Radiographic and Histopathologic Evidences of Movement versus Immobilization Effects on Enthesis Formation in Rabbits

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Abstract

Enthesis joins tendon, ligament, or joint capsule to the bone, which are two very different tissues. As a result, it is more prone to stress and increased risk of rupture. The purposes of this study were to evaluate the role of limb movement or immobilization in enthesis healing after surgical repair and found out if radiography could be a reliable method for determining its prognosis. Fourteen New Zealand white rabbits were randomly divided into two groups. After detaching the Achilles tendon from the bone surface, the tendon was re-attached to the bone with a modified Kessler suture pattern. The limb of group 1 rabbits was externally fixed with the fiberglass cast. In group 2, however, no cast was used. Radiographs were taken after surgery and in weeks 2, 4, 6, and 8 post-operations. Samples were taken from the area after 8 weeks and stained by the hematoxylin-eosin method. The results were reported descriptively. Bone growth, increased chondrocytes in the cartilaginous region, and increased fibroblasts and fibrocytes were seen in group 1. While erosion, collagen fibers regularity, and tissue maturity were more predominant in group 2. Although early limb movement during the enthesis healing period helps to increase the regularity of collagen fibers in the tendinous zone of the enthesis, it does not increase the amount of callus in the bone and increases the risk of bone erosions and even fractures. While stabilizing the limb in the first two months of enthesis healing protects the bone against the forces and helps bone formation, which has a positive role in healing the enthesis.

Introduction

Enthesis is where a tendon, ligament, or joint capsule connects to a bone, which is clinically important because it is a common site of injury due to overuse of the limb.¹ Depending on the nature of the tissue at the junction of the tendon or ligament to the bone, the entheses are divided into fibrous or fibrocartilaginous types. Fibrous entheses are usually found where the tendons or ligaments attach to the diaphysis or metaphysis of a long bone. This type has probably received much less attention than...
fibrocartilaginous ones because they are less involved in enthesopathy. Fibrocartilaginous entheses are more common and are predominant in the epiphyses and apophyses of long and short bones. In this type of connection, four regions are generally distinguishable: the tendon region, which has regular type I collagen fibers and small amounts of decorin proteoglycan; the fibrocartilage region, the site of tissue transformation from tendon to bone, which is composed of type II and III collagen fibers and smaller amounts of type I, IX, and X collagen fibers and aggrecan and decorin proteoglycans; the mineralized fibrocartilage region, which is mainly made of type II collagen fibers and large amounts of type X collagen fibers and aggrecan; and finally, the osseous area that has type I collagen fibers and relatively high mineral content. Because of its role in the initial absorption of stress, enthesis is vulnerable to either chronic injury due to overuse or acute traumatic injuries. In addition, this structure is invaded in rheumatic autoimmune conditions. Noise et al. have identified three major sites of tendon rupture years ago: rupture of the tendon itself, avulsion of the bone below the enthesis, and rupture of the tendon-to-bone junction. After surgical healing or repair in adults, these structures do not regenerate in their original form. Postoperative rupture, which can occur in 94% of elderly patients, usually occurs at the junction and is a major challenge in orthopedic surgery.

Healing of the entheses involves the formation of a soft callus that allows cartilage cells to invade the tendon graft during the first 3 weeks. The tendon is then attached directly to the bone by collagen fibers. About the third month, the tendon stiffly attaches to the newly formed bone around the bone tunnel. Restoration of the enthesis can be affected by the use of growth regulators during surgical repair. Bone morphogenetic protein-2 (BMP-2) has been shown to increase the rate of attachment of semitendinous muscle grafts in rabbits in which anterior cruciate ligament repair is performed. The use of mesenchymal stem cells along with the hamstring tendons autograft in rabbits encourages fibrocartilage to form an enthesis that closely resembles the normal one. A similar study in sheep showed that osteogenic protein 1 increases bone integrity at the junction and healing rate. The use of transforming growth factor-β (TGF-β) has increased the collagen fibers synthesis at the tendons-to-bone junction and mechanical properties in dogs. Similarly, alpha-2 macroglobulin increases fibrocartilage formation and collagen synthesis at the tendons-to-bone junction and decreases collagen degeneration.

The role of mechanobiology in the healing of the tendon-to-bone junction is not clear. Although all three components of the junction (tendon, fibrocartilage, and bone) respond to loading in healthy conditions, their response to load during tendon-to-bone healing requires further study. Current restorations rely mainly on mechanical stabilization and are unable to promote regeneration of the soft tissue junction with bone. The effect of early movement on the healing of the tendon itself has been investigated. Fifteen minutes of mechanical loading from the third day of tendon rupture is sufficient to increase tendon strength, and a weak correlation has been observed between the duration of loading and the effect on healing. Additionally, four short loading episodes improved the strength and histological properties of the healed Achilles tendon in rats. Loading also has a positive effect during the initial inflammatory phase. However, complications such as re-rupture are also possible.

In this study, considering the role of movement in the new bone formation in cases of bone fractures, and on the other hand, considering that movement is essential for the natural healing of tendons, it was hypothesized that limb movement can help in enthesis healing. It has also been assumed that radiography may be a reliable method for determining the prognosis of injuries in the tendon-to-bone junction.

Materials and Methods

Animals

Fourteen adult 6-month-old male New Zealand white rabbits (Oryctolagus cuniculus) weighing 2.6 ± 0.25 kg were used in this study. Rabbits were given a week to acclimatize to the environment (18 ± 2.5°C environmental temperature and 12 h light/dark intervals) before surgery. They were fed a combination of laboratory animals’ pellets, lettuce, and carrots. Drinking water was also freely available. Treatment of animals was based on the statement by the World Medical Association on the use of animals in biomedical research. The study was approved by the Ethics Committee in Biomedical Research of the University of Tabriz under the code IR.TABRIZU.REC.1398.006.

Grouping and Surgery

Rabbits were randomly divided into two groups of
including group 1 (limb stabilization) and group 2 (free to move). On the day of surgery, rabbits were anesthetized by intramuscular injection of ketamine (35 mg/kg, Alfasan, Woerden, Holland) and xylazine (5 mg/kg, Alfasan, Woerden, Holland). Intramuscular penicillin G procaine (150,000 IU, Proca-Pen, Daana Pharma Co., Tabriz, Iran) was also administered to prevent infection. After applying hair removal cream to the right leg of the animals, aseptic preparation of the area was performed using 7.5 and 10% Povidone-Iodine solutions, respectively. The Achilles tendon was exposed by incising the caudal surface of the right limb above the tarsus joint. The tendon was then completely detached from the calcaneus bone with a scalpel blade. The exposed surface of the calcaneus was gently scraped with a blade to remove the cartilaginous part. Although two or three holes were made in the bone in other studies, a single 1.1 mm diameter hole was drilled in the lateromedial cortices of the calcaneal tuberosity in this study to keep the strength of the bone. The tendon was then re-attached to the bone using a modified Kessler suture with 2-0 monofilament nylon material (Monofil Polyamide, SUPA, Iran) so that the suture material passes through the tendon in a Kessler-like fashion and within the bone in a simple pattern (Figure 1A-D). Subcutaneous tissues and skin were then closed in one layer with a simple continuous pattern using 4-0 nylon suture material. Ketoprofen (2 mg/kg, Ketoject 10%, Nasr Pharmaceutical Co. Fariman, Iran) was injected intramuscularly for three days. The limbs of rabbits in group 1 were externally stabilized for immobilization from toe to mid-thigh using 3-inch fiberglass casts after surgery (Robo Cast, Daroo Sahel Co., Babolsar, Iran) so that the stifle joint was bent at an angle of 135° and the tarsus was bent backward so that the gastrocnemius and soleus muscles were in their shortest position. In this position, passive tendon stretching is prevented. In group 2, however, no casts were used, and the animals were allowed to move freely inside the cage during the study.

Radiographic Evaluation

The tarsal mediolateral radiographs were obtained the same day after surgery (day 0) and in 2, 4, 6, and 8 weeks following surgery using a radiography machine (Mobile drive AR30, Italy) under 50 KVp and 2 mAs projection conditions (Figure 2). The radiographs were examined by a radiologist who was blinded to the groups, and the osseous changes at the tendon-to-bone junction were evaluated in groups and compared with each other. Radiographic results were reported descriptively.

Pathologic Evaluation

At the end of the eighth week, according to the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition, the animals underwent cervical dislocation after injection of the aforementioned anesthetics combination, and samples of the Achilles tendon and calcaneus bone attached to it were taken. The samples were fixed in 10% buffered formalin solution for two weeks. Decalcification was done using a 20% of ethylene diamine tetraacetic acid (EDTA) solution for two weeks to prevent soft tissue and tendon damage during the process. After molding, 5 μm sections were prepared. The sections were stained by the hematoxylin-eosin method. The healing properties of the tendon-to-bone junction were examined by a pathologist under a light microscope, and the results were reported descriptively.

Results

Radiographic Findings

At the beginning of the study, the radiographs of
both groups were normal and no signs of bone changes were observed. All radiographs showed a hole made in the calcaneus for suture placement. In the 2nd week, a slight irregular bone formation was observed at the junction of the Achilles tendon in two cases of group 1 and one case of group 2. Also, a small amount of erosion was observed at the junction site in a case of group 1 and two cases of group 2. A fracture was observed in one radiograph of group 2 at the site of the calcaneus tuberosity on this day. In the 4th week, new bone formation was observed in two cases of group 1 and erosion in two cases of group 2 at the junction site. Although the rate of bone formation was higher than in the 2nd week, bone erosion was not much higher. Calcaneus deformity was observed due to fracture in one case in group 2. In both the 6th and 8th weeks, new bone formation at the tendon-to-bone junction was the most important finding in group 1 (Figure 3A). No bone erosion was observed and callus caused by the fracture in the calcaneus tuberosity was the most important radiological finding in one case in the 6th week and in two cases in the 8th week in group 2 (Figure 3B).

In general, it can be said that new bone formation was the most important radiological finding in group 1, the amount of which was increasing during the study. Erosion at the site of the tendon-to-bone junction was the most important radiological finding in group 2 until the 4th week. In addition, free movement of the animal and perforation of the calcaneus, which led to the weakening of the bone at that site, led to fractures in some cases in group 2, especially in the final weeks. In both study groups, no suture holes were obvious in the calcaneus from the 4th week onward.

Pathologic Findings

The tendon was healing and there was a lot of ground substance in group 1. Collagen fibers were formed in some places, but they did not have much regularity. In addition, collagen fibers had not yet formed into collagen bundles. The number of chondrocytes in the fibrocartilage region was high. Fibroblasts and fibrocytes were abundant. Some inflammatory activity was seen in the newly formed connective tissue. There were also many blood vessels (Figure 4).

In Contrast, the tendon was healing and the ground substance was lower in group 2. Collagen fibers and bundles were formed and arranged in most places. The number of chondrocytes in the fibrocartilage region was high. Fibroblasts and fibrocytes were abundant. Some inflammatory activity was seen in the newly formed connective tissue. There were also many blood vessels (Figure 4).

In Contraste, the tendon was healing and the ground substance was lower in group 2. Collagen fibers and bundles were formed and arranged in most places. The number of chondrocytes in the fibrocartilage region was moderate and less than the previous group. The number of bone-forming cells was low, and the bone showed no evidence of repair. Fibroblasts and fibrocytes were lower than the previous group and the tissue was more mature. Inflammatory activity in tissues was very rare. Finally, the vessels were lower than the previous group (Figure 5).
Figure 3. Mediolateral radiographs of the tarsus joint of rabbit after Achilles’ tendon reattachment to the calcaneal tuberosity in the 2nd, 4th, 6th, and 8th weeks of the study in group 1 (A, limb stabilization) and group 2 (B, free to move). A: Marked bone formation at the junction of the Achilles tendon to bone (arrows) is increasing during these weeks. B: A fracture in the proximal calcaneus is seen. An increase in callus size is observed with increasing study weeks (arrows).

Discussion

Since the enthesis or tendon-to-bone junction is composed of four areas including tendon, fibrocartilage, mineralized fibrocartilage, and bone, the healing of traumatic injuries in this area is controversial. It is generally believed that active or passive movement regulates the collagen fibers and increases the quality of the healing tissue. In contrast, many studies have emphasized the importance of immobilization during tendon healing to bone. Therefore, in this study, the role of immobilization or movement in the healing of enthesis was evaluated. All fibrocartilages of the natural enthesis associate with the lack of blood vessels, leading to poor healing at the junctions. Attention has not yet been paid to the factors that control angiogenesis during enthesis formation, and we are currently relying on information from fibrocartilage studies elsewhere. Here, angiogenesis is controlled by a variety of angiogenic and anti-angiogenic factors. Whether a normal fibrocartilage enthesis can be repaired after surgical reattachment of the tendon or ligament is of considerable clinical importance. Although few authors have reported failures at natural junction site reattachment, there are numerous reports claiming reconstruction of the enthesis structure after surgery. However, researchers often report poor repair quality in reconstructions, slow recovery, or the longer time necessary for enthesis remodeling to regain its mechanical properties. In the present study, suturing of the tendon to the bone was able to connect them after two months, confirming the results of researchers who accept the possibility of reconstruction of the enthesis. However, it should be noted that the biomechanical test was not performed in this study, which may be one of the limitations of this study. However, pathological evaluations showed some stages of tendon repair and angiogenesis, especially in group 1. It should also be noted that the type of surface to which the graft is attached has an effect on the healing outcome. According to Soda et al., when the ligament is reattached to the dense bone rather than spongy bone, its regeneration is more successful. However, St
Pierre and coworkers had previously reported no difference. Aoki et al. investigated the effect of graft attaching to spongy bone instead of calcified fibrocartilages. They attributed the poor junction in the canine infraspinatus tendon enthesis to the blocking effect of the calcified fibrocartilage layer, which inhibits angiogenesis. The calcaneal tuberosity is histologically composed of a very thin layer of cortical bone, with the subcortical spongy system just below it. However, the cartilaginous covering of the tuberosity surface compensates for the thinness of the cortical tissue. In this study, the fibrocartilage surface of the calcaneal tuberosity was scratched before attaching the tendon, and the severed tendon was sutured directly to the cortical bone surface; therefore, the negative effects of tendon attachment to both cancellous bone and fibrocartilage layer were avoided.

Although cartilage tissue, especially in group 1, had been formed to some extent in our study, the four areas of enthesis were not yet completely separable. Despite some researchers have reported that the healed junction shows the natural structure of the four regions, the importance of these regions in the repair process is disputed. In one study, the healed site of tendon-to-bone attachment was examined pathologically 24 weeks after surgery, and it was concluded that structural integrity at the repair site was weak and lacked a fibrocartilage region, indicating that the four-zone structure may not matter for obtaining good healing. Cell proliferation and differentiation, the presence of some growth factors, and a healthy scaffold contribute greatly to tissue regeneration. Hibino and coworkers showed that the presence of a tendon stump near the bone stimulates the growth of bone callus, which is probably due to the release of factors involved in osteogenesis by the tendon stump. In addition, mechanical stress is a key factor in osteogenesis. The calcified or bony tissue callus involves the attached tissue fibers, and this connection is important for the remodeling of tendon-to-bone attachment. Radiography is not practiced widely for enthesis problems. Benjamin et al. obtained radiographs to evaluate the trabecular network and enthesis in various parts of the human cadavers. They found that some features such as the orientation of the trabeculae along the tendon long axis, and the greater number of cancellous bone spicules at the site are more evident radiographically compared to histology. We used radiography to evaluate the healing process of the enthesis in this study. Radiological findings in group 1, in which applying a cast following the Achilles tendon to the calcaneus suturing prevented intense movements in the tendon, showed that the new bone formation on the calcaneus was greater than the other group. Bone formation increased over time and was at its highest in the 8th week. In contrast, bone erosion at the attachment site was greater in group 2 until the 4th week, in which the limb was allowed to move. In addition, the presence of two cases of fractures in the calcaneal tuberosity can be a complication of limb...
movement in this group. However, no fracture was observed in group 1. In both study groups, no suture holes were observed from the 4th week onwards, which is consistent with the bone repair process. Therefore, with increasing callus formation in group 1 based on radiographic images, it is likely that healing is better, and the attachment is stronger in this group. Four weeks of immobilization improved collagen organization and 16 weeks of immobilization were required to improve biomechanical properties in tendon-to-bone attachment in the rotator cuff of the rat shoulder.\textsuperscript{35} In a similar study, eight weeks of immobilization improved biomechanical properties.\textsuperscript{36} Immobilization after removal of the middle third of the rabbit patellar tendon has increased the tensile strength and quality of collagen organization.\textsuperscript{37} Hetrich et al. similarly concluded that immobilization of tendon-to-bone restoration may have a positive effect on the postoperative healing environment.\textsuperscript{38} Immobilization of middle ligament injuries in rabbits stifle also led to greater collagen regularity.\textsuperscript{39} In contrast, early intermittent passive movements can both protect healing tissue and prevent adhesions.\textsuperscript{40} More recent researches have shown that after rotator cuff repair surgery, delayed onset of postoperative activity reduced range of motion compared to rats in normal cage activity.\textsuperscript{41} Muscle tension has been shown to be essential for the formation of the postnatal enthesis. Four weeks of muscle paralysis with botulinum toxin in neonatal rats had a negative effect on biomechanical properties, organization of collagen fibers, and mineral composition of the enthesis.\textsuperscript{42} Although immobilization provides the opportunity for healing of tendon-to-bone attachment, complete immobilization of the joint may cause weakness and atrophy of the surrounding structures.\textsuperscript{43} The results of our study also showed that the regularity of collagen fibers was greater and tendon tissue was more mature in group 2 than the other group. However, the number of fibroblasts and fibrocytes, and blood vessels were higher in group 1. Less inflammatory activity in the newly formed connective tissue in group 2 also shows the advantage of movement during healing.

According to the results of the present study, it can be concluded that although early limb movement during the enthesis healing period helps to increase the regularity of collagen fibers in the tendinous area of the enthesis, it does not increase the amount of callus in the bony area. In addition, it increases the risk of bone erosion and even fractures. While stabilizing the limb in the first two months, healing of the enthesis protects the bone against the forces and helps bone formation, which has a positive role in enthesis healing. Therefore, it can probably be concluded that initial stabilization and subsequent delayed limb movement may help to regenerate the tendon area while giving the opportunity for the regeneration of the bony and fibrocartilage areas of the enthesis.

**Conflict of Interest**

The authors declare no conflict of interest regarding the contents of this publication.

**References**


