Knee ligament mechanical properties are not influenced by estrogen or its receptors

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1Department of Physical Therapy, School of Health and Rehabilitation Sciences, Indiana University; Departments of 2Anatomy and Cell Biology and 3Orthopaedic Surgery, Indiana University School of Medicine; and 4Department of Biomedical Engineering, Purdue School of Engineering and Technology, Indiana University-Purdue University Indianapolis, Indianapolis, Indiana

Submitted 8 August 2005; accepted in final form 22 November 2005

Warden, Stuart J., Leanne K. Saxon, Alesha B. Castillo, and Charles H. Turner. Knee ligament mechanical properties are not influenced by estrogen or its receptors. Am J Physiol Endocrinol Metab 290: E1034–E1040, 2006. First published November 29, 2005; doi:10.1152/ajpendo.00367.2005.—Women are at greater risk of tearing their knee anterior cruciate ligament (ACL) than men participating in similar athletic activities. There is currently no conclusive explanation for this disparity; however, as ACL injuries in women have been linked with estrogen fluctuations during the menstrual cycle, one hypothesis is that estrogen has a direct detrimental effect on knee ligament mechanical properties. This study investigated the influence of estrogen and its receptors (ERα and ERβ) on knee ligament mechanical properties. This was achieved by testing the viscoelastic and tensile mechanical properties of knee medial collateral ligaments (MCL) and ACLs from: 1) male Sprague-Dawley rats treated with either estrogen (17α-ethynylestradiol; 0.03 mg/kg) or an ERα-specific agonist (propyl pyrazole triol; 2 mg/kg), and 2) female mice with a null mutation of the gene encoding for ERβ. Estrogen treatment had no significant effects on the viscoelastic or tensile mechanical properties of the rat MCL or ACL. Similarly, pharmacological stimulation of ERα using a selective agonist in rats and genetic modulation of ERβ by null mutation of its gene in mice did not influence MCL or ACL properties. These data indicate that estrogen does not have a major direct effect on ligament mechanical properties. Energies for the prevention of the disproportionately high rate of knee ligament injuries in women may be better spent focusing on more established and modifiable risk factors, such as abnormalities in neuromuscular control about the knee.

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reduced ACL ultimate force in rabbits. In contrast, Räsänen and Messner (42) found 5 mo of estrogen treatment in normal growing rabbits to have no effect on MCL ultimate force or elastic modulus; however, estrogen treatment did significantly reduce MCL cross-sectional area. Most recently, Strickland et al. (52) found estrogen to have no influence on knee MCL or ACL mechanical properties in sheep. Given these contrasting results, a need persists to establish the direct effect of estrogen on knee ligament mechanical properties.

The aim of this study was to further investigate the influence of estrogen on knee ligament mechanical properties and investigate the contribution of each ER. This was achieved using a multidirectional approach by 1) assessing ligaments from male rats treated with either estrogen (nonspecific ER agonist), an ERα-specific agonist, or a vehicle solution; and 2) assessing ligaments from female mice with and without a null mutation of the gene encoding for ERβ.

MATERIALS AND METHODS

Animals. Estrogen and ERα effects were investigated in 24 male Sprague-Dawley rats purchased at 6 wk of age from Harlan Sprague Dawley (Indianapolis, IN). Male rats were selected to minimize the effects of endogenous estrogen production. ERβ effects were investigated in female ERβ homozygous mutant (ERβ−/−) mice (BERKO) with a C57BL/6 background (N = 10; gift from P. Chambon, MD, Institut de Génétique et de Biologie Moléculaire et Cellulaire, France). These animals were generated by inbreeding of heterozygotes for ERβ disruption (ERβ+/-), as described by Dupont et al. (11). Control mice consisted of female wild-type (ERβ+/-) littermates (WT; N = 10). Progeny genotype was determined from tail-derived DNA by polymerase chain reaction amplification using established primers (11). Animals in both studies were maintained under standardized environmental conditions with ad libitum access to standard mouse or rat chow and water. All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Indiana University.

Interventions. To investigate estrogen and ERα effects, rats were acclimatized until 8 wk of age and randomly divided into three intervention groups (N = 8/group): 1) estrogen treated (EE2); 2) propyl pyrazole triol treated (PPT); and 3) vehicle-treated controls (VEH). The EE2 group was treated via oral gavage with 17α-ethynylestradiol (0.03 mg/kg; Sigma-Aldrich, St. Louis, MO), which is a synthetic estrogen that is used in oral contraceptive formulations. The dose was chosen as it has been shown to correct the body weight and skeletal effects of estrogen deficiency in rats (12), whereas higher doses have been found to be detrimental to development in young male rats (53). The PPT group was treated via subcutaneous injection with propyl pyrazole triol [4-propyl-1,3,5-Tris(4-hydroxyphenyl)-pyrazole, 2 mg/kg in 50% DMSO-50% Dulbecco’s phosphate-buffered saline (PBS); Sigma-Aldrich]. PPT is an ERα-specific agonist that has a binding affinity that is 410-fold greater for ERα than ERβ (51), and the chosen dose of PPT corrects the bone loss caused by estrogen deficiency in rats (19). The VEH group received via an oral gavage vehicle solution (0.25 ml of 20% hydroxypropyl-β-cyclodextrin; Sigma-Aldrich). All rats were treated 7 days/wk for 5 consecutive weeks and killed at 13 wk of age. To investigate ERβ effects, mice were aged until 20 wk before being killed. There was no specific intervention in these animals.

Specimen preparation. Immediately after death, the right hindlimb was removed from each animal at the hip joint, placed intact in a plastic bag, and stored at −80°C. Postmortem storage by freezing does not change ligament properties during destructive mechanical testing (57). On the day of mechanical testing, the hindlimbs were allowed to thaw to room temperature in PBS. Dissection of extraneous tissue exposed the knee joint, and, with further careful dissection, femur-ACL-tibia and femur-MCL-tibia complexes were created. Because rats were concurrently being used to investigate estrogen effects on the skeleton, only one knee joint was available from these animals for ligament testing. To permit testing of both the MCL and ACL in the same knee in these animals, a Dremel drill (Robert Bosch Tool, Mount Prospect, IL) with attached diamond-embedded wafer blade (Super Flex Diamond Disc; Miltex, York, PA) was used to cut the femur and tibia sagittally. This was not performed in the mouse knees, as there were sufficient numbers to test the MCL and ACL in different animals. At all times, the ligament and insertion sites were kept hydrated with PBS.

MCL thickness and width were measured optically, and the MCL area was estimated using an elliptical geometry (40). Geometry of the ACL was not determined due to its dual bundles and intimate position within the knee joint. The femoral and tibial portions of the femur-ACL-tibia and femur-MCL-tibia complexes were placed in a custom-built testing jig, and the knee was positioned at 90° and 0° flexion for testing. To permit testing of both the MCL and ACL in the same knee in these animals, a Dremel drill (Robert Bosch Tool, Mount Prospect, IL) with attached diamond-embedded wafer blade (Super Flex Diamond Disc; Miltex, York, PA) was used to cut the femur and tibia sagittally. This was not performed in the mouse knees, as there were sufficient numbers to test the MCL and ACL in different animals. At all times, the ligament and insertion sites were kept hydrated with PBS.

Fig. 1. Representative images of the setup for rat medial collateral ligament (MCL; A) and anterior cruciate ligament (ACL; B) testing. The knee joint was positioned in 0° and 90° of flexion for MCL and ACL testing, respectively. The femur and tibia were held in place by Wood’s low-melting-point metal. Bone slippage and/or growth plate avulsion were avoided by designing fixtures that supported (“cupped”) the distal femur and proximal tibia. To permit more space within the knee joint during MCL testing, the proximal tibial growth plate was carefully removed before mounting. At the completion of testing, the MCL (C) and ACL (D) were visibly ruptured.
ACL and MCL testing, respectively (Fig. 1, A and B). The femoral
and tibial portions were embedded in Wood’s low-melting-point
metal (Bismuth alloy LMA-117; Small Parts, Miami Lakes, FL) for
fixation, and the jig was coupled to an electromagnetic material-
testing device (TestBench 200 N ELF LM-1; EnduraTEC Systems
Group, Bose, Minnetonka, MN). A 50-N load cell was used to
measure forces during testing of rat ligaments, which resulted in a
force and displacement resolution of 0.01 N and 0.001 mm, respec-
tively. A 5-lb (22 N) load cell was used to measure forces during
testing of mouse ligaments, which resulted in a force and displace-
ment resolution of 0.001 N and 0.001 mm, respectively. A preload of
0.05 N was applied to each ligament, and the ligaments were precon-
ditioned in displacement control by cyclically loading at 1 Hz for 10
cycles to ~1% strain to rectify any alteration in low-load properties
of the ligament due to deep freezing (57). The ligaments were subse-
quently unloaded and allowed to recover for 10 min before testing of
viscoelastic properties.

Ligament viscoelastic properties. Viscoelastic properties were
determined via stress relaxation tests. After reestablishment of preload
(0.05 N), ligaments were stretched to ~5% strain and held for 100 s.
To achieve this strain, rat MCLs and ACLs were lengthened 0.32 and
0.12 mm, respectively, whereas mouse MCLs and ACLs were length-
ened 0.05 and 0.03 mm, respectively. A strain magnitude of 5% is
below the threshold for structural damage in ligaments (39), and
testing for 100 s enables accurate estimation of the viscoelastic
behavior of ligaments (34). Loading was ramped at a strain rate of
~10%/s, and data were collected at 20 Hz during testing. Force-time
data were plotted on both linear-linear and log-log scales. The percent
force reduction from the peak force was calculated from linear-linear
plots [(peak force – final force)/peak force × 100] (Fig. 2A). Log-log
scales were curve fitted with a power law, \( n \) (Fig. 2B). The dimen-
sionless parameter \( n \), which is the slope on a log-log plot, was
considered as the “rate” of relaxation (34, 38).

Ligament tensile mechanical properties. After viscoelastic testing,
ligaments were unloaded and allowed to rest for 1,000 s (16–17 min)
while being kept hydrated with PBS. Experiments subjecting liga-
ments to displacements <5% tissue strain have shown that a recovery
time equal to 10 times the test time allows the initial preload
gauge length (38). Following tissue recovery, preload (0.05
N) was reestablished, and the ligaments were pulled to tensile failure
in displacement control at a strain rate of ~10%/s (Fig. 1, C and D).
To achieve this strain rate, rat MCLs and ACLs were lengthened at 0.8
and 0.5 mm/s, respectively, whereas mouse MCLs and ACLs were
lengthened at 0.5 and 0.3 mm/s, respectively. During testing, force
and displacement data were collected at 100 Hz. From the force-
displacement curves, the mechanical properties of ultimate force and
stiffness were obtained (Fig. 2C). Ultimate force data from the MCLs
were divided by MCL area to obtain ultimate stress.

Statistical analyses. Statistical comparisons were performed with
the Statistical Package for Social Sciences (SPSS 6.1.1; Norusis/
SPSS, Chicago, IL), with a level of significance set at 0.05. Differ-
ces between VEH, PPT, and EE2 rat groups for body weight,
viscoelastic properties, and ultimate stress were assessed by using
one-way ANOVAs followed by Fisher’s protected least significant
difference test for pairwise comparisons. Analyses of covariance were
used to assess for rat group differences in ligament ultimate force
and stiffness with body weight as the covariate. Differences between WT
and BERKO mice were assessed using unpaired t-tests. All data are
presented as means ± SD.

RESULTS

Animal characterization. Body weight did not differ be-
tween rat groups at baseline (\( P > 0.05 \)). However, after 5 wk
of intervention, the groups differed significantly (\( P = 0.02 \))
with the PPT group (304.5 ± 18.2 g) being lighter than both
the EE2 (333.4 ± 33.4 g; \( P = 0.04 \)) and VEH (342.3 ± 23.0 g;

Fig. 2. Representative force-time (A and B) and force-displacement (C) curves
from rat medial collateral ligament viscoelastic and tensile mechanical tests,
respectively. Force-time data were plotted on both linear-linear (A) and log-log
(B) scales to determine ligament viscoelastic properties. Derived properties
included the peak force, percent force reduction, and “rate” of relaxation
during the test. Force-displacement data were plotted on linear-linear scales to
determine ligament tensile properties. Derived properties included the ultimate
force (peak of the curve on the y-axis) and stiffness (slope of the linear portion
of the curve).
stress relaxation tests in rat MCLs and ACLs

Table 1. Peak force and percent force reduction during stress relaxation tests in rat MCLs and ACLs

<table>
<thead>
<tr>
<th>Group</th>
<th>MCL Force, N (S.D.)</th>
<th>Force reduction (%)</th>
<th>ACL Force, N (S.D.)</th>
<th>Force reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEH</td>
<td>2.37±0.65</td>
<td>66.4±9.0</td>
<td>0.76±0.33</td>
<td>39.5±5.1</td>
</tr>
<tr>
<td>EE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>2.93±0.50</td>
<td>61.4±10.8</td>
<td>0.83±0.35</td>
<td>37.4±9.0</td>
</tr>
<tr>
<td>PPT</td>
<td>2.94±0.72</td>
<td>61.1±7.0</td>
<td>0.99±0.66</td>
<td>42.0±4.4</td>
</tr>
<tr>
<td>P values</td>
<td>0.15</td>
<td>0.86</td>
<td>0.65</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Values are means ± SD. MCL, medial collateral ligament; ACL, anterior cruciate ligament; VEH, vehicle-treated controls; EE<sub>2</sub>, estrogen treated; PPT, propyl pyrazole triol treated.

Table 2. Peak force and percent force reduction during stress relaxation tests in mouse MCLs and ACLs

<table>
<thead>
<tr>
<th>Group</th>
<th>MCL Force, N (S.D.)</th>
<th>Force reduction (%)</th>
<th>ACL Force, N (S.D.)</th>
<th>Force reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.27±0.08</td>
<td>32.9±9.6</td>
<td>0.31±0.13</td>
<td>32.8±15.5</td>
</tr>
<tr>
<td>BERKO</td>
<td>0.26±0.06</td>
<td>39.6±8.4</td>
<td>0.32±0.10</td>
<td>23.6±8.5</td>
</tr>
<tr>
<td>P values</td>
<td>0.86</td>
<td>0.18</td>
<td>0.78</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Values are means ± SD.

$P = 0.01$) groups. The reduction in weight gain in the PPG group is consistent with feminization of male rats, similar to that produced by high-dose estrogen (53). Genotype in the mice did not influence body weight ($P = 0.92$), with WT and BERKO mice weighing $19.2 \pm 2.6$ and $19.1 \pm 1.7$ g, respectively.

Viscoelastic properties. There were no significant group differences in the peak force obtained or percent force reduction that occurred during the stress relaxation tests in either rat (Table 1) or mouse (Table 2) ligaments. The rate of stress relaxation in rat MCLs and ACLs was not influenced by intervention group ($P = 0.27$ and 0.12, respectively; Fig. 3A). Similarly, mouse genotype did not influence the rate of stress relaxation in MCLs and ACLs ($P = 0.10$ and 0.13, respectively; Fig. 3B).

Tensile mechanical properties. Estrogen and ERα-specific agonist treatment did not influence knee ligament strength in rats, with no differences being found between the VEH, EE<sub>2</sub>, and PPT groups for MCL ($P = 0.62$) or ACL ($P = 0.30$) ultimate force (Fig. 4A). Similarly, no differences were found between groups for knee ligament stiffness (all $P > 0.29 – 0.41$; Fig. 4B) or MCL ultimate stress (all $P > 0.44$; Fig. 4C).

Null mutation of the gene encoding for ERβ by null mutation of its gene in mice did not influence knee ligament stiffness in mice, with no differences being found between WT and BERKO groups for MCL ($P = 0.49$) or ACL ($P = 0.29$) ultimate force (Fig. 5A). Similarly, no genotype differences were for knee ligament stiffness (all $P > 0.29 – 0.52$; Fig. 5B) or MCL ultimate stress (all $P = 0.39$; Fig. 5C).

DISCUSSION

A possible cause for the disproportionately high rate of knee ligament injuries in women is a direct detrimental effect of estrogen on knee ligament mechanical properties and strength. This study used controlled animal models to investigate the influence of estrogen and its receptors on knee ligament mechanical properties. Estrogen treatment had no significant effects on the viscoelastic or tensile mechanical properties of the rat MCL or ACL. Similarly, pharmacological stimulation of ERα using a selective agonist in rats and genetic modulation of ERβ by null mutation of its gene in mice did not influence MCL or ACL viscoelastic or tensile mechanical properties. These data indicate that estrogen and its receptors do not directly influence the mechanical properties of these knee ligaments.

The current findings indicate that direct ligament effects of estrogen may not be responsible for the disproportionately high rate of knee ligament injuries in female athletes. However, statistical power and the potential of committing a Type II statistical error needs to be explored in any study that observes a nonsignificant finding. Power calculations for ultimate force indicated that there was $80\%$ probability of detecting a mouse genotype effect of 10 and 21% for the MCL and ACL, respectively. Similarly, there was 80% probability of detecting a difference of 15–24% in ligament ultimate force between VEH, PPT, and EE<sub>2</sub> rats. Although post hoc power calculations need to be interpreted cautiously (24), these minimal detectable differences are considered to be clinically relevant and indicate that we addressed our research question with sufficient statistical power.

It is possible that estrogen and its receptors had effects smaller than could be statistically detected. Previous studies have shown estrogen to negatively impact the collagen content of a variety of connective tissues (16, 47). As collagen fulfills the major load-bearing role in ligaments, these studies suggest that estrogen may also influence the mechanical properties of ligaments.

Fig. 3. Effect of estrogen and the estrogen receptors on the rate of stress relaxation in rat (A) and mouse (B) ligaments. VEH, vehicle-treated control rats; EE<sub>2</sub>, estrogen-treated rats; PPT, propyl pyrazole triol-treated rats; WT, wild-type littermate mice; BERKO, estrogen receptor-β homozygous mutant mice. Bars represent means ± SD.
ligaments. However, this has been confirmed by only one study, which showed that estrogen treatment following ovariectomy reduced ACL ultimate force in rabbits by 11.3% (48). This small effect was generated in animals exposed to high levels of estrogen, with the serum estrogen level being two times higher than normal and equivalent to that experienced by rabbits during pregnancy. Such an effect was not observed in the present study using more physiological levels of estrogen, nor has it been confirmed by previous studies (42, 52). Also, the removal of estrogen does not result in the opposite effect of an increase in ligament mechanical strength and subsequent decrease in injury risk (50, 54). These data indicate that any direct detrimental effect of physiological levels of estrogen on knee ligament mechanical properties is, at most, small.

The present study was unable to find any effect of estrogen or its receptors on knee ligament mechanical properties with continuous exposure to intervention. Rats were treated daily for 5 wk with either estrogen or an ERα-selective agonist, and mice had life-long disruption of ERβ. These exposures are somewhat unphysiological, as they deviate from the clinical scenario where ligaments and their ERs are exposed to phasic levels of estrogen during the menstrual cycle. It is possible that estrogen may produce a different effect when introduced intermittently as opposed to continuously (41) and that intermittent doses may significantly alter ligament mechanical properties within a matter of days, as collagen turnover can occur rapidly (3–5%/day in adult rat skin) (27). However, normal physiological fluctuations in estrogen during the estrous cycle have not been found to alter the failure properties of the rat ACL (43), and clinically assessed knee ligament mechanical properties (laxity and stiffness) do not differ across the different phases of the menstrual cycle (5, 6, 8, 25). As such, we believe that intermittent intervention would not have altered the findings in the present study and that the absence of an effect, despite constant exposure to intervention, actually strengthens the conclusions. The latter theory is based on the

![Fig. 4. Effect of estrogen and an estrogen receptor-α-specific agonist on ultimate force (A), stiffness (B), and ultimate stress (C) in rat ligaments. Bars represent means ± SD.](image)

![Fig. 5. Effect of null mutation of the gene encoding estrogen receptor-β on ultimate force (A), stiffness (B), and ultimate stress (C) in mouse ligaments. Bars represent means ± SD.](image)
finding that ligament fibroblasts exhibit a dose-dependent response to estrogen, with increasing estrogen concentrations resulting in progressive declines in fibroblast proliferation (31, 59, 60). This indicates that continuous intervention may magnify estrogen effects, resulting in a larger and more detectable effect than when estrogen levels vary phasically with intermittent intervention.

A possible further limitation of our study is the multidirectional approach used to investigate estrogen and ER effects. The effect of combined ERα and ERβ stimulation, isolated ERα stimulation, and no ER stimulation was investigated by treating rats with EE2, PPT, and VEH, respectively, whereas isolated ERβ effects were investigated by comparing mice with and without a null mutation in the gene encoding ERβ. While we did not investigate isolated ERβ effects in rats, as no selective agonist of this receptor was available at the time of investigation. It is possible to use diarylpropionitrile for the stimulation, and no ER stimulation was investigated by comparing mice with and without a null mutation in the gene encoding ERβ. We did not investigate isolated ERβ effects in rats, as no selective agonist of this receptor was available at the time of investigation. Consequently, it was elected to investigate isolated ERβ effects in genetically modified mice. This multidirectional approach to address the research question may be viewed as a limitation; however, it is actually a strength. Irrespective of the model used, no effect of estrogen or the ERs was found. Further investigation of ERβ effects in rats and ERα effects in genetically modified mice may more completely answer the research question; however, we do not believe that data from these studies would alter our conclusions.

As direct ligament effects of estrogen may not explain the disproportionately high rate of knee ligament injuries in female athletes, other mechanisms need to be considered. The risk of knee ligament injury is multifactorial and results from a combination of extrinsic and intrinsic factors (4). Women have been shown to exhibit a number of intrinsic risk factors that have been linked with ACL injuries. These have been adequately reviewed elsewhere (10) and include anatomic features, such as a narrower intercondylar notch (46), smaller ACL size (9), and altered lower extremity alignment (32), as well as neuromotor features. The latter are of particular interest as they are potentially modifiable and may be influenced by the differing levels of estrogen during each phase of the menstrual cycle. Women have been found to utilize different muscular activation patterns compared with men during activity (23). This results in significant sex differences in mechanics, with women displaying increased hip internal rotation and a larger valgus at the knee (13, 21, 35, 58). This “dynamic valgus” knee motion has prospectively been shown to be a significant risk factor for ACL injury in female athletes (22), suggesting that clinical approaches targeting neuromuscular control issues may be useful in preventing ACL injuries in women. Supporting this, training programs for lower extremity neuromuscular control have been found to significantly decrease knee injury rates (20, 33, 36).

In summary, this study found in two separate animal models that estrogen and its receptors did not significantly influence the mechanical properties of the knee ligaments. While an effect smaller than could be statistically detected may exist, the clinical relevance of such an effect is questionable. Combined with previous studies, these data indicate that estrogen does not have a major direct effect on ligament mechanical properties. Energies for the prevention of the disproportionately high rate of knee ligament injuries in women may be better spent focusing on more established and modifiable risk factors, such as abnormalities in neuromuscular control about the knee.

REFERENCES