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Influence of mechanical unloading on histological changes of the patellar tendon insertion in rabbits

Hirotaka Mutsuzaki [a,*], Hiromi Nakajima [b], Yasuyoshi Wadano [a], Hikaru Takahashi [b], Masataka Sakane [c]

[a] Department of Orthopaedic Surgery, Ibaraki Prefectural University of Health Sciences, 4669-2 Ami, Inashiki-gun, Ibaraki 300-0394, Japan
[b] Department of Agriculture, Ibaraki University, 3-21-1 Chuo, Ami, Ibaraki 300-0393, Japan
[c] Department of Orthopaedic Surgery, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8575, Japan

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ABSTRACT

Background: The purpose of this study was to clarify the influence of mechanical unloading on histological changes of the patellar tendon (PT) insertion in rabbits.
Materials and methods: The PT was completely released from stress by drawing the patella toward the tibial tubercle with a stainless steel wire installed between the patella and tibial tubercle (mechanical unloading group, n = 28). The animals of the sham group underwent the same surgical procedure; however, the wire was not tightened (n = 28). The average thickness of the Safranin O-stained glycosaminoglycan (GAG) area, chondrocyte apoptosis rate and chondrocyte proliferation rate of the cartilage layer at the insertion were measured at one, two, four, and six weeks.
Results: The chondrocyte apoptosis rate in the mechanical unloading group was significantly higher than that in the sham group at one and four weeks ($p < 0.05$). The chondrocyte proliferation rate in the mechanical unloading group was significantly lower than that in the sham group at four and six weeks ($p < 0.05$). The average thickness of the GAG-stained area in the mechanical unloading group was significantly lower than that in the sham group at six weeks ($p < 0.05$).
Conclusion: Mechanical unloading significantly affected the increase in the chondrocyte apoptosis rate, decrease in the chondrocyte proliferation rate, and decrease in the GAG layer thickness at the PT insertion for up to six weeks in rabbits.
Clinical relevance: We suggest that more than 6 weeks of mechanical unloading should be avoided to prevent degeneration at the PT insertion.

1. Introduction

The patellar tendon (PT) insertion and anterior cruciate ligament (ACL) insertion comprise the following four distinguishable transitional tissue layers: tendon/ligaments, uncalcified fibrocartilage, calcified fibrocartilage, and bone [1–3]. Gradual changes in the hardness of different tissues reduce the stress concentration at the insertion site [1,3]. The inclusion of glycosaminoglycans (GAGs) in the cartilage zone provides water absorbability and flexibility to the ligaments [4]. The GAG and fibrocartilage layers are presumed to resist tensile, compressive, and shear stresses at the insertion site [3]. The GAG layer is important for load transmission at the insertion site. An understanding of the structural properties and extracellular matrix of the tendon-to-bone insertion site is necessary for the early management of injury and regeneration of the soft–hard tissue interface after tendon/ligament reconstruction and repair.

An increase in the chondrocyte apoptosis rate and degenerative histological changes of the cartilage layers were observed in our previous studies of the post-rupture human ACL tibial insertion [5,6]. We also reported that an increase in the chondrocyte apoptosis rate preceded a decrease in the average thickness of the Safranin O-stained area at the ACL insertion after ACL resection in rabbits [7,8]. After ACL rupture and resection, chondrocyte apoptosis can lead to degenerative changes of the cartilage layers in the insertion. After ACL rupture and resection, the insertion may be characterized by mechanical unloading. However, knee joint instability remains after ACL rupture and resection. Therefore, we used an experimental model in which the PT was slackened by drawing the patella toward the tibial tubercle without knee joint instability [9–12]. No investigators have yet studied the influence of mechanical unloading on the histological changes of the PT insertion. We hypothesized that mechanical unloading may increase the chondrocyte apoptosis rate and decrease the chondrocyte proliferation rate at the PT insertion. This may subsequently lead to a decrease in the average thickness of the stained GAG area at the PT insertion site. Thus, clinically, limiting the period of mechanical unloading after PT repair may be beneficial in order to reconstitute the normal histology of the PT

* Corresponding author. Tel.: +81 29 888 4000; fax: +81 29 840 2301.
E-mail address: mutsuzaki@ipu.ac.jp (H. Mutsuzaki).

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insertion site. The purpose of this study was to clarify the influence of mechanical unloading on the histological changes of the PT insertion in rabbits.

2. Materials and methods

2.1. Surgical procedure

Fifty-six skeletally immature male Japanese white rabbits (weight range, 2.5–3.0 kg, 14 weeks old) were used for this study. The rabbits were maintained in accordance with the guidelines of the Ethical Committee of the University of Tsukuba and the National Institutes of Health guidelines for the care and use of laboratory animals (NIH Pub. No. 85-23 Rev. 1985). The rabbits were divided into two groups (mechanical unloading group, n = 28; sham group, n = 28). Although the PT tension in the mechanical unloading group was completely released in the same fashion as that reported previously, we created a modified experimental mechanical unloading model using a rabbit PT [9–12]. After intravenously injecting a barbiturate (40 mg/kg body weight), an anterior skin incision was made on the right knee. The medial and lateral retinacula were incised longitudinally along both PT edges. Drill holes of one-millimeter diameter were created to open both the patella and tibial tubercle perpendicularly to the tibial axis. A stainless steel wire (0.97-mm diameter) was installed through both holes. The PT was slackened by pulling the stainless steel wire. The distance between the distal aspect of the patella and proximal aspect of the tibial tubercle was shortened by approximately five millimeters. Both edges of the wire were firmly fixed (Fig. 1). We confirmed that the PT was relaxed at all knee flexion angles and that no stress was applied to the PT throughout the experimental period. The same surgical procedure was performed on the animals of the sham group; however, the wire installed between the patella and the tibial tubercle was not tightened. The incision area was sewn with 2-0 nonabsorbable sutures. After the operation, each animal was allowed free movement in a cage without receiving any antibiotics. Seven animals in each group were euthanized by deep anesthesia at four time periods (one, two, four, and six weeks) after surgery. These time periods were the same as in our previous studies [7,8].

2.2. Histomorphological analysis

Patella–PT complexes were obtained from each animal's hind limb, which was trimmed to determine the sagittal plane of the central region of the insertion site. The specimens were fixed in 10% paraformaldehyde (pH 7.4) for one week. After fixation, all specimens were decalcified using 10% EDTA (pH 7.4) and embedded in paraffin. For each specimen, five-micrometers-thick serial sections of the sagittal plane of the insertion site were stained with hematoxylin and eosin (HE). Safranin O staining was conducted to observe GAGs, and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) was performed to detect apoptotic cells in the PT insertion [5–8] (Fig. 2a). Proliferating cell nuclear antigen (PCNA) was used to detect proliferating cells in the PT insertion [6] (Fig. 2b).

TUNEL was carried out in accordance with the instructions included in the Apoptag® Plus Peroxidase *In Situ* Apoptosis Detection kit (Merck Millipore, Billerica, USA) with the exception of counterstaining. Using the Apoptag method, the sections were incubated in an equilibrium buffer for 10 min at room temperature. Terminal deoxynucleotidyl transferase (TdT) and digoxigenin-labeled nucleotides were then incubated for 60 min at 37 °C in a humid chamber. Next, a peroxidase-conjugated digoxigenin antibody was incubated with the sections for 30 min at room temperature in the humid chamber. After the immunoreaction product was developed in diaminobenzidine, the sections were counterstained with Mayer's hematoxylin for 30 s. TUNEL-positive nuclei stained dark brown, and TUNEL-negative nuclei stained blue [5–8].

PCNA immunostaining was performed in accordance with the instructions included in the Histofine® SAB-PO(M) kit (Nichirei Biosciences Inc., Tokyo, Japan). Deparaffinized sections were rinsed in PBS for five minutes. They were then immersed in three percent hydrogen peroxidase (H_2O_2) in methanol for 10 min to block endogenous peroxidase. After the slides were rinsed in PBS for five minutes, they were preblocked with a solution of 10% normal rabbit serum at room temperature for 10 min and incubated at 4 °C for 12 h with a monoclonal antibody to PCNA (PC-10, Code No. M0879; Dako, Denmark), which was diluted 100 times. For the negative control, Antibody Diluent (Code No. S0809; Dako) was used as the primary antibody [6].

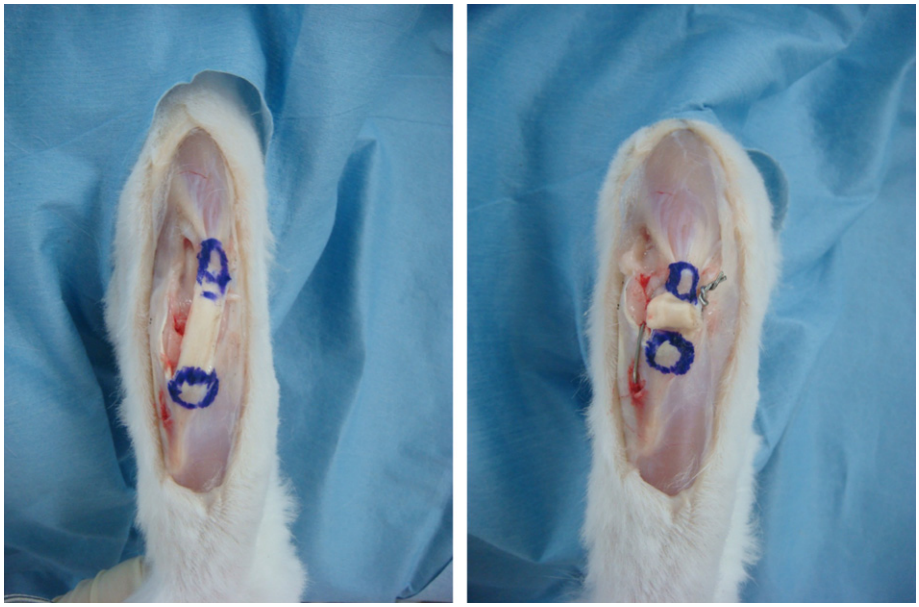

Fig. 1. Right knee of a rabbit. (a) Patellar tendon before mechanical unloading. (b) The patellar tendon was completely released from stress by drawing the patella toward the tibial tubercle with a stainless steel wire installed between them (mechanical unloading). Upper blue circle is the patella. Lower blue circle is the tibial tubercle.