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Estrogen inhibits lysyl oxidase and decreases mechanical function in engineered ligaments

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Lee CA, Lee-Barthel A, Marquino L, Sandoval N, Marcotte GR, Baar K. Estrogen inhibits lysyl oxidase and decreases mechanical function in engineered ligaments. *J Appl Physiol* 118: 1250–1257, 2015. First published April 2, 2015; doi:10.1152/japplphysiol.00823.2014.—Women are more likely to suffer an anterior cruciate ligament (ACL) rupture than men, and the incidence of ACL rupture in women rises with increasing estrogen levels. We used an engineered ligament model to determine how an acute rise in estrogen decreases the mechanical properties of ligaments. Using fibroblasts isolated from human ACLs from male or female donors, we engineered ligaments and determined that ligaments made from female ACL cells had more collagen and were equal in strength to those made from male ACL cells. We then treated engineered ligaments for 14 days with low (5 pg/ml), medium (50 pg/ml), or high (500 pg/ml) estrogen, corresponding to the range of in vivo serum estrogen concentrations and found that collagen within the grafts increased without a commensurate increase in mechanical strength. Mimicking the menstrual cycle, with 12 days of low estrogen followed by 2 days of physiologically high estrogen, resulted in a decrease in engineered ligament mechanical function with no change in the amount of collagen in the graft. The decrease in mechanical stiffness corresponded with a 61.7 and 76.9% decrease in the activity of collagen cross-linker lysyl oxidase with 24 and 48 h of high estrogen, respectively. Similarly, grafts treated with the lysyl oxidase inhibitor β-aminoproprionitrile (BAPN) for 24 h showed a significant decrease in ligament mechanical strength [control (CON) = 1.58 ± 0.06 N; BAPN = 1.06 ± 0.13 N] and stiffness (CON = 7.7 ± 0.46 MPa; BAPN = 6.1 ± 0.71 MPa) without changing overall collagen levels (CON = 396 ± 11.5 μg; BAPN = 382 ± 11.6 μg). Together, these data suggest that the rise in estrogen during the follicular phase decreases lysyl oxidase activity in our engineered ligament model and if this occurs in vivo may decrease the stiffness of ligaments and contribute to the elevated rate of ACL rupture in women.

ACL; exercise; tendon; ultimate tensile strength

FEMALE ATHLETES PARTICIPATING in cutting and jumping sports have a four to six times greater chance of tearing their anterior cruciate ligament (ACL) than their male counterparts (1). Often, this results from a noncontact injury involving rapid changes of direction or landing following a jump (4). The pathophysiology of this injury is currently unknown but is likely multifactorial involving anatomic, neuromuscular, and hormonal influences.

Because of the obvious difference in circulating hormones in men vs. women, researchers have theorized that elevated estrogen levels in females may be a potential cause for the increased incidence of ACL injury (10). In fact, a meta-analysis of the existing studies on estrogen and knee laxity showed a significant relationship (41). From the meta-analysis, it was clear that the greatest knee laxity occurred between *days 10* and *14* of the cycle (highest estrogen), with *days 15–28* (moderate estrogen) showing lower laxity and *days 1–9* showing the least laxity (and lowest estrogen levels). Shultz and colleagues (33, 34) have repeatedly shown that not only does the laxity of the knee increase following the rise in estrogen (34) but this decrease in knee stiffness is repeated monthly in a very predictable manner (33), likely underlies the greater laxity in the female knee, and leads to changes in landing mechanics that predispose women to ACL injury (35). In support of this finding, a second meta-analysis showed that the increased laxity due to estrogen resulted in an increase in the rate of ACL injury in women (14). Wojtys et al. (38) have shown that as estrogen levels rise there is a concomitant increase in the number of ACL injuries in athletic women. This is a somewhat surprising finding since it suggests that the mechanics of ligaments like the ACL can be altered over a very short time scale even though collagen turnover occurs very slowly (13).

Estrogen receptors have been identified in the human ACL in both men and women (21), suggesting that estrogen may directly modulate fibroblast function and overall ligament mechanical properties. Indeed, ACL-derived fibroblasts are responsive to estrogen in vitro (19, 22, 39, 40), with both increased (19) and decreased (36, 39, 40) collagen production being reported. However, ligament stiffness is determined not only by collagen content but also by the density of cross-links between the collagen molecules (7, 25). The collagen content of a tendon or ligament changes very slowly, with the core of the tendon/ligament having the same collagen from the age of 17 until death (13). It would therefore be surprising if the collagen content of a ligament could change throughout the menstrual cycle.

In contrast to the stable nature of collagen, the chemical cross-links within the matrix are added and removed from the collagen in a more dynamic fashion (2, 24). The primary enzyme that produces collagen cross-links is lysyl oxidase (LOX), an amine oxidase produced by fibroblasts (16). LOX is a copper (Cu)-dependent enzyme that catalyzes the cross-linking of lysine residues in neighboring collagen molecules thereby increasing collagen stiffness and ligament strength (16). Blocking LOX activity is known to decrease the stiffness of developing tendons without affecting the production of collagen (24), and this has been used in horses to improve adult

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tendon recovery (8). Furthermore, LOX mRNA is increased in response to exercise (12), suggesting that LOX may be dynamically controlled by physiological interventions that alter sinew function.

At a mechanistic level, the molecular processes that regulate tendon and ligament function are poorly understood. This is largely due to the difficulty in obtaining human samples as well as the density of the collagen matrix and the paucity of cells in the mature tissue. To overcome these difficulties, we have recently developed a three-dimensional (3D) engineered ligament model that recapitulates many aspects of normal tendon development (3, 17, 27–29). Like developing tendons/ligaments (25), these engineered tissues have a much higher ratio of cells to matrix proteins, and the density of the cell-secreted matrix increases rapidly over time (5). However, unlike native ligaments the engineered ligaments express more developmental proteins, such as type III, XII, and XIV collagens (3), have a significantly lower collagen content, fewer mature cross-links, and as a result are much weaker (29). In spite of these differences, the engineered ligaments respond the same way to nutrients (29), growth factors (9), and exercise (23, 30) as the native tissue; can also be mechanically tested to determine their function; and therefore might be a good tool for understanding the molecular processes that regulate tendon and ligament development and function.

The purpose of the current study was to first determine whether there were differences between ACL cells isolated from male and female donors. Second, we determined the effect of either continuous or phasic estrogen treatment, designed to mimic the estrous cycle, on engineered ligament function, mRNA expression, and LOX activity. We hypothesized that an acute rise in estrogen would decrease the mechanical properties of ligaments by inhibiting collagen synthesis.

METHODS

Materials. All materials were purchased from Sigma unless indicated otherwise.

Cell isolation. The University of California Davis Institutional Review Board approved all procedures and protocols. Human anterior cruciate ligaments were collected from discarded tissue during standard ACL reconstruction surgeries. Before surgery, all subjects (7 subjects: 3 male, 4 female) signed informed consent forms. The female donors had a mean age of 20 ± 1.9 yr, whereas the male donors averaged 23.3 ± 0.58 yr (not statistically different). The time between injury and the reconstruction was similar (1–4 mo) for all but two donors who had injured their ACL more than 4 mo prior. However, there was no relationship between the time since rupture and the strength of the resulting ligaments (not shown).

The ligament remnants were digested as previously described (29). Briefly, ligaments were washed five times in sterile PBS and then placed in a 5% antibiotic/antimycotic (ABAM) solution for 2 h. The tissues were digested in 0.1% collagenase type II dissolved in DMEM containing 20% FBS and 1% penicillin overnight at 37°C. The resulting freed fibroblasts were collected by centrifugation (1,500 *g* for 5 min), washed three times with growth media (DMEM containing 10% FBS and 1% penicillin), and then plated and cultured as normal. All experiments were performed on cells before passage 5.

Ligament formation. Sinew constructs were engineered as previously described (17, 29). Briefly, 2.5×10^5 cells were suspended in growth media containing 5.8 U thrombin, 20 μg aprotinin, and 2 μg aminohexanoic acid. Seven-hundred fourteen microliters of this cell/thrombin solution were dispersed onto each plate containing two cylindrical brushite anchors pinned 12 mm apart. The fibrin gel was formed by adding 286 μl of a 20 mg/ml fibrinogen solution. The plates were then incubated at 37°C with 5% CO_2 for 15 min to allow gelation. Following gel formation, 2 ml of growth media supplemented with 5 ng/ml transforming growth factor-β1 (Peprotech, Rocky Hill, NJ), 200 μM ascorbic acid, and 50 μM proline were added to each plate. The constructs were cultured for 14 days with media changes every other day. The cells contracted the fibrin resulting in the formation a cylindrical tissue between the two brushite anchors.

Mechanical testing. Mechanical testing was performed as described previously (9). Briefly, the width and length of each construct were determined using a digital caliper, and the ligaments were then placed in a custom-built tensile tester. The sample was kept submerged in phosphate-buffered saline and loaded to failure without preconditioning. The tensile test used a constant elongation rate of 0.4 mm/s. From the resulting deformation and load data, the maximal tensile load (MTL) was calculated as the maximal load (Newtons) achieved during the test. The load and deformation data were also normalized to the cross-sectional area and initial length of the graft to generate stress and strain data. From the slope of the linear region of the stress-strain curve, we obtained the Young's modulus, and the maximal stress value was used for ultimate tensile strength (UTS).

Collagen. Collagen content of the sinews was determined using a hydroxyproline assay (37). After mechanical testing to failure, the constructs were removed from their brushite anchors and dried in an oven for 30 min at 110°C. Each sample was then weighed and hydrolyzed in 200 μl of 6 N HCl at 130°C for 1.5 h and then dried for 1.5 h. The resulting dehydrated pellet was resuspended in 200 μl of hydroxyproline buffer and then further diluted 1:8 in that buffer. One-hundred fifty microliters of chloramine T solution were added to each sample, mixed, and left at room temperature for 20 min. One-hundred and fifty microliters of aldehyde-perchloric acid were then added to each tube before being vortexed and incubated at 60°C for 15 min. The tubes were then cooled for 10 min and read at 550 nm on an Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, VT). Hydroxyproline was converted to collagen mass assuming that collagen contains 13.8% hydroxyproline (26). The collagen fraction was determined by dividing the collagen content by the dry mass of the tissue.

Estrogen treatments. For the continuous treatments, the growth media were supplemented with 5 (low), 50 (medium), and 500 pg/ml (high) of 17β-estradiol at each feeding. These levels of estrogen were selected to mimic the levels seen during the follicular phase (5 pg/ml) and following the luteinizing hormone surge (500 pg/ml) with the central concentration selected as a middle point to give a consistent 10-fold scale (38). To mimic the estrogen cycle, 5 pg/ml 17β-estradiol were given to the control group for the entire 14-day period, whereas the 24-h group received 13 days of 5 pg/ml and 24 h of 500 pg/ml 17β-estradiol and the 48-h group received 12 days of 5 pg/ml and 48 h of 500 pg/ml 17β-estradiol. This treatment protocol was used for subsequent experiments assayed for LOX activity and gene expression.

LOX activity. LOX activity was determined using the LOX activity kit (Abcam, Eugene, OR) in constructs from control and 24- and 48-h high estrogen groups (n = 8). Briefly, constructs were placed in a 2-ml Eppendorf tube with 200 μl of RIPA buffer and a metal bead. The samples were homogenized by shaking at 50 Hz for 3 min followed by a 2-min rest and another 2 min at 50 Hz. The beads were removed the samples were centrifuged (10,000 *g* for 1 min) and the supernatant was collected and analyzed following the manufacturer's instruction. Fluorescence was read (excitation = 540 and emission = 590) on a SpectraMax M2 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA) using recombinant LOX (R&D Systems, Minneapolis, MN) between 25 and 200 ng as a control.

Gene expression. Following treatment with estrogen, the constructs were washed twice in ice-cold PBS, blotted dry, placed in RNAlater

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Table 1. *Experimental samples and replicates for each experiment*

Figure	Donors Used	Biological Replicates per Donor/Group	Independent Experiments
1	7	8	2
2	2	8	2
3	2	8	2
4	2	8	2
5	2	8	2
6	2	8	4

The number of anterior cruciate ligament (ACL) donors used for each experiment together with the biological replicates (number of ligaments made from that donor for the given group and experiment), and the number of times that the experiment was performed.

(Life Technologies, Grand Island, NY), and stored at 4°C until analyzed. Total cellular RNA was extracted from the engineered ligaments using TRIzol reagent (Invitrogen, Carlsbad, CA), per the manufacturer's instructions. Briefly, tissues were placed in a 2-ml Eppendorf tube with 250 μl of TRIzol and a metal bead. The samples were homogenized by shaking at 50 Hz for 3 min followed by a 2-min rest and another 2 min at 50 Hz. The beads were removed, the samples were centrifuged, and RNA was isolated by chloroform:isopropanol precipitation. Total RNA was quantified and 1 μg of RNA from each sample was reversed transcribed for analysis of gene expression.

Quantitative RT-PCR was performed with primers to LOX (forward: CGGCGGAGGAAAACTGTCT; reverse: CTTGGTCGGCTGGGTAAGAA), collagen 1A1 (forward: CCCACCAATCACCTGCGTACAGA; reverse: TTCTTGGTCGGTGGGTGACTCTGA), insulin-like growth factor (IGF)-1 (forward: ATGTATTGCGCACCCCTCAA; reverse: GCACTCCCTCTACTTGCGTT), and tenascin C (forward: ATTCTGGGAAGCTGCTGGAC; reverse: GCTTGGTTTGATGCCCTTGG). Gene expression analysis was based on the Pfaffl method (31), using GAPDH (forward: ACAGCCTCAAGATCATCAGC; reverse: ATGAGTCCTTCCACCGATACC) as a reference. The absolute C_T values of GAPDH were not different in any of the experimental groups.

β-Aminoproprionitrile treatment. The LOX inhibitor β-aminopropionitrile (BAPN) was added at a final concentration of 1 mM to the normal growth media for the last 24 h of the 14 days in culture, and the mechanical properties and collagen content of the resulting ligaments were determined as described above at *day 14*, compared with untreated controls. A second set of constructs treated with the same protocol was thoroughly rinsed several times with growth media at *day 14*, cultured for an additional 6 days, and then assessed for mechanical properties and collagen content.

Statistical analysis. Data are reported as means ± SE. Differences in mean values were compared between groups by ANOVA and Tukey's honestly significant difference test was used for post hoc comparisons (Brightstat.com). Statistical analyses and the type I error was maintained at $\alpha < 0.05$ for all comparisons. Table 1 lists the number of ACL donors, biological replicates, and independent trails performed for each experiment.

RESULTS

Comparison of ligaments engineered using cells originating from female or male donors. Cross-sectional area and collagen content for the engineered sinews did not differ between male- vs. female-derived ligament cells (Fig. 1). However, collagen fraction by dry mass for the male sinews was significantly less compared with the female constructs (11.1 ± 0.19 vs. 13.90 ± 0.72% dry mass; $P = 0.014$). Despite the difference in collagen fraction, no differences were found in mechanical properties of MTL or modulus. However, UTS tended to be lower in male (0.66 ± 0.05 MPa) vs. female constructs (0.86 ± 0.08 MPa), with female sinews tending towards withstanding higher stress ($P = 0.065$).

Effects of physiological levels of estrogen on the mechanical properties of engineered ligaments. Engineered sinews treated in low (5 pg/ml), medium (50 pg/ml), and high (500 pg/ml) estrogen levels resulted in no difference in construct cross-sectional area (Fig. 2). However, low and medium dose estrogen treatment did significantly increase collagen production

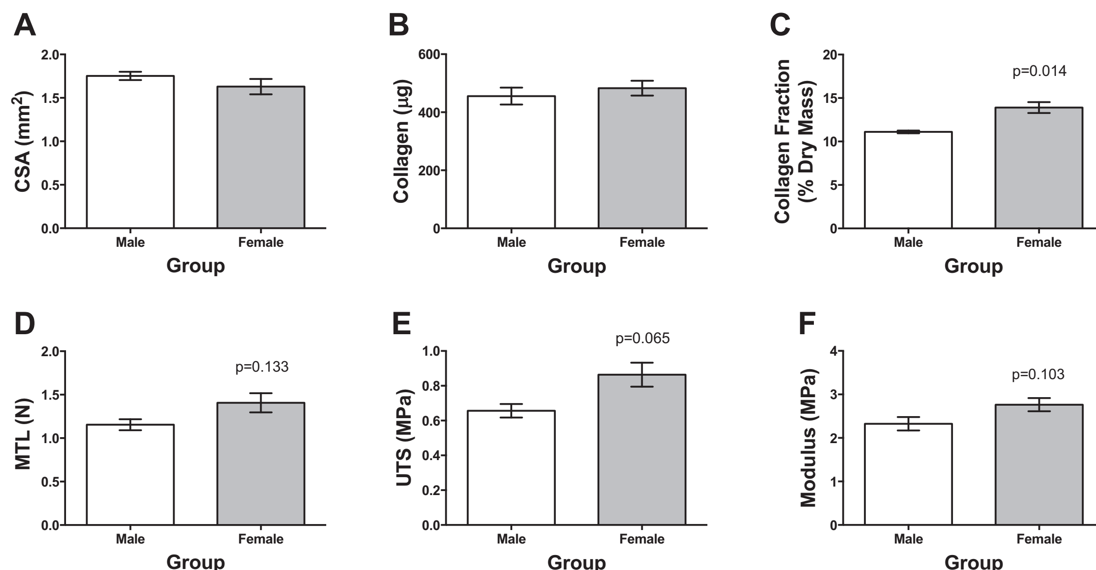

Fig. 1. Engineered ligaments show little variation attributable to the sex of the donor. Engineered ligaments were formed using cells originating from male (n = 3) or female (n = 4) donors and the cross-sectional area (CSA; *A*), total collagen mass (*B*), collagen fraction (*C*), maximum tensile load (MTL; *D*), ultimate tensile strength (UTS; *E*), and modulus of the constructs (*F*) were quantified. The data are representative of 2 independent experiments using $n = 8$ constructs from each donor. Data are presented as means ± SE. *$P < 0.05$, significant difference.

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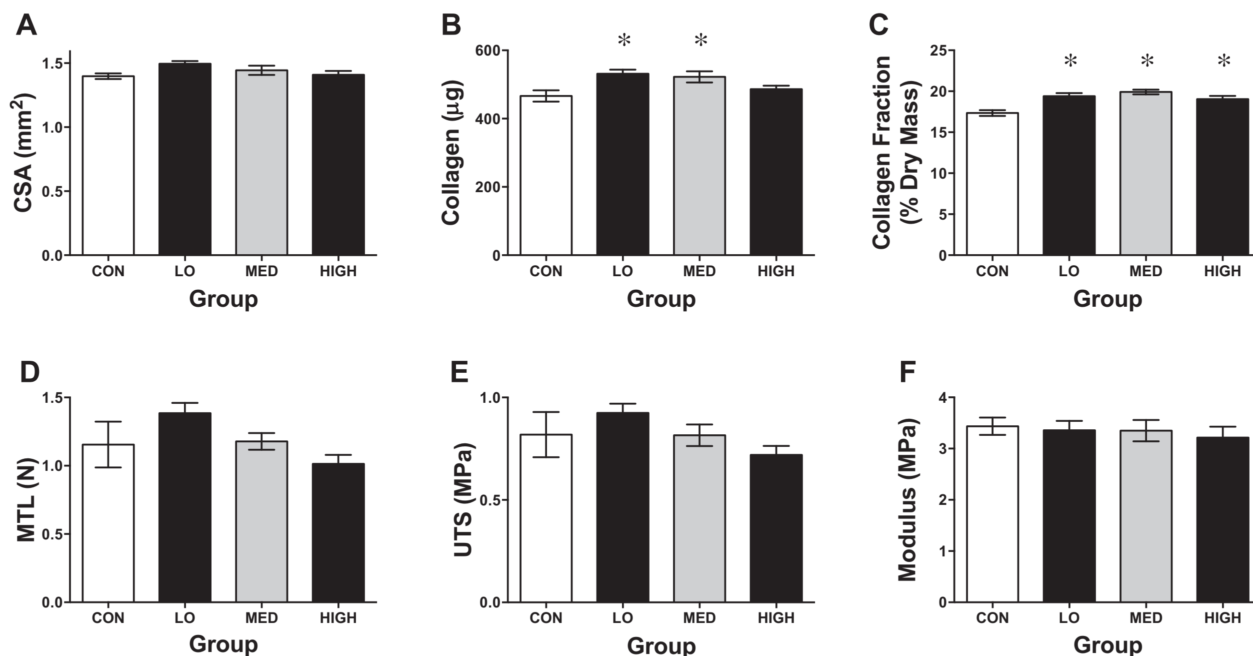

Fig. 2. Low estrogen environment improves collagen content of engineered ligaments. Ligaments were engineered using female cells and treated with 0 pg/ml [control (CON)], 5 pg/ml (low), 50 pg/ml (medium), and 500 pg/ml (high) of 17β-estradiol. The CSA (*A*), total collagen mass (*B*), collagen fraction (*C*), MTL (*D*), UTS (*E*), and modulus of the constructs (*F*) were quantified. The data are representative of 2 independent experiments using $n = 8$ constructs for each level of estrogen. Data are presented as means ± SE. $^*P < 0.05$, significant difference.

compared with control ($P = 0.006$ and $P = 0.03$, respectively). When examining the collagen fraction, all concentrations of estrogen increased collagen production significantly. Although collagen production was significantly increased by estrogen treatment, the mechanical properties of MTL, UTS, and modulus were not significantly affected, suggesting that estrogen treatment results in dissociation of collagen content and mechanical strength especially at high concentrations of estrogen.

Short-term exposure to high dose estrogen–mechanical properties. To physiologically mimic the menstrual cycle estrogen peak just before ovulation, we exposed the ligament constructs to 500 pg/ml of estrogen for the final 24 or 48 h of a 14-day culture (Fig. 3). Short-term high estrogen levels did not change the cross-sectional area, collagen content, or collagen fraction of the constructs. However, the UTS and modulus were significantly decreased following 48-h exposure to high estrogen ($P = 0.02$ and $P < 0.001$, respectively). Since this decrease in tensile strength and stiffness is not attributable to a decrease in collagen, we sought to determine whether this was due to altered cross-linking activity.

Estrogen and LOX activity. Ligament constructs treated with physiologically high levels of estrogen for the last 24 or 48 h of a 14-day culture exhibited a 62% ($P = 0.0084$) and 77% ($P = 0.0021$) decrease in LOX activity, respectively (Fig. 4).

Gene expression in ligament homeostasis. To determine whether the decrease in LOX activity was the result of a direct effect on existing LOX protein or a decrease in LOX expression, LOX gene expression was determined following treatment of ligament constructs with 500 pg/ml estrogen for 24 or 48 h. LOX mRNA decreased 25% in the presence of high estrogen over 24 h ($P = 0.03$) but returned toward baseline by 48 h (Fig. 5). High levels of estrogen did not affect the expression of collagen Ia1 and IGF-1 over 24 or 48 h. Tenascin C expression tended to decrease with high estrogen levels getting progressively lower over the 48 h of treatment. However, this did not reach statistical significance.

BAPN–mechanical properties. To determine whether the inhibition of LOX could lead to the changes in mechanics that we had observed with high estrogen supplementation, constructs were treated for the last 24 h of the 14-day culture period with the irreversible inhibitor of LOX BAPN. BAPN treatment resulted in a significant decrease in all of the mechanical properties of the ligament without affecting the collagen content of the grafts (Fig. 6). To ensure that the effect of BAPN was not due to cytotoxicity, we performed a BAPN recovery experiment in which constructs were treated with BAPN for 24 h on *day 13* after which the BAPN was rinsed away and the grafts were allowed to recover for an additional 6 days. At the end of the 6-day recovery period, both the untreated controls and BAPN-treated grafts showed greater MTL, UTS, modulus, and collagen content then the 14-day-old constructs (Fig. 6), indicating that the BAPN did not permanently damage the cells within the constructs.

DISCUSSION

Women sustain a significantly higher number of ACL injuries compared with men participating in the same sports, in part due to an increase in knee laxity as a result of cyclic rises in estrogen levels (14, 33–35, 38, 41). Using our 3D in vitro engineered ligament model, we have been able to reproduce the effects of cyclical hormonal variations on ligament function

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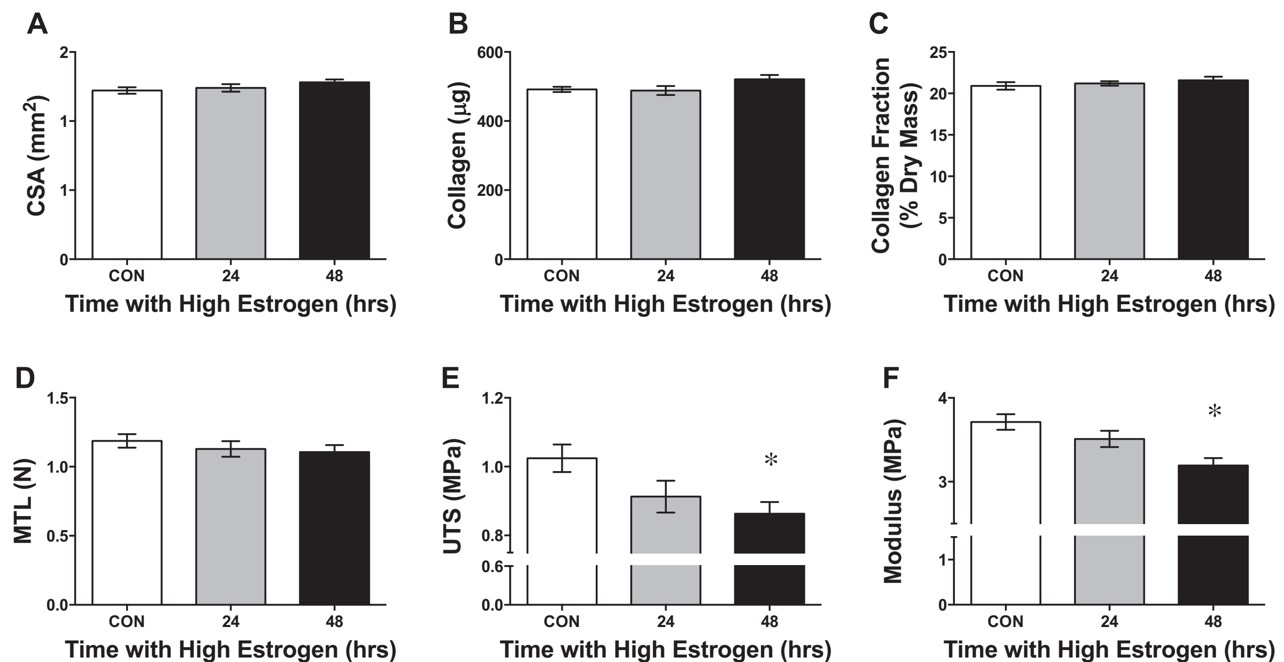

Fig. 3. Transiently increasing estrogen levels in culture decreases the material properties but not the collagen content of engineered ligaments. Engineered ligaments were cultured in 5 pg/ml (low) estrogen for 14 days as a control while additional ligaments were subjected to an increase to 500 pg/ml (high) estrogen in the last 24 or 48 h of culture. The CSA (*A*), total collagen mass (*B*), collagen fraction (*C*), MTL (*D*), UTS (*E*), and modulus of the constructs (*F*) were quantified. The data are representative of 2 independent experiments using $n = 8$ constructs for each level of estrogen. Data are presented as means ± SE. $*P < 0.05$, significant difference.

that contribute to the greater risk of ACL injury during the late follicular phase. We found that even though ligaments derived from female ACL cells showed no inherent difference in mechanical properties, acute high estrogen levels resulted in a decrease in LOX activity that preceded a decrease in the modulus of the ligament. Furthermore, inhibiting LOX with BAPN showed a similar decrease in engineered ligament function (~20%) compared with 48-h treatment with high estrogen (~15%), suggesting that the ability of estrogen to inhibit LOX may underlie the greater risk for ligament injury in women.

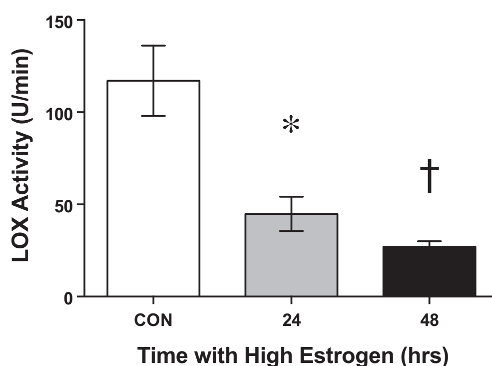

Fig. 4. Lysyl oxidase (LOX) activity decreases in a time-dependent manner with exposure to high estrogen. Engineered ligaments were cultured in low (5 pg/ml) estrogen for 14 days as a control (CON) while additional ligaments were subjected to an increase to high (500 pg/ml) estrogen in the last 24 or 48 h of culture and assayed for LOX activity. Data are presented as means ± SE $* P < 0.02$, $†P < 0.004$, significant differences from the control group (n = 8 constructs for each condition).

The function of a ligament is dependent on the content and the cross-linking of the collagen within the tissue (7, 25). Within a ligament, fibroblasts produce both the collagen and the enzymes that cross-link the collagen. We have previously shown that musculoskeletal cells are epigenetically programmed and these differences can be maintained in culture (15). Therefore, we first sought to determine whether there were any intrinsic differences between cells isolated from males or females. While subtle differences were evident, these differences could not explain the different rate of injury, suggesting that intrinsic differences between male and female cells were not the basis for this disparity.

We next sought to determine the effect of estrogen on the collagen content of our ACL grafts. Receptors for estrogen have been identified on the human ACL in both males and females (21). However, there have been conflicting reports of the effects of estrogen on collagen production, with some showing inhibition of collagen synthesis (36) and fibroblast proliferation (22) and others showing upregulation (19) or no effect (32). We found increased collagen production by human ACL cells exposed to estrogen for 14 days and a trend toward increased collagen Ia1 mRNA following 48 h of elevated estrogen. There are a number of potential reasons for the differences between the current work and other work in this area. First, most of the previous studies have been performed on cells isolated from animals such as rabbit (22), pig (20), or sheep (32). Second, most previous cell culture work has been performed using short-term 2D culture. The current study is

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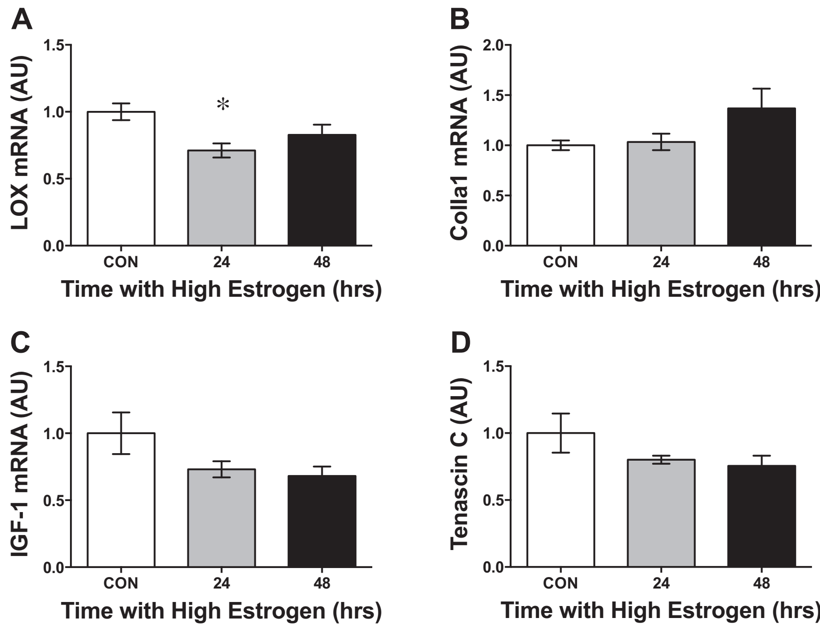

Fig. 5. Gene expression of LOX (*A*), Col 1A1 (*B*), IGF-1 (*C*), and tenascin C (*D*) following exposure to high dose estrogen. Engineered ligaments were cultured in low (5 pg/ml) estrogen for 14 days as a control (CON) while additional ligaments were subjected to an increase to high (500 pg/ml) estrogen in the last 24 or 48 h of culture before collection, RNA isolation, and quantitative RT-PCR. AU, arbitrary units. Data are presented as means ± SE. **P* < 0.05, significant difference (*n* = 8 constructs for each condition).

unique in that it focuses on human-derived cells and a 3D culture model to produce a more physiologic environment that can be studied over a longer time period. Our data suggest that fluctuations in estrogen concentration do not negatively affect mechanical properties via altered collagen production.

Since the stiffness of a ligament is directly related to the load at failure (18), numerous investigators have attempted to link hormonal fluctuations with the stiffness of the ACL. Even though the individual studies are equivocal, meta-analysis of the resulting data shows that in humans as knee laxity increases with the rise of estrogen there are more ACL ruptures (14). This suggests that the effect of estrogen is to decrease ligament function but that the effect is small and hard to detect in mature ligaments in vivo. To determine whether we could reproduce

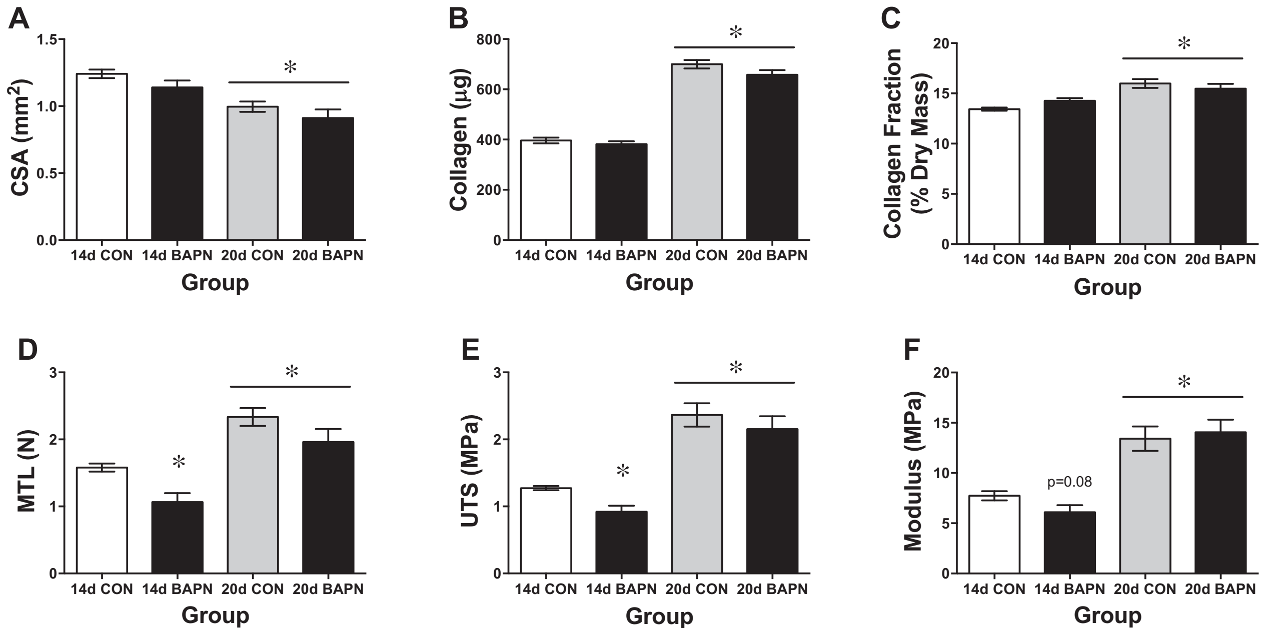

Fig. 6. Inhibition of LOX using 1 mM of β-aminoproprionitrile (BAPN) on the mechanical properties of engineered ligaments. Constructs were left untreated for 14 days (14d CON), treated for 24 h with 1 mM of BAPN (14d BAPN), left untreated for 20 days (20d CON), or treated with 1 mM BAPN for 24 h on day 13 and then given 6 days to recover (20d BAPN). The CSA (*A*), total collagen mass (*B*), collagen fraction (*C*), MTL (*D*), UTS (*E*), and modulus of the constructs (*F*) were quantified. The data are representative of 4 independent experiments using *n* = 6 constructs for each condition. Data are presented as means ± SE. **P* < 0.05, significant difference.

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the effect of high estrogen in an in vitro model, we used ligaments engineered from cells isolated from human ACL remnants and by simulating the estrogen surge saw that the modulus and ultimate tensile strength were decreased 15% by 48 h with no change in the amount of collagen in the tissues. It is likely that the magnitude of change is greater in our model than in vivo. However, the similarities between what we observed and that seen in vivo (33, 34, 41) suggest that we could use our model to try to understand the mechanism underlying this physiological change in ligament function.

A decrease in ligament function with no change in collagen content suggested that the elevated estrogen was affecting the cross-linking of the tissue. The primary enzymatic collagen cross-linker is LOX, an amine oxidase that catalyzes the formation of lysine cross-links between adjacent collagen fibrils (16). In support of that hypothesis, we found that physiologically high estrogen levels resulted in a 77% decrease in LOX activity over 48 h. In spite of the dramatic decrease in LOX activity, the high estrogen levels only modestly affected LOX mRNA, suggesting that the effect of estrogen was to directly inhibit the activity of existing LOX. Furthermore, the high estrogen level did not significantly alter other markers of ligament health such as IGF-1 or collagen 1a1, suggesting that short-term high estrogen levels did not significantly alter the global expression profile of the ligaments. It is interesting to note that the load-responsive gene tenascin C (6) tended to decrease over the 48 h of high estrogen treatment, suggesting a decrease in the mechanical load within the tissue. To determine whether directly inhibiting LOX activity could alter the mechanics of our constructs, we inhibited LOX with BAPN and noted that, much like with estrogen, BAPN inhibition of LOX resulted in a decrease in the mechanical properties of the engineered constructs without affecting collagen levels. The decrease in function as a result of BAPN treatment was transient, in that the ligaments were able to increase in stiffness in the 6-day washout period. However, the transient loss of LOX activity tended to decrease the UTS and MTL of the ligaments at the 20-day test point. This suggests that monthly inhibition of LOX could result in the greater laxity seen in female knees (34). The greater effect of BAPN compared with estrogen on ligament mechanics could reflect the fact that estrogen decreased LOX activity only 80% whereas BAPN is known to completely inhibit the enzyme (25). It is possible that cross-link turnover is higher in our developmental model than in adult tendons. However, BAPN has been used to treat adult horse tendons because it rapidly improves the appearance of bowed tendons and together with a controlled exercise program improves tendon recovery (8). This suggests that cross-link turnover can also be high in adult tendon since inhibiting LOX results in a rapid change in the mechanics of an adult tendon.

In conclusion, our data suggest that physiologically high estrogen levels, such as those in the 3–4 days before ovulation, decrease the activity of LOX in human ACL cells resulting in a decrease in the stiffness of ligaments. The inhibition of LOX could contribute to the increased laxity seen in the female knee (34) and the resulting higher level of ACL rupture seen in normally menstruating women (14). Even though our data suggest that inhibiting the estrogen surge using oral contraceptives may be beneficial for ligament function, work from Copenhagen suggests that this may alter the response of ligament cells to exercise (11), indicating that the effect of estrogen on ligament function is far more complex and will require much more work to completely understand.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.A.L. and K.B. conception and design of research; C.A.L., A.L.-B., L.M., N.S., and G.M. performed experiments; C.A.L., A.L.-B., G.M., and K.B. interpreted results of experiments; C.A.L. and K.B. drafted manuscript; C.A.L., A.L.-B., L.M., N.S., G.M., and K.B. edited and revised manuscript; C.A.L., A.L.-B., L.M., N.S., G.M., and K.B. approved final version of manuscript; A.L.-B., L.M., N.S., G.M., and K.B. analyzed data; K.B. prepared figures.

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