Journal of Athletic Training 2010;45(1):22–28

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Sex, Collagen Expression, and Anterior Cruciate Ligament Strength in Rats

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Context: Sex-specific responses to steroid sex hormones have been suggested as a potential cause for the disparate anterior cruciate ligament (ACL) injury rates between male and female athletes. Type 1 collagen (T1C) and type 3 collagen (T3C) are crucial structural components that define the ligament's ability to withstand tensile loads. Messenger RNA (mRNA) is an important mediator of downstream collagen synthesis and remodeling, but the sex-specific mechanisms of collagen mRNA expression and ACL strength are unknown.

Objective: To examine the influence of sex on T1C and T3C mRNA expression and mass-normalized stiffness and peak failure load in the ACLs of skeletally mature rats.

Design: Observational study.

Setting: Basic sciences and biomechanical testing laboratories.

Patients or Other Participants: Nineteen 12-week-old male (n = 9) and female (n = 10) Sprague Dawley rats.

Main Outcome Measure(s): We used real-time polymerase chain reaction to determine T1C and T3C mRNA expression and a hydraulic materials testing device to measure ACL stiffness and failure load. Nonparametric Wilcoxon rank sum tests were used to compare the groups.

Results: Female rats had lower amounts of T3C mRNA expression and higher normalized ACL tangent stiffness and failure load than male rats.

Conclusions: These findings suggest that sex-specific differences in T1C and T3C mRNA expression may play an important role in the downstream mechanical properties of the ACL.

Key Words: knee injuries, women's health, real-time polymerase chain reaction

Key Points

- Compared with male rats, female rats demonstrated less type 3 collagen messenger RNA (mRNA) expression and greater normalized anterior cruciate ligament stiffness and load to failure.
- Sex-specific differences in type 1 and type 3 collagen mRNA expression may influence the mechanical properties of the anterior cruciate ligament.

The anterior cruciate ligament (ACL) provides as much as 86% of the passive anterior restraint of the tibia on the femur.[1] As a result, ACL injuries often require surgical reconstruction and extensive rehabilitation to restore knee stability and ensure a full functional recovery. The long-term consequences of ACL injuries include accelerated knee joint degeneration; laxity; osteophyte formation; and type 2 collagen degradation, which may lead to early onset of osteoarthritis.[2–5]

Most ACL injuries result from high tensile loads applied to the ligament during sudden changes of direction and jumping.[6] Whereas sex differences in landing and running biomechanics may place the ACL at risk,[7,8] the structure's ability to withstand those tensile loads likely depends upon several factors, including ligament size, rate of collagen remodeling, and collagen isoform.[9–11] Type 1 collagen (T1C) accounts for up to 87%[9,12] of the ligament's collagen content[9,12–14] and, along with type 3 collagen (T3C), acts to resist the loads applied to the ACL.[9,12] Type 3 collagen is also believed to play an important role in healing.[13–15] The extracellular matrix (ECM), including elastin, proteoglycans, glycosaminoglycans, and glycoproteins, maintains the ligament's limited elasticity and its ability to distribute stresses uniformly.[12,16] Given similar shapes, larger ligaments with similar collagen composition and fibril size would be expected to be stronger than smaller ones. The ratio of T1C and T3C isoforms is one variable that contributes to differences in ligament scarring, healing,[9,11,13,17] and mechanical strength[15,17] among individuals and among different connective tissues (Figure 1).

Collagen synthesis and remodeling follow the transcription of T1C and T3C messenger RNA (mRNA) and the conversion of procollagen into collagen fibers.[9,18–20] Extracellular matrix metalloproteinases and their inhibitors help to regulate collagen remodeling and play an important role in determining the composition and mechanical properties of the ACL.[12,21–23] As a result, the expression of collagen mRNA does not necessarily correspond with downstream changes in collagen protein expression and ACL strength. However, any sex-specific differences in ACL strength and remodeling are likely to begin with initial differences in the transcription of collagen mRNA. The potential influence of sex on the relationship between T1C and T3C mRNA expression and the tensile properties of the ACL is still unknown. Thus, the purpose of our study was to examine the influence of sex on T1C and T3C mRNA expression and the

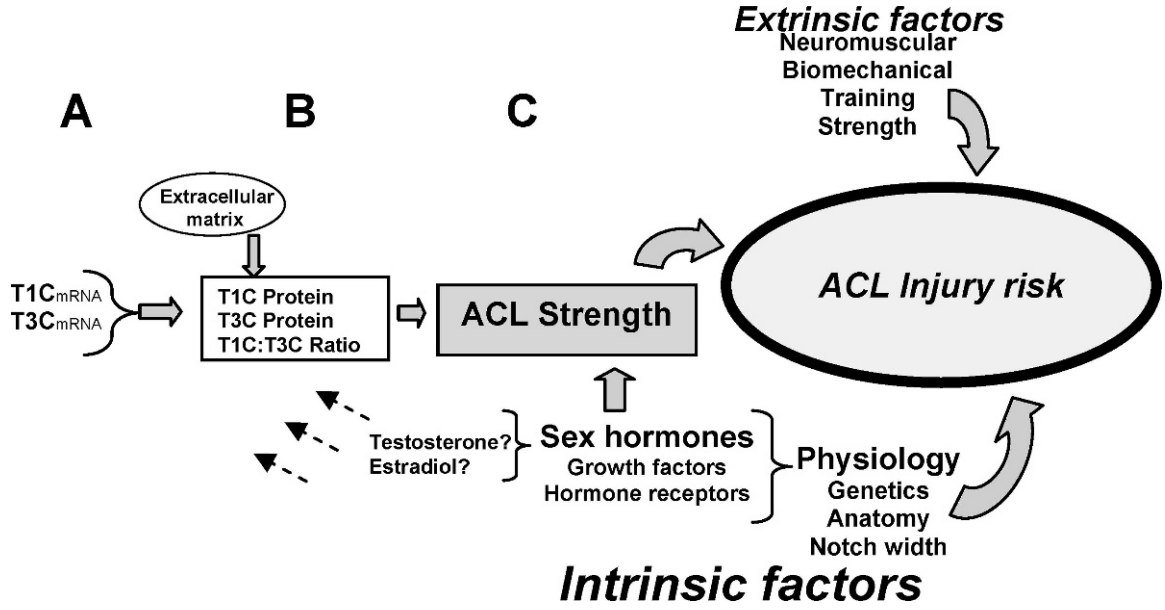

Figure 1. Sex hormones regulate collagen remodeling and anterior cruciate ligament (ACL) strength. Extrinsic factors can be modified to limit the athlete's exposure to dangerous postures or movements. Intrinsic factors, such as physiology and sex hormones, may contribute to the risk of injury by regulating the mechanical strength of the ACL. A, Cyclic, short-term concentrations of testosterone and estradiol influence the acute messenger RNA (mRNA) expression of collagen within 24 to 48 hours. This acute expression is the first of several steps in the formation and remodeling of type 1 collagen (T1C) and type 3 collagen (T3C) protein. B, ACL strength is determined by the amount and ratio of T1C to T3C protein. Acute changes in mRNA expression and exposure to proteinases within the extracellular matrix in A then result in longer-term, downstream changes in the synthesis and remodeling of T1C and T3C in B. After repeated acute exposures to fluctuating concentrations of sex hormones over several reproductive cycles, a baseline level of collagen formation and remodeling determines the strength of the ACL, C, and thus the potential risk for injury.

mass-normalized stiffness and peak failure load in the ACLs of skeletally mature rats.

METHODS

Nineteen 12-week-old male (n = 9, mean weight = 423.4 ± 33.0 g) and female (n = 10, mean weight = 266.0 ± 23.0 g) Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were studied.

Animal Preparation

All protocols were carried out 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 with access to tap water and pellet food ad libitum. In order to help ensure that the female rats were examined during the same stage of the estrous cycle, starting at day 14, we examined them at 8:00 AM using vaginal lavage with phosphate-buffered saline solution. Cells were placed on a glass plate and viewed with a light microscope to determine the estrous cycle stage per the technique of Borgeest et al.[24]

Tissue Collection

The left ACLs of the male rats were harvested at 2:00 PM on day 16 after delivery and from all of the female animals on the same afternoon that they were determined to be in the diestrous stage of their estrous cycle, when estradiol (E2) concentrations are usually at their lowest levels.[25] Tissues were placed in 125 μL of RNA-stabilizing reagent (USA-Qiagen, Valencia, CA) and stored at −20°C until analyzed.

The right lower extremity was disarticulated at the hip and the surrounding muscle excised, with the joint capsule and all ligaments and menisci initially kept intact. Dissected specimens were wrapped in saline-soaked gauze and stored in double–zip-top plastic bags at −70°C. The frozen femur and tibia of each specimen were placed into plastic 0.375-in- (0.95-cm)-diameter bone pots (ie, plastic drinking straws) filled with polymethyl methacrylate cement (Fastray; Harry J. Bosworth Co, Skokie, IL), so that the knee was kept in 90° of flexion. Potting in cement provides support to the bones and a more uniform structure to fit into the grips of the material testing unit. Specimens were kept moist with saline and longitudinally aligned while the cement cured. Immediately before the mechanical testing, the specimen was allowed to thaw, and we isolated the ACL in each specimen by excising the remaining soft tissues, ligaments, and menisci. Specimens were wrapped in saline-soaked gauze to prevent dehydration until testing 15 to 20 minutes later.

One milliliter 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 E2 and testosterone concentrations were determined via enzyme-linked immunoassay (Calbiotech Inc, Spring Valley, CA). All samples were assayed in duplicate and according to the manufacturer's instructions.

Tissue Preparation

The mRNA from the ACL tissue was isolated using the Qiagen QIAshredder kit and RNeasy Mini kit (fibrous tissue) according to the manufacturer's protocol (USA-Qiagen). We homogenized the tissue by placing it and three

2.4-mm zirconia beads into a 1.5-mL specimen tube filled with the RNA lysis buffer RLT provided with the isolation kit. Specimen tubes were placed in a bead mill (Mini-Beadbeater-8; BioSpec, Bartlesville, OK) to violently shake the beads for 2 minutes and break up the dense ligament tissue. Afterward, the isolation samples were measured via spectrophotometry (A260/A280 ratio) to determine the total RNA in each sample (0.44–2.7 μg) and purity (1.6–2.0).

Quantitative Polymerase Chain Reaction Analysis

The RNA was reverse transcribed into complementary DNA (cDNA) using an Omniscript RT kit (USA-Qiagen) with random primers per the manufacturer's protocol. From the cDNA sample, 3 μL was briefly stirred with a vortexer and incubated for 60 minutes at 37°C. A reagent blank prepared using the reverse transcription blank was included in all polymerase chain reaction (PCR) runs. Primer sequences for T1C[26] (5′GCGAGGACATGAGGAG-TAGC3′ [forward] and 5′CCTGTGACCAGGGATGTC-TT3′ [reverse]) and T3C[27] (5′TGCTGCCATTGCTGGAG-TTGGA3′ [forward] 5′TGGCAGAATTTCAGGTCTC3′ [reverse]) were derived from published reports and prepared by our university's Biopolymer Laboratory. Because mRNA extraction yield from the small quantities of dense ligament tissue may be low, cDNA samples were run on a 1.5% agarose gel and electrophoresed at 100 V for 25 minutes. Signal at 269 (T1C) and 201 (T3C) base pairs was present for all samples before quantitative PCR (qPCR) analysis.

A real-time PCR protocol to measure the amount of PCR product by using a SYBR Green kit and a Chromo4 Real-Time Detector (MJ Research, Waltham, MA) and Opticon Monitor software (Opticon Inc, Orangeburg, NY) was followed according to the manufacturers' instructions and was consistent with previous work completed in our laboratory.[28,29] The Chromo4 quantifies the amount of PCR product by measuring the fluorescence of the SYBR Green when it is bound to double-stranded DNA. In order to analyze the genomic value of cDNA, a standard curve was generated from 5 serial dilutions of 1 of the samples.

The T1C and T3C mRNA was used with temperatures varying for each individual primer. Quantitative analysis was carried out with the SYBR Green kit and Chromo4 Real-Time Detector. As an internal control, 18S was used, and each sample was run in duplicate with a qPCR protocol at temperatures as follows. For all samples, initial denaturation was 95°C for 10 minutes, followed by 40 cycles of denaturation at 94°C for 10 seconds, a variable annealing temperature for 10 seconds, and an extension of 72°C for 10 seconds. Each cycle was followed by a plate read, and at the conclusion of 40 cycles, samples underwent a final extension for 10 minutes at 72°C. Melting curves were calculated from 55°C to 90°C every 0.4 seconds, with a hold for 1 second. Melting curve analysis was done from 55°C to 95°C at increments of 0.4°C. Only a single peak was seen at 80°C. Annealing temperatures were 55°C for 18S and T3C and 62°C for T1C.

The average of 2 replicates of each sample was used in the statistical analysis. The 18S was the comparison reference for the T1C and T3C expression in each sample. Differences in mRNA expression were determined based on relative difference (or fold change) in cycle number (ΔCt) necessary to achieve the same level of fluorescence as the 18S reference control. The T1C:T3C mRNA ratio was determined by dividing the average fold change value for T1C by the average fold change value for T3C.

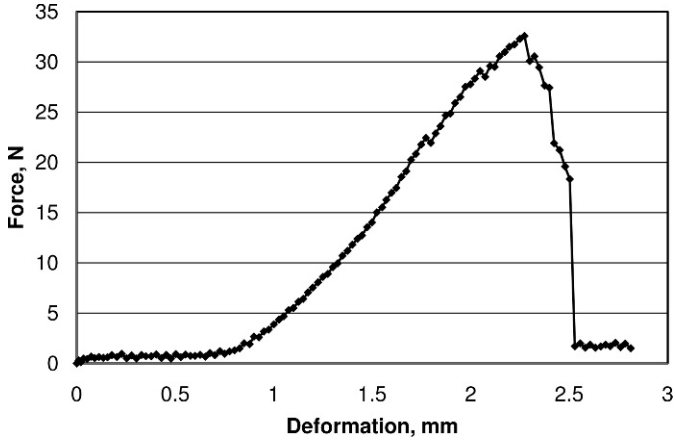

Figure 2. Example of force-deformation curve generated by materials testing unit used to determine anterior cruciate ligament stiffness and failure load.

Materials Testing

The potted specimen was affixed to our materials testing machine (model 858; MTS Systems Corp, Eden Prairie, MN) and aligned with the tibia and femur at a 90° angle, so that the long axis of the ligament passed through the axis of the actuator and the load cell (100 lb [45.36 kg]; Sensotec, Columbus, OH). The ACL was unloaded ("zeroed"), and the specimen was stretched to failure at a grip-to-grip rate of 0.25 mm/s. Force versus deformation data were recorded at 10 Hz (Figure 2). Tangent stiffness (N/mm) and failure load (N) were calculated. Failure load was the peak load recorded before ACL failure. Each outcome measure was divided by the rat's body mass to yield normalized tangent stiffness (N/mm-g) and normalized failure load (N/g).

Statistical Analysis

Distributions of all variables were examined. Because of the inherent variability in mRNA expression among animals, many variables were not normally distributed, and thus did not meet the criteria for parametric analysis. For this reason and because sample sizes were small, we used nonparametric analysis (version 9.1; SAS Institute Inc, Cary, NC). Wilcoxon rank sum tests were conducted to compare distributions of the 2 sex groups. A P value of less than .05 was considered statistically significant. Median values by sex and P values for comparison are presented in the Table. Stiffness and peak load ACL values were normalized by dividing the values by each rat's body mass (g) before analysis.

RESULTS

Mean hormone levels (concentration ± SD) were E2 in females, 27.27 ± 11.16 pg/mL, and testosterone in males, 1.0 ± 0.92 ng/mL. The cycle number (Ct) values for all samples were below 40. Males had an approximately 7-fold greater median expression of T3C mRNA and 2-fold greater median expression of T1C mRNA (Figure 3A and B). Although the females had a 4-fold greater ratio of T1C to T3C mRNA, this difference was not significant ($P = .11$; Figure 3C). No differences were noted between unadjusted

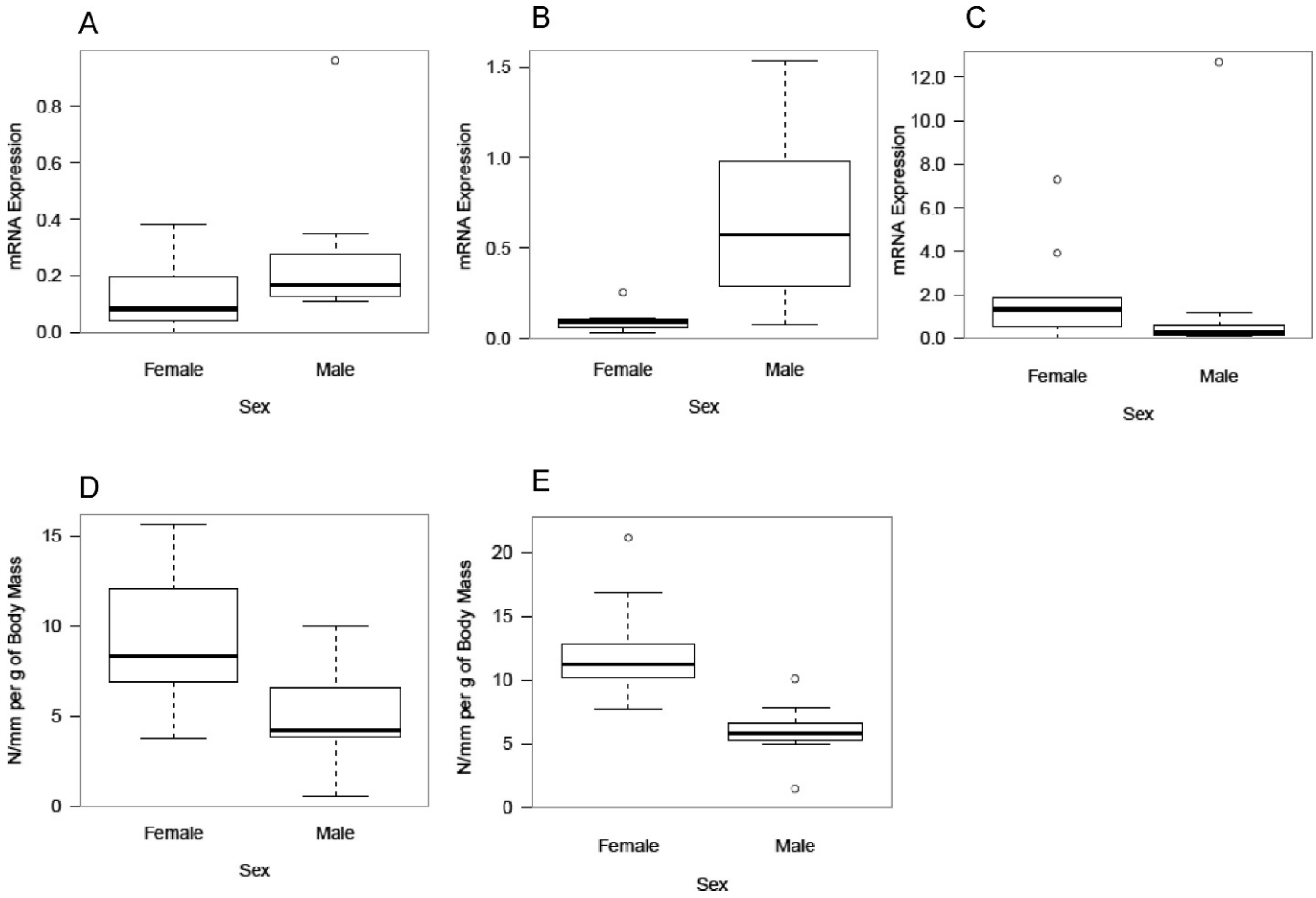

Figure 3. Box plots of A, type 1 ($P = .08$); B, type 3 ($P = .005$); C, type 1 to type 3 messenger RNA expression ratio ($P = .11$); D, anterior cruciate ligament stiffness ($P = .05$); and E, failure load ($P = .003$) normalized for animal body mass in male and female rats. The circles represent data points that are outliers.

male and female stiffness ($P = .56$) or failure load ($P = .55$). However, when normalized for animal weight, stiffness and failure load in the female ACLs were higher than for the males (Table; Figure 3D and E).

DISCUSSION

We examined the sex differences between 2 components of the ACL ultrastructure (T1C and T3C mRNA) and the stiffness and failure load of the ACL. Because mRNA is the precursor to procollagen and the collagen protein fibers that constitute a high percentage of the ultrastructure of the ligament,[9,14,18] we expected that sex-specific differences in T1C and T3C mRNA expression would translate to downstream differences in ACL stiffness and failure load. The rats that we studied demonstrated sexually dimorphic patterns in T3C mRNA expression that were accompanied by differences in normalized ACL stiffness and failure loads.

In our model (Figure 1B), T1C and T3C are 2 proteins that help determine how the ACL withstands tensile loads.[9,12] Although the collagens play a key role in tensile strength, elastin, proteoglycans, glycosaminoglycans, and glycoproteins within the ECM maintain the ligament's limited elasticity and ability to distribute stresses.[9,12,16] Moreover, matrix metalloproteinases and their inhibitors help to determine the composition and mechanical properties of the ACL by helping to break down and synthesize collagen[12,21–23] (Figure 1B). We focused on the beginning and ending points of the developmental cascade of collagen expression and ACL strength: T1C and T3C mRNA expression and ACL stiffness and failure load. We present our data with the understanding that several other entities within the ECM may somehow modulate the expression of mRNA and its downstream conversion to collagen protein and affect the ability of the ligament to withstand tensile loads (Figure 1C).

Table. Types 1 and 3 messenger RNA (mRNA) Expression, Expression Ratio, Normalized Stiffness, and Normalized Anterior Cruciate Ligament Failure Load (Median Value and Wilcoxon Rank Sum Comparison *P* Value)

Sex	Type 1 Collagen	Type 3 Collagen	Type 1:Type 3 Collagen Ratio	Normalized Anterior Cruciate Ligament Stiffness	Normalized Anterior Cruciate Ligament Load to Failure
Males (n = 9)	0.167	0.574	0.290	4.23	5.76
Females (n = 10)	0.084	0.081	1.350	8.35	11.18
P value	.08	.005	.11	.05	.003

Our female rats had a more than 4-fold greater ratio of T1C to T3C, although the difference was not significant, and greater normalized stiffness and load to failure. Greater normalized ACL stiffness and load to failure were present, even though male rats demonstrated higher amounts of both T1C and T3C mRNA than females. This finding suggests that the ratio of T1C to T3C mRNA may play an important role in ACL structure and compliance, as it does in other animal[13,30,31] and human[9,15,32] tissues.[9,11,13,17] However, even though the proportion of T1C to T3C has been shown to play a role in ACL and medial collateral ligament healing,[9,30] further studies of its effect on the mechanical properties of the ligament tissue in larger populations must be completed to confirm this relationship. Why our male and female rats had different T1C:T3C mRNA expression ratios is unclear. It is possible that these differences may have been due to sex differences in the collagen remodeling cascade or a potentially disparate influence on the role of testosterone and E2 on the relative mRNA expression of the 2 types of collagen.

Our findings suggest sex-specific differences in mRNA transcription that may modulate the downstream expression of collagen and the mechanical properties of the ACL. However, why these sex-specific differences in mRNA expression exist is still unknown. As presented in Figure 1, sex hormones are only 1 of many factors that have been suggested to play a role in the sex disparity in ACL injuries. Estradiol has been shown to modulate changes in collagen mRNA expression,[19,33] turnover, and synthesis[32,34] in human and animal models but had no significant effect in male rats and female mice.[35] Testosterone has not been studied in the same detail as E2, but it has also been shown to influence collagen remodeling and structure[36] to potentially protect the ligament.[37–40] Even if sex hormones are found to influence collagen mRNA expression, remodeling, and strength of the ACL, it is important to note that sex hormones are only 1 of many potential factors that may predispose women to ACL injuries and that additional intrinsic and extrinsic factors must also be considered[6] (Figure 1).

Human and rodent males are generally larger and heavier than females and have larger and stronger knee ligaments than their female counterparts.[11,41] Because differences in ligament size may play a role in the strength of knee ligaments, several methods, including computer modeling,[11] measurement from magnetic resonance imaging,[41] and micrometers,[42] have been used to account for various ligament sizes when calculating tensile and mechanical properties. Although these methods have routinely been used on larger human ligaments, the small size and structure of the rat ACL makes similar methods difficult to employ.[35,43] Tipton et al[44] showed that the rat's body mass was linearly related to the separation force applied to the medial collateral ligament and ACL. Like Tipton et al,[44] we divided the stiffness and failure load values by the animal's body mass to account for the various sizes of animals and ACLs in a method used by previous authors.[45–49]

It is important to note that although our female rats demonstrated greater stiffness and failure load, when normalized for animal weight, no differences were seen in these variables when animal mass and, thus, the size of the ligament was accounted for. In humans, women have smaller ACLs than men, and both this size and potential differences in the remodeling and ultrastructure of the ligament are believed to be possible factors in the disparate injury rates between men and women.[11,41,50,51]

Some limitations existed to the present study. First, although the rat estrous and human menstrual cycles are cyclic and have similar midcycle estrogen surges[52–55] and the rat has been validated and widely used to investigate the influence of sex hormones on the synthesis and proliferation of collagen in reproductive[25,56] and musculoskeletal tissues,[57,58] some species-specific differences between animal and human models[59] are likely present. Our protocol provided a practical mechanism to examine the effect of sex hormones on mRNA expression and ACL stiffness and failure load in an in vivo model; however, caution must be taken when applying our results to the human condition.

Second, our study was limited to ACL stiffness and failure load and the expression of T1C and T3C mRNA. Although T1C and T3C collagen mRNA are precursors to the procollagen and collagen protein that make up most of the ultrastructure of the ACL, their expressions are not necessarily a direct indication of subsequent protein content, collagen fibril size, or ligament strength. We measured collagen mRNA expression and compared it with ACL failure load and normalized stiffness in order to begin to define the potential mechanisms of sex-specific changes in ACL strength. Additional studies into the effects of sex hormones and sex on collagen protein formation, the ECM and matrix metalloproteinases, their inhibitors, growth factors, and the aromatases that have been shown to influence collagen remodeling in ligament and other collagen-rich tissues also warrant further investigation.[19,22,23,27,58,60–63]

Athletic trainers commonly work with athletes who are at risk for tearing or have torn their ACLs. As a result, it is important for athletic trainers to understand the potential risk factors and mechanisms of injury. We attempted to identify some of the intrinsic factors that may place women at higher risk for ACL injuries than men. A better understanding of these factors will help athletic trainers explain the potential causes of and risk factors for ACL injuries and assist them in designing appropriate prevention and rehabilitation programs for their players.

In summary, sex-specific differences in T1C and T3C mRNA expression were accompanied by differences in ACL stiffness and failure load. Although based on small groups of animals and just 2 points in a longer cascade, these findings suggest that sex differences in collagen mRNA expression may play a role in the downstream development of ACL stiffness and failure load.

ACKNOWLEDGMENTS

We acknowledge David Russ for his help with manuscript preparation, Jessica Witherspoon for her assistance with data collection, Reginald Dunn for his assistance with the figures, and Dr Rupesh Gupta, Dr Chuck Greenfeld, and the laboratory of Dr Jodi Flaws for their assistance with real-time PCR analysis. This study was supported in part by National Institute of Child Health and Human Development grant 5 K12 HD-043489, National Institute of Arthritis and Musculoskeletal and Skin Diseases grant 1R03AR54573-1A1, and United States Public Health Service Health Disparities Program MD00532-02.

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