

# Effects of unloading and return to normal load conditions on the mouse Achilles tendon enthesis

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## 1. Introduction

The insertion site of a tendon on a bone is called the enthesis<sup>1</sup>. The Achilles tendon inserts onto the calcaneus through fibrocartilage (FC). This fibrocartilaginous insertion is composed of an uncalcified fibrocartilage zone (UFC) rich in type I, II and III collagens and proteoglycans (PG), and a calcified fibrocartilage zone (CFC), additionally composed of minerals. This composition allows a gradual transition from tendon (soft tissue) to bone (hard tissue), which is why the achillean enthesis (ATE) is referred to as a functionally graded material. This particular structure allows the insertion to withstand a complex mechanical environment, combining tensile, compressive and shear stresses. Like other musculoskeletal tissues, the enthesis is sensitive to variations in mechanical load. The microgravity conditions to which astronauts are subjected during their mission are a hypoactivity situation, where the mechanical environment is altered.

The purpose of this study was to characterize the effects of 14 days of simulated microgravity and 7 days of return to normal load conditions on the structure, composition and mechanical response of the ATE in mice. Through an interdisciplinary approach, tissue remodeling and the mechanical behavior of the enthesis were characterized.

## 2. Methods

### 2.1 Animal Model

The Morey-Holton model<sup>2</sup> was used to simulate microgravity conditions. 26 mice were hindlimb unloaded (HU) for 14 days. After the suspension, 13 were euthanized (HU group) and 13 were unsuspended and moved freely for 7 days (HUR group). 13 other mice, unsuspended, were kept as control (CTL group).

### 2.2 Biological characterization

The right paw of each mouse was dedicated to histological experiments. The samples were prepared and embedded in paraffin. 7- $\mu$ m thick cuts were made and whether stained with Toluidine Blue (TB) for FC visualization and collagen organization (under polarized light), or Alcian Blue (AB) for PG characterization.

### 2.3 Mechanical characterization

The left paw of each mouse was frozen at  $-80^{\circ}\text{C}$ . 7 samples by group were used for tensile tests. The 6 other samples by group were used for compressive cyclic tests. Mechanical tests were performed on an Instron 5566A device equipped with a 100N sensor.

For tensile test<sup>3</sup>, samples were tested to failure at a 2mm/min speed. Maximum strength was determined from the strength-displacement curves and the local

displacement and the local stiffness of the enthesis were determined from local displacement on video recordings with Kinovea software.

For compressive cyclic test<sup>4</sup>, 2500 cycles were applied at 1Hz from 0 to 5N with a flat tip of 2mm in length. These parameters aimed to mimic the repetitive solicitations applied at the ATE during 1 day of walk for a laboratory mouse at 60% of maximum force. Stiffness, displacement and hysteresis area were determined from strength-displacement curves with Matlab software. Variations of these parameters between the beginning and the end of the test were calculated.

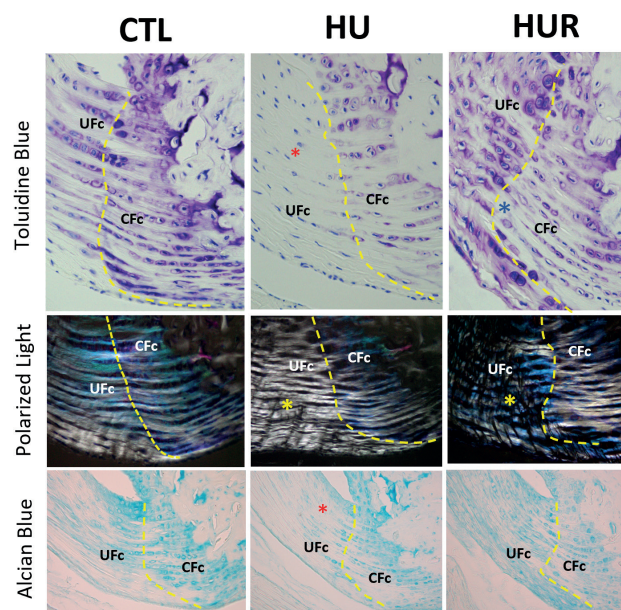
### 2.4. Statistics

For tensile test, data were compared with a non-parametric Kruskal-Wallis test and a pairwise Conover-Iman test. For compressive test, a 2-factors mixed ANOVA was performed followed by a pairwise t-test if a significant effect was highlighted. Significance was set at  $p < 0.05$ .

### 3. Results and discussion

The FC of CTL ATE is composed of PG (purple in TB and blue in AB) and well-aligned collagen fibres (polarized light) (Fig. 1).

After 14 days of suspension (HU), we observed a loss of PG and a collagen disorganization in the UFc. In traction, while these changes led to a decreased stiffness, the strength at failure was not affected (Table 1). Indeed, as we showed that the gradient of mechanical properties at the ATE was not affected by HU, the remodeling that occurred in the FC strengthened the ATE in traction<sup>3,5</sup>. However, this remodeling does not protect the ATE response in compression since it led to a quick mechanical fatigue and a loss of elasticity (Table 1).



**Figure 1.** Biological remodeling at the ATE after HU and return to normal load. Red arrow: loss of staining in the UFc. Blue arrow: Newly mineralized CFc. Yellow arrow: Collagen disorganization.

7 days of reloading (HUR group) resulted in a return of PG in the UFc, but the collagen fibers were still crimped and disorganized (Fig. 1). We also observed an advance of the CFc that led to an increased CFc area<sup>3</sup>. However, it appeared that the presence of PG in the UFc was sufficient for the ATE to recover its tensile properties (Table 1). In the same way, the remodeling in HUR ATE allowed a partial recovery of the compressive behavior. The hysteresis loss was still high in the HUR group, as well as a higher gain in stiffness after 2500 cycles. This probably highlight the role of collagen fibers in the mechanical behavior of the ATE as well as its resistance to compressive stress<sup>5</sup>.

**Table 1.** Tensile and Compressive behavior of the ATE. § different from CTL, # different from HUR ; \* values at 2500 cycles statistically different from 100 first cycles.

Condition	Tensile Behavior			Compressive Behavior	
	Maximum strength (N)	Stiffness (N/mm)	Displacement Loss (%)	Hysteresis Loss (%)	Stiffness Gain (%)
CTL	7.85 ± 0.89	15.12 ± 3.62	-16.7	-16.2	+14.2
HU	9.80 ± 1.07 §	9.86 ± 2.17 §	-27.2 *	-26.9	+45.2
HUR	9.60 ± 1.32	16.32 ± 3.04	-11.7	-28.5 *	+25.7 *

#### 4. Conclusions

The ATE undergoes remodeling in response to variation in mechanical load. This biological remodeling has an impact on the tissue's ability to withstand mechanical stresses and may alter its function. While the enthesis behavior under various stresses has been characterized individually, it is important to consider the impact of the enthesis remodeling in all the solicitations axis. Indeed, in addition to the risk of failure in traction, changes in compressive behavior may result in additional complications such as insertional tendinopathies.

In addition, future work should focus on how to counterbalance these changes and minimize the risks of injury to prevent the health of astronauts during their missions and when they return to Earth.

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