Subsequently, rats were kept warm on a warmed pad of a water-heating circulator for a few hours (WBC 3044-PR, COMMAT, Ankara, Turkey). Carprofen at 5 mg/kg subcutaneously (Rimadyl, Pfizer) was given postoperatively.

2.3. Total excision of Achilles tendon

At 2 weeks (n = 15) and 4 weeks (n = 15) after surgical operation, all rats were euthanized with CO_2 inhalation. The skin overlying the Achilles tendon was shaved and a longitudinal incision was made above the outline of the tendon. Surrounding connective tissues were removed and transverse cuts were made below the musculotendinous junction and above the calcaneal bone.

2.4 Histopathological evaluations

Tissue specimens were fixed in 10% formaldehyde for 24 h and then washed under tap water for another 24 h for further histopathological analysis. Specimens were dehydrated through ascending concentrations of ethanol before being embedded in paraffin (Merck, Darmstadt, Germany). A Leica RM 2125 microtome was adjusted at 5 µm. The sections were placed on slides. The slides were then stained with hematoxylin eosin and examined blind independently by 3 observers with an Olympus BX51 system light microscope. The photographs were captured with Bs200ProP software.

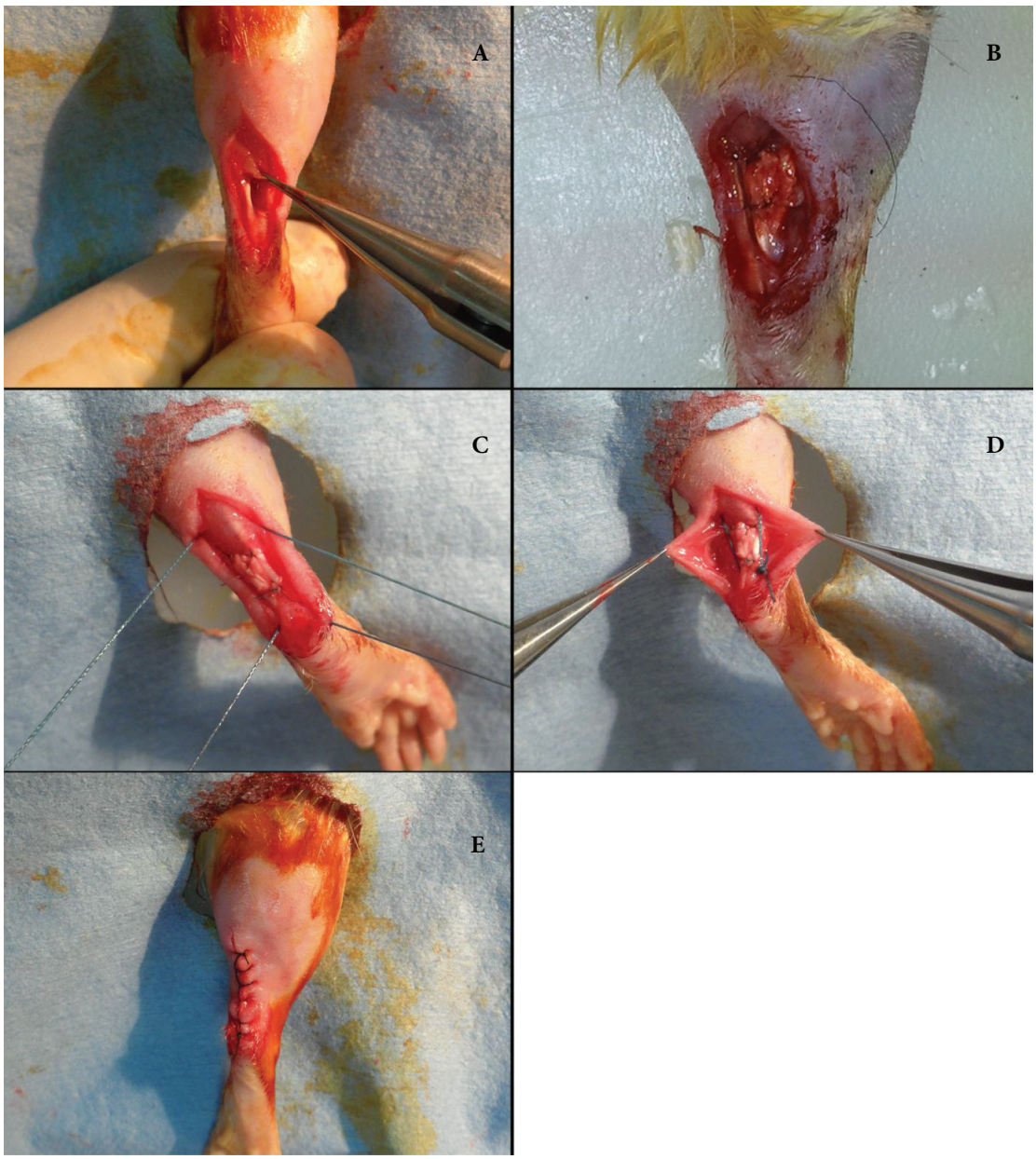

Figure 1. A) The excision of the mid-3/1 section (3 mm long) of the Achilles tendon; B) an immediate interposition of excised tendon part with 8-0 absorbable suture material; C) needle was passed 3 mm proximally and distally around excised tendon through the gastrocnemius muscle and close to the calcaneal bone; D) a knot was applied close to the calcaneal bone; E) skin was closed with continuous, Ford interlocking suture pattern using 6-0 nonabsorbable suture material.

3. Results

3.1. Gross observations

All tendons showed the first intention of healing by attachment to proximal and distal parts close to the incision zones without any evidence of swelling or sepsis. The evaluation at the end of the postoperative 4th week after surgery was satisfactory in the immobilization group. No difference was observed in the means of muscle atrophy when compared to the contralateral intact muscle. No ulcer, sore, or foot swelling was observed in any of the animals. The tendons were intact.

3.2. Histopathological examinations

The native rat Achilles tendon consisted of closely packed collagen fibril bundles and few tenocytes lying in between the fibrils in a longitudinal arrangement (Figure 2A). Fiber bundles were well organized with parallel arrangement.

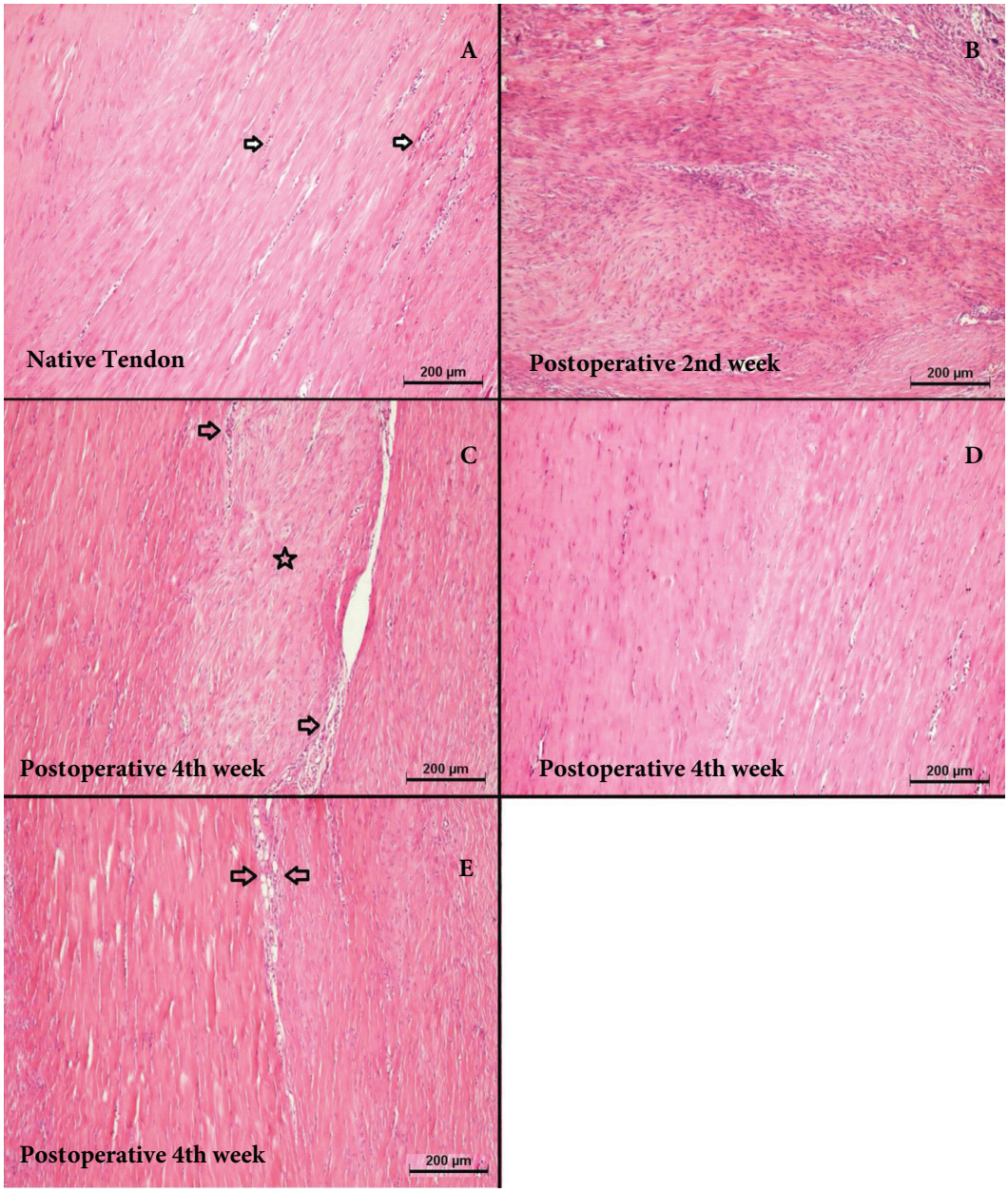

Figure 2. A) Closely packed collagen fibril bundles and tenocytes lying in between the fibrils in longitudinal arrangement; B) a cell and hypertrophic collagen fibril rich granulation tissue at the end of the 2nd week after operation; C) decreased amount of inflammatory cells and regression of granulation tissue at the end of the 4th week after operation; D) intact fiber bundles at the end of the 2nd week; E) small amounts of lipid droplets and adipocyte accumulation.

Tenocytes were in a spindle shape with basophilic nuclei and massive amounts of cytoplasm (Figure 2A, black arrows). It was hard to identify and obtain round-shaped tenoblasts.

At the end of the postoperative 2nd week, a cell-rich and hypertrophic collagen fibril-rich granulation tissue was visible around the incision zone when compared to the native tendon (Figure 2B, arrow). Tendons were disorganized as a result of space-filling tissue ingrowth. Massive infiltration of polymorph nuclear leucocytes, neutrophils, and platelets representing an inflammatory healing reaction was also observed. The number of tenoblasts with more round nuclei was greater when compared to the spindle-shaped tenocytes. Cell density was increased when compared to the native tendon specimens. The space between collagen fibril bundles was

increased, as a typical dense connective tissue network.

At the end of the postoperative 4th week, the major difference between the 2nd week's group was the decrease in the amount of inflammatory cells and regression of granulation tissue (Figure 2C). Major histological improvement was observed as the restoration of more organization in collagen fibril structure (Figure 2C, star). There were still small amounts of inflammatory cells (Figure 2C, arrows). However, it was detectable that the healing process was still not complete when compared to native tendon specimens. The number of tenoblasts was also decreased. A decrease in cell density was another important finding. Fiber bundles were more intact with each other when compared to the 2nd week's specimens (Figure 2D, star). There was no fibrocartilage formation between bundles; however, small amounts of lipid droplets and adipocyte accumulation were observed (Figure 2E, arrows).

4. Discussion

Achilles tendon injuries are commonly seen in humans and in veterinary orthopedics. The ability to restore functional healing in the treatment of Achilles tendon injuries is greatly improving with the development of better surgical techniques. There have been several attempts at immobilization techniques during healing process. In spite of these, however, the outcome of surgical repair is often less than desirable.

It has been previously reported that the movement during regeneration causes a delay in healing (15), which leads surgeons to apply immobilization techniques following surgical repair. However, no attempt has been made to partially immobilize the repair zone that may potentially be applied for Achilles tendon reconstruction. Considering the importance of the Achilles tendon in body function, we set up a novel partial immobilization technique, different from previous studies, to explore tendon recovery after rupture. The technique applied in this study seems to be effective in applying adequate strength to protect the defect area from muscle tensile stress of the gastrocnemius during the recovery period. Consequently, we can say that the partial immobilization technique provided significant stability without restricting limb functions and causing any infection or accumulation of natural dirt. The experiment carried out here is also reproducible.

Other approaches of tendon immobilization during the healing process led to reports that mechanical loading stimulates and improves the formation of remodeling. However, mechanical loading on the healing zone is seldom used because of the fear of overloading and distracting the newly forming tendon (20). During the healing process, animals usually exert considerable contraction forces, which results in loading on the injured site. According to our histological results, partial immobilization had a stimulatory effect on tendon repair by enabling the animal to move freely and preventing overloading without damaging the surgical repair zone.

Another aspect of immobilization is the adverse effect on protein synthesis of different muscle fibers (30). As in other studies, unloading of the rat hind limbs for 6 days by tail cast suspension led to atrophy and reduced growth of the gastrocnemius and plantaris muscles (31,32). It was also reported that due to decreased activity caused by oxidative enzymes, muscle fibers with oxidative stress are more vulnerable to muscle activity (33). Considering our approach, depending on the fact that the animal is functional and enabled to use its limb, the formation of muscle atrophy and oxidative stress is not expected. Our technique does not cause any reduction of muscle mass as well as an increase in connective tissue amount.

Another important factor is that partial immobilization allowed the animal to keep good adaptation to its environment postoperatively without promoting any skin lesions or edema. Since the formation of these changes in gross appearance occurs slowly, these discrepancies with our results may be due in part to the fact that our period of immobilization was not too long of a duration; also, our approach with internal fixation does allow for skin repair without infection.

Histopathological results also supported the gross morphological examinations. The histopathological results revealed that the healing process was successful in all specimens obtained from treatment legs. In addition, histology of specimens belonging to the treatment legs showed a similar and structurally well-organized tissue composition when compared with the control specimens at the end of the 4th week. In a previous study conducted with partial tendon defect in rabbits, it was reported that 6 weeks after tendon surgery, most of the specimens had a looser extracellular matrix (ECM) combined with hypercellularity and hypervascularity (34). In contrast, these findings were mostly observed in the 2-week group. The 4-week group showed a more organized histological structure, such as dense ECM, consisting of parallel orientated collagen fibers and longitudinal aligned tenocytes. Consequently, it is possible to say that partial immobilization of the Achilles tendon positively affects histological remodeling.

Our findings indicate that with partial immobilization it is possible to generate a protective environment for the Achilles tendon during the healing period. Establishment of such an approach is important when considering the potential for clinical application. Therefore, the approach developed by this study may provide insight into the future clinical repair of tendon defects by employing such

a partial immobilization. To our knowledge, this is the first report of the partial immobilization of the Achilles tendon complex. Developments in our understanding of tendon healing at cellular, histological, and molecular levels are promising to enable surgeons to modulate the operative repair and improve immobilization techniques. Although immobilization is being extensively studied, there is still a requirement for further research.

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