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# Testosterone may increase rat anterior cruciate ligament strength☆

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ABSTRACT

*Background:* Women are more likely than men to injure the anterior cruciate ligament (ACL). Human and animal trials have linked circulating estradiol to injury rate and ligament strength. Fewer studies have examined the role of testosterone. The purpose of this study was to determine if male rats with normal testosterone levels would have stronger ACLs than castrated rats.
*Methods:* Eight castrated (group C) and eight normal (group N) 12-week-old, male Sprague–Dawley rats were used for the study. Mean testosterone levels were 0.14 ng/mL (95% CI: 0.10 to 0.17) in group C and 3.54 ng/mL (95% CI: 1.32 to 5.76) in group N. After euthanasia, ACL cross-sectional area was calculated, and a servohydraulic material testing unit was used to measure ligament properties.
*Results:* Specimens from both groups had similar cross-sectional area, but N specimens showed greater mean load-to-failure (34.5 N [95% CI: 31.6 to 37.4] vs 29.2 N [95% CI: 27.9 to 30.6]) and ultimate stress (38.7 MPa [95% CI: 34.1 to 43.3] vs 31.8 MPa [95% CI: 29.8 to 33.8]). Mean energy was 27.7 mJ (95% CI: 23.1 to 32.2) in the N group and 23.4 mJ (95% CI: 18.2 to 28.6) in the C group.
*Conclusions:* Rats with normal circulating testosterone had higher ACL load-to-failure and ultimate stress, indicating that testosterone may influence ACL strength and the injury rate of the ligament.


## 1. Introduction

Women are two to 10 times as likely to injure the anterior cruciate ligament (ACL) as men participating in similar military and athletic activities [1,2]. Several intrinsic and extrinsic explanations have been proposed to account for this disparity [3], including sex-specific differences in how circulating sex hormones may affect ligament remodeling and strength [4–15].

Previous human and animal trials have shown mixed results, linking the circulating estradiol that is predominant in females to injury risk [16–18] and the physical properties of the ligament [4,6,8–12,15–17,19–23]. Androgen receptors have been identified in animal and human ACLs [8–25], suggesting that the ligament may be an androgen-responsive tissue. Testosterone, in particular, has demonstrated antagonistic effects to estradiol, including increasing the collagen expression in prostate [26], urinary [27], capsular [28], and intervertebral disk tissues [29] and protecting against inflammation-induced cartilage degradation [30]. Few studies, however, have examined the potential role of testosterone as an intrinsic factor in collagen remodeling and the physical properties of the ACL [8,11,31,32].

Because altering the normally circulating sex hormones in healthy human subjects is not feasible, several animal models have been developed to examine the potential role of sex hormones on ligament strength and remodeling [6,10,12,32,33]. The purpose of this study was to determine the effect of testosterone on the strength of rat ACLs. If testosterone is, in fact, an antagonist to the potentially detrimental effects of estradiol on ligament tissue remodeling, we would expect that higher levels of testosterone would enhance collagen remodeling and ligament strength. Therefore, we hypothesized that intact male rats with normal levels of circulating testosterone would have stronger ACLs than castrated animals with negligible levels of testosterone.

## 2. Methods

We used sixteen 12-week-old male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA). Eight were sexually intact (normal [N] group; pre-operative mean weight, 484 g [SD: 21.4]), and eight had been castrated by the vendor immediately before shipping to eliminate production of the predominant gonad-produced androgens and estrogens (castrated [C] group; pre-operative mean weight, 474 g [SD: 20.5]).

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### 2.1. Animal preparation

All protocols were in accordance with our institution's Animal Care and Use Organization and the Guiding Principles in the Care and Use of Animals, approved by the Council of the American Physiological Society. Rats were housed in a controlled environment at 22.5 °C and had access to tap water and pellet food ad libitum. Rats were maintained for 35 days after delivery prior to blood draw and euthanasia, similar to the model used by Warden et al. [34].

### 2.2. Testosterone and estradiol determination

Immediately before euthanasia, rats were weighed, and 1 mL of blood was drawn from each rat and centrifuged at 1200 rpm to separate serum from cells. Serum was stored at −70 °C until assay analysis. Serum testosterone and estradiol concentrations were determined via enzyme-linked immunoassay (Calbiotech, Inc., Spring Valley, CA, USA). All samples were assayed in duplicate and according to manufacturer's instructions. C group rats had significantly lower mean levels of circulating testosterone (0.14 ng/mL [95% CI: 0.10 to 0.17] vs 3.54 ng/mL [95% CI: 1.32 to 5.76]) and estradiol (3.11 pg/mL [95% CI: 2.58 to 4.41] vs. 11.53 pg/mL [95% CI: 8.49 to 14.57]) than the N group rats.

### 2.3. Tissue collection

Rats were euthanized 35 days after delivery. Each right lower extremity was disarticulated at the hip, and the surrounding muscle was excised with the joint capsule. All ligaments and menisci were initially kept intact. Dissected specimens were wrapped in saline-soaked gauze and stored at −80 °C in double zip-top plastic bags until material testing.

Immediately before mechanical testing, the specimens were allowed to thaw, and the ACL in each was isolated by excising the remaining soft tissues, ligaments, and menisci. Specimens were wrapped in saline-soaked gauze to prevent dehydration until testing one to two minutes later.

### 2.4. Material testing

To address several previously reported technical challenges in measuring rodent ACLs, including avulsion of the bony origin or insertion and difficulty measuring the size of the ligament [34–36], we developed a lightweight aluminum fixture patterned after a design by Warden et al. [34]. With this fixture, we were able to secure the femur-ACL-tibia complex and prevent epiphyseal avulsion, ensuring that all samples failed within the midsubstance of the ligament.

Each specimen was affixed to our material testing machine (Bose Model 3200, Bose, Framingham, MA, USA) in an aluminum fixture that prevented epiphyseal avulsion by securing the femoral and tibial surfaces (Figure 1). The tibia and femur were aligned at a 90° angle so that the long axis of the ligament passed through the axis of the actuator and the load cell (100 N, Sensotec, Columbus, OH, USA) (Figure 1). Slack in the ACL was removed by manually displacing the actuator until a force of 1 N was registered on the load cell. Each ligament was then cyclically deformed by 0.5 mm at a grip-to-grip rate of 0.25 mm/s for 10 cycles [34,37]. On the last cycle, the displacement was held for 60 s while images of the front and side of the ligament were captured by an 8.1-megapixel camera (Cannon, Lake Success, NY, USA) (Figure 2). The ligament was then returned to its zero displacement and allowed to relax for 600 s, after which the specimen was stretched to failure at 0.25 mm/s, and force-vs-deformation data were recorded at 10 Hz [10,34].

Data were transferred to Microsoft Excel, version 12.0, software (Microsoft Corp., Redmond, WA, USA) for analysis. Load-to-failure was defined as the highest point in the load-vs-displacement curve before ligament rupture. Energy-to-failure was determined by integrating the area under the force-vs-displacement curve with MATLAB, version 12.3, software (MathWorks, Natick, MA, USA). Ultimate stress was calculated by normalizing the load-to-failure value by cross-sectional area (CSA).

**Figure 1.** Side view of the material testing fixture showing restraint of rat femoral condyle and tibial plateau to prevent bone avulsion.
This set-up was based on a design by Warden et al. [34].

### 2.5. Cross-sectional area

A digital camera secured to a stand fixed 27 mm from the specimen with its lens at the height of the exposed ligament was used to capture images at the front and side of the ACL (Figure 3). Before testing, a standard metric ruler was placed in line with the front and side of an ACL specimen, and images of the ruler were captured from each direction. The images were uploaded to ImageJ (National Institutes of Health, Bethesda, MD, USA), and the distance of one millimeter was measured with the straight line function and converted to pixels to determine the number of pixels per millimeter from each direction.

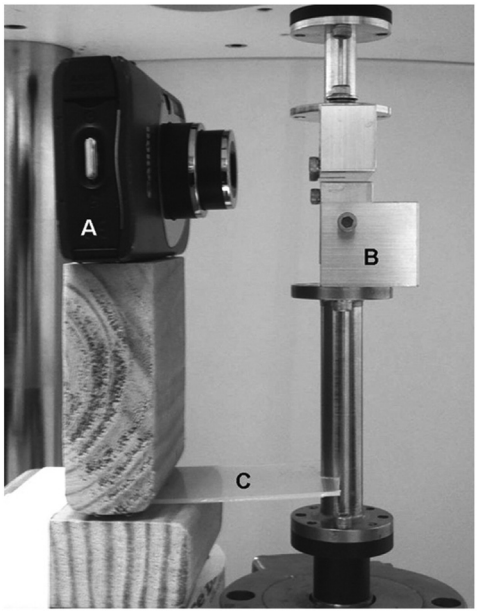


**Figure 2.** Camera and material testing fixture set-up with (A) camera, (B) fixture hardware, and (C) plastic guide fitted to circumference of the fixture base to maintain a 27-mm distance between camera lens and specimen on side and front views.

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