Testosterone may increase rat anterior cruciate ligament strength ☆

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A R T I C L E I N F O

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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.

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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]).
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).

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.
To ensure a consistent point of measurement for each sample, front and side views of the specimen were measured three times each with the ImageJ straight line tool at a level two-thirds of the distance between the tibial and femoral fixation hardware. Averages of the three front and side measurements were converted from pixels to millimeters using the standard measures of the ruler captured before testing. The calculation of the cross-sectional area was based on measurements calculated at two-thirds of the distance between the femoral and tibial restraints of the material testing fixture.

3. Theory/calculation

Differences in circulating levels of sex hormones are one of many possible mechanisms suggested for the sex disparity in ACL injury rates. Whereas considerable work in this area has focused on the predominant female sex hormone, estrogen, previous studies by Romani et al. [10] and Sciore et al. [25] suggest that circulating testosterone in males may also play a key role. Because in vivo alterations of circulating sex hormones and accurate measurement of the ACL are impractical in a human model, it is important to further develop animal models and measurement techniques to better understand the molecular mechanisms of injury risk and the physical properties of the ligament. As a result, the current study used a practical method to further characterize the strength and CSA of the rat ACL to show that normal levels of circulating testosterone are associated with higher rat ACL load-to-failure and ultimate stress. As training and motor control strategies for preventing ACL injuries evolve, it will be important to continue to develop animal models and measurement techniques that help determine predisposing factors for increased ACL injury risk and better enable sports medicine professionals to target the athletes who may benefit most from these interventions.

4. Results

The CSAs for the N and C groups were not significantly different ($p = 0.70$): 0.90 mm$^2$ (95% CI: 0.81 to 1.00) and 0.92 mm$^2$ (95% CI: 0.88 to 0.97), respectively. The N group showed significantly 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]). Differences in mean energy between the N and C groups were not significant ($p > 0.05$): 27.7 mJ (95% CI: 23.1 to 32.2) and 23.4 mJ (95% CI: 18.2 to 28.6), respectively.

5. Discussion

The primary finding of our study was that male rats with intact testes and normal levels of circulating testosterone had ACLs with higher load-to-failure and ultimate stress than castrated rats. This finding was consistent with our hypothesis and suggests that androgens may contribute to the ACL’s ability to withstand tensile loads and may be one of a number of factors responsible for the disparate ACL injury rate between men and women. There were no significant differences in energy under the curve between the groups.

Altering circulating sex hormones and accurately measuring the physical properties and failure of the ACL in an in vivo condition is impractical in a human model. Therefore, several animal models have been developed to address this question [6,10,12,24,32,33]. Large animal models may be similar to the human condition but are commonly limited by cost and low statistical power [38,39]. The rat model is more accessible, has midcycle estrogen surges similar to the human menstrual cycle, [40–43] and has been validated and used to investigate the effect of sex hormones on the synthesis and proliferation of collagen in musculoskeletal tissue [44,45]. Therefore, we chose the rat model for the current study and incorporated a number of advancements over previous methods, including an extended exposure to the experimental and control levels of sex hormones equal to five estrous cycles, the practical measurement of CSA, and material testing with a device that ensured midsubstance rupture of the ACL.

Because male rodents are larger than females of the same age, corresponding differences in ligament size must be considered when determining ligament strength and sex-specific differences in mechanical properties [46–48]. Tipton et al. [48] showed that knee ligament size in male rats was linearly related to body mass. This method of normalization, although useful in males, is not an accurate measure in females or in animals that undergo large fluctuations in mass caused by fluctuations in sex hormones [48]. Therefore, it is important to have a measure of ligament CSA or volume to normalize for differences in rat mass because of age, sex, or hormonal exposure.

In larger animals, measures of ligament length and CSA are common [5,6,39,49]. But, in rodent models, where the ACL is small and hidden by the femoral condyle, investigators have used indices of ligament size...
based on similar animals as a substitute for traditional caliper or imaging measures [35] or refrained from measurement altogether [10,35,48]. Our fixture design and recent advances in digital photography allowed us to capture images consistently in the frontal and sagittal planes of the ACL to determine CSA at approximately one-third of the distance between the tibial and femoral insertions. One of the confounding issues with earlier methods of rodent ACL material testing has been isolating the failure of the ligament at midsubstance rather than at the ligament–bone junction [48]. Our material testing device addressed this factor by using a lightweight aluminum fixture designed to reduce interference with the low load measures of the ligament and to encapsulate the femoral condyle and tibial plateau to prevent avulsion at the epiphyseal plate. The result was that all 16 of the ligaments failed at midsubstance (Figure 1).

If testosterone does, in fact, protect the ACL through increasing the remodeling and strength of collagen, we would expect tissue exposed to normal circulating levels of testosterone to be stronger than tissue in the absence of testosterone. Earlier work has, in fact, linked testosterone to increased collagen expression and strength in several collagen-rich animal and human tissues [33, 45]. In rat models similar to the model used in the present study, Dehghan et al. [50] showed an association between testosterone levels and reduced knee laxity. Despite this earlier work, we have not identified studies specifically targeting levels of testosterone and subsequent ACL strength changes in an in vivo animal model. Our results suggest a downstream association between testosterone and ligament strength that may indicate that testosterone plays a role in the ability of the ACL to withstand tensile loads and in the disparate numbers of ACL injuries between males and females.

Hormone regulation was the result of castration in the C group of rats. Successful castration of our rats was confirmed by blood assay after 35 days of exposure of the ligaments to the altered level of sex hormones before tissue harvest. Although the focus of our study was the role of testosterone on the ACL strength of male rats, it is important to note that castration also resulted in a reduction of circulating estradiol. Estradiol has been implicated as an antagonist to collagen formation and ligament strength [4–15]. It has also been shown to interact with testosterone in breast cancer cells through aromatization and alterations of sex hormone-binding globulin [51, 52] and as the key mediator of testosterone’s association with increases in ACL stiffness in women [8]. With the much higher levels of fluctuating testosterone in males relative to females, and with E2 levels as much as one-third as high as those normally found in females, it is not clear that E2 played the same mediating role in collagen remodeling in males as earlier work in females suggests. This potential relationship is an area for future examination to identify a more complete mechanistic explanation for the findings in the present study.

Although we took steps to improve the way the ligaments in our study were measured compared with previous models based on indices or animal weight [10, 35, 48], there were some limitations to our methods. For instance, the measurement of CSA is an estimate based on the assumption that the rat ACL is elliptical in shape. Some inaccuracies in CSA approximation have been noted in previous three-dimensional imaging of human cadaver ACLs [49]. However, we believe that our technique provides a practical estimation of the smaller ligament size in a rodent model that can be easily measured for each ligament without reliance on indices based on body mass that are commonly confounded by hormone-mediated changes in body weight. Similarly, it is unlikely that the method that was used to calculate CSA and ultimate load-to-failure changes the main finding of this study, i.e., that male rats with intact circulating levels of testosterone had higher ACL loads-to-failure than castrated rats.

As in all animal studies, it is important to note that there are differences from human models that must be considered when extrapolating the findings to humans. Finally, this project examined the strength of the ACL in two hormonal conditions but leaves unanswered the potential mechanism by which sex hormones may affect collagen formation or the expression of matrix metalloproteinases, tissue inhibitor of metalloproteinase, or other processes associated with ligament remodeling and strength. These questions will be part of a future trial using a similar model.

6. Conclusions

Sex hormones are believed to play a role in the remodeling and strength of collagen-rich tissue such as ligaments. Several studies have suggested that estrogen may be a risk factor for ACL injuries in women. Whereas previous work has compared the association between concentrations of the predominant male sex hormone, testosterone, and in vitro tissue remodeling and injury, we used a lightweight aluminum fixture and a practical measure of ligament cross-sectional area to determine the potential effect of testosterone on in vivo ACL strength in male rats. Our primary finding that male rats with intact testes and normal levels of circulating testosterone had ACLs with higher load-to-failure and ultimate stress than castrated rats suggests that androgens may contribute to the ACL’s ability to withstand tensile loads and may be one of multiple factors responsible for the disparate ACL injury rate between men and women.

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