The Effects of Estrogen on the ACL and its Relationship to the Increased Prevalence of ACL Injury in Female Athletes

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A thesis submitted to the Department of Biology in partial fulfillment of the requirements for a degree of Bachelor of Science

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May 3, 2022

I pledge my word of honor that I have abided by the Washington College Honor Code while completing this assignment.

Madison Parker

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Abstract

Female athletes experience an incidence rate 2- to 6- times higher than males for anterior cruciate ligament (ACL) injuries. The ACL is responsible for preventing hyperextension by becoming stiff as the knee extends. Injury is more likely to occur in a knee that has an increase in ligament laxity as the ACL would then be unable to resist stretching and therefore hyperextension. The determining factor on how stiff a ligament is dependent on the density of collagen cross-links found within the ligament. It is possible that the differences in the sexes endocrine system be a factor in why females are more susceptible to ACL injury. The types, levels, and exposure of sex hormones differ between the sexes and one the hormones that largely differs between them is estrogen. There are higher rates of ACL injury in females during the ovulatory phase, when estrogen is high. Additionally, there is also an increase in ACL laxity during this phase. This is due to estrogens ability to inhibit an enzyme that is important in the cross-linking of collagen. The inhibition of the enzyme creates cross-links that are less dense and produce a ligament that is lax and weak. It is crucial that not only the athlete but the whole athletic system be aware of these predisposing factors that make females more prone to certain injuries like those to the ACL. Proper prevention programs that take the female biology into account could drastically reduce the prevalence rate for this severe injury for the sex.

Introduction

The knee is the body's largest joint and is comprised of bones, cartilage, ligaments, and tendons that all work together to support the weight of the body (Figure 1). Due to its ornate structure, it is also easily injured making up around 41% of all injuries sustained by athletes (Sancheti et al., 2010). After the implementation of Title IX, a law that prohibits the discrimination on the basis of sex in any federally funded program, knee injury rates in athletes began to rise (Hewett, 2000). Researchers noticed that the increase in the number of knee injuries coincided with the influx in female athletic participation from Title IX. This was the first bit of evidence that suggested that females may be more susceptible to knee injury than their male counterparts. Many of the athletes were sustaining their knee injuries during jumping and cutting sports like volleyball, basketball, and soccer (Hewett, 2000). In these sports the motion known as cutting is common and refers to movements where the knee moves in the opposite direction of the planted foot, like when a runner makes a quick change in direction. Motions like this require the knee to work fast to supplement for the quick shift in force, so if any part of the knee was not performing properly injury could occur. When compiling the data researchers found that when competing in the same jumping and cutting sports, females are 4- to 6- times more likely to suffer a knee injury than males (Hewett, 2000).

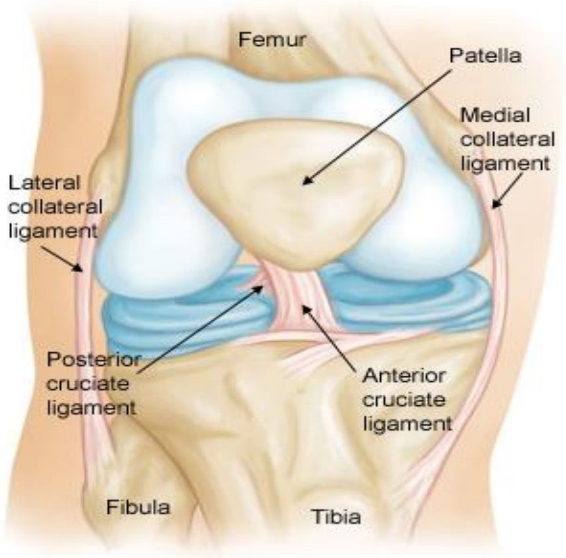

Figure 1. Normal knee anatomy. Taken from American Academy of Orthopaedic Surgeons, 2014.

Within their own sex, it is predicted that about 1 in every 10 female athletes will sustain a debilitating knee injury in any given year of collegiate participation (Hewett, 2000). Currently in the National Collegiate Athletic Association (NCAA) there are about 216,318 females participating in varsity athletics (NCAA, 2021). Of that, approximately 21,632 athletes are predicted to sustain a serious knee injury. A majority of these knee injuries are season ending and require surgical repair in addition to intense physical therapy. Consequently, injuries like this can result in an athlete's loss of a scholarship and be detrimental to the athlete's mental health.

Of these serious knee injuries, rupture of the anterior cruciate ligament (ACL) is the most common. Females experience an incidence rate 2- to 6- times higher than males for ACL injuries (Chidi-Ogbolu and Baar, 2019). The disproportionate prevalence in females is concerning when considering the recovery and reoccurrence rate for females. Compared to their male counterparts, females experience significantly less strength and improvement 1 year post ACL reconstruction (Kim and Park, 2015). Additionally, recurrent ACL ruptures make up about 14% of all ACL injuries in females in comparison to making up only 10% in males (Gans et al., 2018). The ACL is one of two cruciate ligaments found on the inside of the knee and is attached inferiorly to the tibia at the intercondylar eminence (Figure 2) (Betts et al., 2017). The cruciate ligaments are named for where they attach to the tibia, either anteriorly or posteriorly. Additionally, the term cruciate means cross, which describes the anatomy of the ligaments inside the knee (Betts et al.,

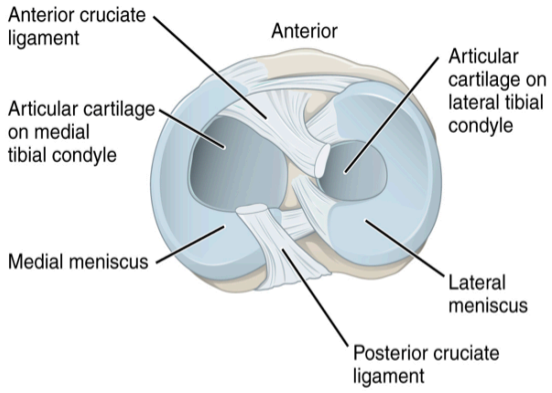

Figure 2. Superior view of the right tibia in the knee joint, showing the menisci and cruciate ligaments. Taken from Betts et al., 2017.

2017). The two ligaments run diagonally upward to attach to the inner part of a femoral condyle where they overlap to form an X-shape (Figure 2) (Betts et al., 2017).

While ligaments are responsible for connecting bone to bone, the specific role of the ACL is to prevent hyperextension by becoming stiff when the knee extends (Betts et al., 2017). Ligaments are naturally stiff in the human body, which means they are not easily bent. A knee that is hyperextended is one that is bent beyond its normal range, which is typically 180 degrees. Consequently, injuries to the ACL are often sustained when hyperextension of the knee is produced. For example, hyperextension can be the result of a forceful blow to the anterior knee or when an athlete makes a sharp cut (Betts et al., 2017). Hyperextension is more likely to occur in a knee that has an increase in ligament laxity. The opposite of stiffness, laxity can be defined as looseness hence, a lax ligament is more susceptible to injury because it is unable to resist hyperextension. In a scenario where the ACL loosens, the knee would be able to hyperextend and in doing so rupture the ligament (Figure 3). Therefore, keeping a stiff ligament and reducing ligament laxity is paramount in maintaining ideal ACL function and reducing the risk of injury.

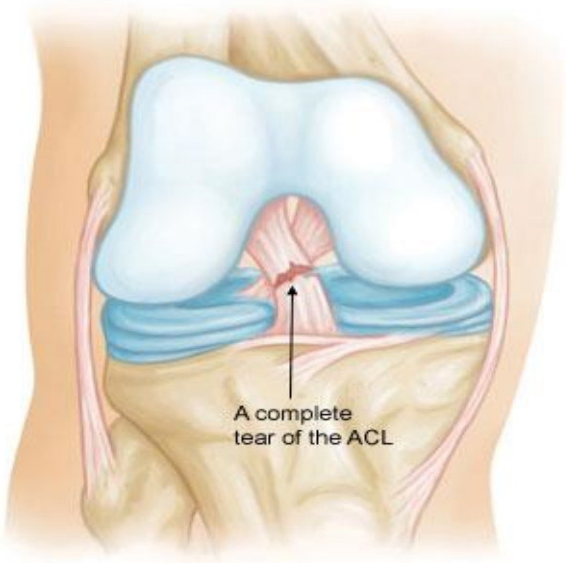

Figure 3. Complete tear of ACL. Taken from American Academy of Orthopaedic Surgeons, 2014.

Musculoskeletal Importance

Ligaments are made of connective tissue, a type of tissue that is responsible for the connecting and supporting of tissues (Betts et al., 2017). Like most tissue structures, connective

tissue is composed of cells within a matrix made up of extracellular material, which is produced from the cells found in it (Betts et al., 2017). There are three categories of connective tissue, and each is classified based on the features of their ground substances and the kind of fibers found within its matrix (Betts et al., 2017). Connective tissue proper is one of these categories and includes the subcategories dense connective tissue and loose connective tissue. In connective tissue proper the most prominent cell is the fibroblast (Betts et al., 2017). The release of polysaccharides and proteins from fibroblasts combines with extracellular fluids to create a viscous ground substance that then merges with embedded fibrous proteins to form the extracellular matrix (Betts et al., 2017). Of the two types of connective tissue proper, ligaments are made up of dense connective tissue. This particular tissue is supported by bundles of fibers that offer tensile strength, elasticity, and protection (Betts et al., 2017). More specifically ligaments are made up of regular dense connective tissue. Within this tissue are fibers that run parallel to one another increasing tensile strength and resistance to stretching in the direction of the fiber orientation (Betts et al., 2017).

Fiber type is important in the classification, strength, and resistance of connective tissues and in dense connective tissue one of the primary fibers is collagen. This particular fiber type is one of the three main fibers produced by fibroblasts and is composed of fibrous protein subunits that are linked together to create a long and straight fiber (Betts et al., 2017). Most of the dry weight found in ligaments is collagen, making up roughly 75% of the overall weight (Chidi-Ogbolu and Baar, 2019). The reason most of ligamentous tissue is composed of collagen is in part due to the fiber not only being flexible, but also because it has great tensile strength and resistance (Betts et al., 2017). Collagen fibers are the reason for the typical resiliency and strength found in dense connective tissue and therefore ligaments.

The primary functions and properties of ligaments are greatly affected by collagen cross-linking, a method that chemically joins molecules by covalent bonds. Collagen fibers are able to be cross-linked in two ways: enzymatically and non-enzymatically (Chidi-Ogbolu and Baar, 2019). Enzymatic cross-links of collagen are primarily produced by lysyl oxidase (LOX), an enzyme that is secreted by fibroblasts (Lee et al., 2015). The copper (Cu)-dependency of LOX allows for the cross-linking of lysine residues in neighboring collagen molecules, which increases collagen stiffness and therefore ligament strength (Lee et al., 2015). Non-enzymatic cross-links of collagen can also occur through advanced glycation end-products (AGE), a Maillard reaction between a sugar and amino acid (Chidi-Ogbolu and Baar, 2019). Although both ways of cross-linking increase tissue stiffness, AGE decreases collagen turnover rate, which can overtime effect ligament function (Chidi-Ogbolu and Baar, 2019).

Estrogen's Importance

Apart from its role as a sex hormone, estrogen also plays substantial roles in the development, maturation, and aging of extragonadal tissue, which includes connective tissues and therefore ligaments (Chidi-Ogbolu and Baar, 2019). In females, the hormone is produced from cholesterol through reactions that take place within the ovaries (Chidi-Ogbolu and Baar, 2019). There are three types of estrogens: estradiol, estrone, and estriol. The most abundant type of estrogen is 17ß-estradiol and is the primary circulating estrogen in non-pregnant mature females. Due to it being a steroid hormone, estrogen is able to freely pass through the plasma membrane and move into the nucleus (Chidi-Ogbolu and Baar, 2019). Once it is inside the nucleus, estrogen binds to its nuclear receptors and can modify gene expression (Chidi-Ogbolu and Baar, 2019). Estrogen receptors can be found in ligaments, such as the ACL, and within the

tissue, and is known to regulate metabolism (Chidi-Ogbolu and Baar, 2019). Additionally, estrogen has the ability to directly inhibit specific enzymes (Chidi-Ogbolu and Baar, 2019). Estrogen levels in females vary considerably and this due to the menstrual cycle and its different phases.

During the menstrual cycle estrogen naturally fluctuates, increasing 10- to 100- fold over the span of the cycle (Chidi-Ogbolu and Baar, 2019). The purpose of the menstrual cycle is to prepare the uterus for potential pregnancy and usually begins around the age of 13 and lasts until menopause (Carmichael et al., 2021). A normal menstrual cycle is classified as one that occurs regularly and lasts between 21 and 35 days (Carmichael et al., 2021). However, menstrual cycles are often observed/studied as a 28-day cycle with two distinct phases, follicular and luteal (Figure 4). Within these two phases are what are called subphases which includes ovulation between the follicular and luteal phases. The follicular phase begins on the first day of menstruation and continues until ovulation, typically lasting 13 days (Carmichael et al., 2021). During this phase, estrogen levels gradually increase, from 5 picogram/milliliter (pg/ml) to a peak of 200-500 pg/ml right before ovulation (Chidi-Ogbolu and Baar, 2019). The rise in estrogen is in response to the maturation of ovarian follicles that contain the eggs (Carmichael et al., 2021). The ovulatory phase always occurs 14 days before menstruation, thus typically occurring on day 14 of a 28-day cycle. Ovulation immediately follows the peak in estrogen seen during the follicular phase and is characterized by the rupture of mature ovarian follicles and the release of the egg into the uterus (Carmichael et al., 2021). The luteal phase proceeds ovulation and continues until menstruation, typically lasting 14 days (Carmichael et al., 2021). Immediately following ovulation there is a dramatic decrease in estrogen and then mid-way

through the phase there is a second rise in estrogen that prepares the endometrium for fertilized egg implantation (Carmichael et al., 2021).

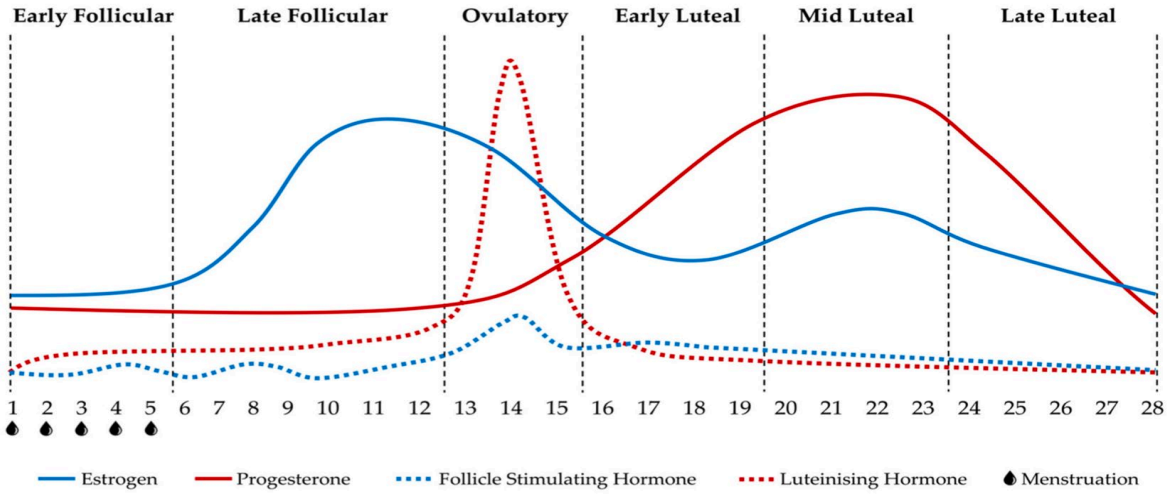

Figure 4. Hormonal events and phases in a eumenorrheic 28-day menstrual cycle. Taken from Carmichael et al., 2021.

Cause for the Prevalence of ACL Injuries in Female Athletes

Females may be predisposed to injuries, like ones sustained to the ACL, because of their own biology. Structural differences between the sexes have been discovered that suggest anatomical causes for the increase in ACL injury rates in females (Park et al., 2009). However, males and females also differ in their endocrine systems. The two sexes vary considerably in the types, levels, and recurring exposure of circulating sex hormones (Shultz et al., 2005). Furthermore, females undergo periodic fluctuations in their hormone levels over the course of the menstrual cycle, where males tend to have hormone levels that remain fairly constant (Shultz et al., 2005). It is possible that the differences in the male and female endocrinology play an important role in the difference of prevalence in ACL injuries between the two sexes. This capstone will explore the possibility that these differences in endocrinology, and more specifically estrogen, be the reason for the higher prevalence of ACL injuries in females.

Research conducted by Wojtys et al., 2002, Park et al., 2009, and Lee et al., 2015 support the theory that estrogen is key in the increase prevalence of ACL injuries in females.

NOTE: When referring to male and females in this paper it is in terms of biological sex and not gender. Furthermore, any discussion of the differences in sex is strictly biological.

Chapter 1: Menstrual Cycle vs ACL Injuries

As mentioned earlier, females are 2- to 6- times more likely to sustain an ACL injury than males and this disproportionate prevalence of ACL injuries could be explained by their biological sex differences. Wojtys et al., 2002 sought to explore the relationship between the menstrual cycle phase and the distribution of ACL injuries in female athletes. They hypothesized that there is an association between cycle phase and when ACL injuries occur, therefore affecting a female athlete's susceptibility to ACL rupture (Wojtys et al., 2002).

Wojtys et al., 2002 tested the hormone levels of females who sustained an ACL tear within 24 hours of injury and the start of their next menstrual cycle. The hormone levels for total estrogen metabolite (E1G), progesterone metabolite (PDG), and luteinizing hormone metabolite (LH) were measured using a urine analysis (Wojtys et al., 2002). The results from the analysis showed that all the hormone levels of the athletes were within normal clinical range (Wojtys et al., 2002). However, when the hormone metabolite levels were compared, there was a significant difference in the levels seen at day of injury and the levels seen at day 1 in cycle (Table 1). The hormone metabolite levels collected at the time of injury were significantly higher than the levels collected at the beginning of the athlete's next menstrual cycle (Table 1). This suggests a connection between elevated hormone concentrations and when ACL injuries occur.

Table 1. Mean (SD) Normalized Hormone Metabolite Levels at Specified Days in Subjects Cycle. Taken from Wojtys et al., 2002.

	Day 1 in Cycle	Day of Injury
Total Estrogen (μg/24 hrs)	45.5 (5.8)	80.2 (13.0)
Progesterone (mg/24 hrs)	1.1 (0.2)	2.0 (0.4)
Lutenizing Hormone (IU/24 hrs)	6.7 (1.4)	19.5 (5.1)

A chi-squared analysis was used to compare the expected and observed frequency of ACL ruptures in each phase of the menstrual cycle. The number of days in that specific phase was divided by the total number of days in the menstrual cycle to determine the expected rate,

and then the actual number of injuries to occur in each phase was divided by the total number of injuries to determine the observed rate (Wojtys et al., 2002). They identified the menstrual cycle phase that each injury occurred in by looking at the hormone metabolite levels recorded within 24 hours of injury for each athlete and compared them to clinical phase hormone levels (Wojtys et al., 2002). The chi-square analysis showed a significant relationship (indicated by the *) between the observed rate of when ACL injuries occurred and menstrual cycle phase (Table 2). 43% of the ACL injuries were sustained during ovulation, compared to 23% during the follicular phase and 34% during the luteal phase (Table 2). The observed number of ACL injuries during the ovulatory phase were more than 2.5 times higher than what was expected in that phase (Wojtys et al., 2002). This implies that ACL injuries are menstrual cycle phase dependent.

Table 2. Chi-Squared Analysis of Overall ACL Injuries. Taken from Wojtys et al., 2002.

Phase	Expected Rate	Observed Rate*
1 (Follicular)	9/28=32%	15/65=23%
2 (Ovulatory)	5/28=18%	28/65=43%
3 (Luteal)	14/28=50%	22/65=34%

*χ^2=27.7, p<0.001

These results suggest that there is a relationship between hormone levels, menstrual cycle phase, and ACL injury occurrence. The number of observed injuries was highest during the ovulatory phase compared to the luteal and follicular phases. As mentioned earlier, ovulation follows the peak of estrogen thus the presence of high levels of the hormone during this phase.

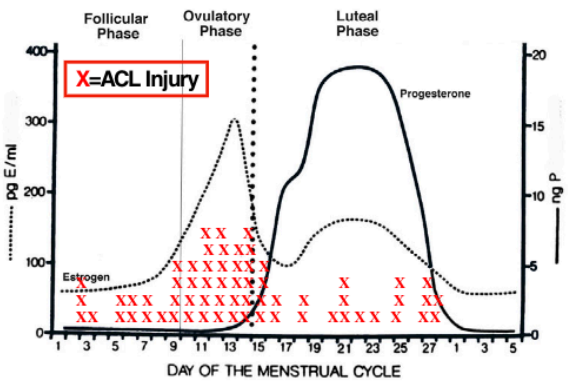

Figure 5. Histogram of observed ACL injuries plotted by day of cycle. Taken from Wojtys et al., 2002.

When looking at the histogram of ACL injuries versus the day in menstrual cycle, there appears to be a pattern between when injuries happen and estrogen levels (Figure 5). As estrogen levels (pg/ml), demonstrated by dotted

line) increase so do the number of observed injuries (demonstrated by X), the peak of estrogen seen in the ovulatory phase coincides with the peak in injuries, then as estrogen levels decrease so do observed injuries (Figure 5). So, although all hormone levels were significantly higher at the time of injury, based off where the injuries were occurring in the menstrual cycle it is likely that estrogen plays a key role in ACL injuries.

Chapter 2: Menstrual Cycle vs Knee Laxity

As discussed in chapter 1, Wojtys et al., 2002 concluded that there is a relationship between ACL injury occurrence and the menstrual cycle, their research showed a higher risk for ACL injury during the ovulatory phase of the menstrual cycle. During this phase, estrogen is high, and when looking at the distribution of ACL injuries across the menstrual cycle, it mimics the levels in estrogen. This implies there is a connection between estrogen and ACL injuries.

As previously mentioned, there are estrogen receptors in the ACL, and it is possible that estrogen could impact the overall function of the ligament itself. It is the responsibility of the ACL to prevent hyperextension and it does so by becoming stiff as the knee extends. Consequently, if the ACL were to loosen then the knee would be able to overextend resulting in rupture. Any alteration that could potentially occur to the ligament could be critical. Park et al., 2009 set out to determine if the changing hormone levels during the menstrual cycle have any influence on knee joint laxity of the noncontractile knee joint. They hypothesized that there would be an increase in laxity from follicular phase to the ovulatory phase because of the rise in estrogen seen during ovulation then decrease from the ovulatory phase to the luteal phase because of the decline in estrogen during the luteal phase (Park et al., 2009). Park at al., 2009 drew blood at 3 times, all at different periods during the menstrual cycle, for all the subjects. The blood samples were used to quantify hormone levels for estradiol and progesterone, as well as used to determine the appropriate menstrual cycle phase for testing (Park et al., 2009). The overall phase effect shows that the hormone levels, for both estradiol and progesterone, significantly differed (indicated by the * or **) across all 3 tested phases (Table 3). The highest levels for both hormones were found during the luteal phase. It is important to note that the highest levels of estrogen during this study were seen during the luteal phase due to where in the

Table 3. F, follicular phase; O, ovulation; L, luteal phase. One-way repeated measure analysis of variance was performed at α = .05. Independent 2-tailed *t* test with a Bonferroni adjustment for multiple comparisons (*P* was multiplied by the number of comparisons [n = 3]) was performed at α = .05. **P* < .05. ***P* < .001. Taken from Park et al., 2009.

Summary of Hormone Levels at 3 Test Intervals in All Subjects[a]

Outcome Measure	Follicular Phase, mean (SD)	Ovulation, mean (SD)	Luteal Phase, mean (SD)	Overall Phase Effect, F(*df*) [*P*]	Bonferroni-Adjusted *P* Value		
					F vs O	O vs L	F vs L
Days of test, d	6.12 (1.40)	16.08 (2.99)	22.85 (3.22)	343.277(2,50) [.000**]	.000**	.000**	.000**
Estradiol, pg/mL	44.49 (23.77)	80.84 (40.49)	137.49 (85.82)	19.103(2,50) [.000**]	.000**	.012*	.000**
Progesterone, ng/mL	0.99 (0.46)	3.24 (2.37)	11.43 (7.01)	45.952(2,50) [.000**]	.000**	.000**	.000**

cycle they decided to quantify hormone levels. It is expected that the highest levels of estrogen by seen during ovulation, but their testing for this phase occurred 24- to 48- hours after the estrogen peak. Estradiol (demonstrated by solid line) was on the decline when the ovulation phase (#2) was tested for, and therefore higher levels were recorded during the luteal phase (#3) where estrogen has a second, but minor, surge (Figure 6). The reason behind this delay in testing could be due to estrogens delayed effects on laxity. Shultz et al., 2005 study revealed that laxity (demonstrated by ■) mirrors estrogens (demonstrated by ◆) fluctuations but lags behind (Figure 7). Therefore, an explanation

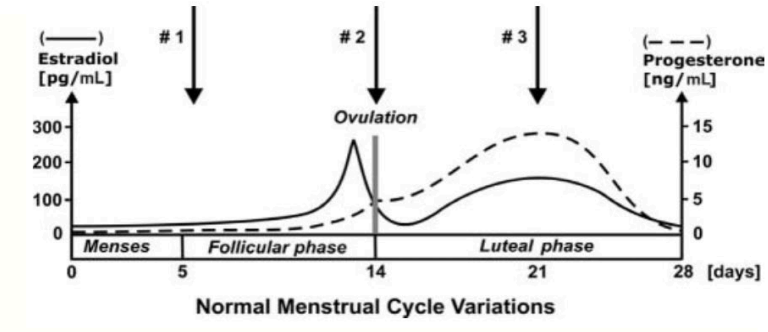

Figure 6. Chart indicating the timing of hormone level testing during the menstrual cycle. #1, follicular phase; #2, ovulation; #3, luteal phase. Taken from Park et al., 2009.

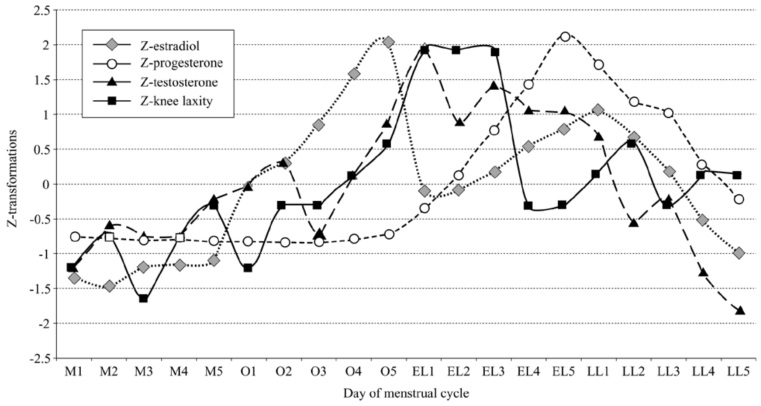

Figure 7. Graphic representation of changes in serum hormone levels and knee laxity values in females across the 20 days of the menstrual cycle. Each value was converted to Z-scores to allow equivalent scaling. M1 – −M5 =: days of menses; O1 – −O5 =: days of the initial estradiol rise near ovulation; EL1 – −EL5 =: days of the early luteal phase (rise in progesterone <2 ng/mL); LL1–LL5=: days of the late luteal phase (decline in progesterone levels). Taken from Shultz et al., 2005.

for testing 24- to 48- hours after the estrogen surge for the ovulatory phase was to ensure laxity was tested at its highest during ovulation.

After each blood sample was collected the subjects had their knee laxity tested using a standard arthrometer (Park et al., 2009). They measured the displacement, or misalignment, of the knee joint at 89 Newtons (N) and at its maximum to determine laxity (Park et al., 2002). Testing revealed that over the menstrual cycle the greatest knee laxity at a displacement of 89 N (DIS89) was seen during the ovulatory phase which was significantly higher (indicated by *) than the knee laxity seen during the luteal phase (Figure 8). At this displacement, knee laxity during ovulation was 11.3% higher than what was seen during the luteal phase (Park et al., 2009). Maximum displacement (MAX) was the greatest during ovulation compared to both the follicular and luteal phase but was significantly greater (indicated by *) than laxity during the follicular phase (Figure 8).

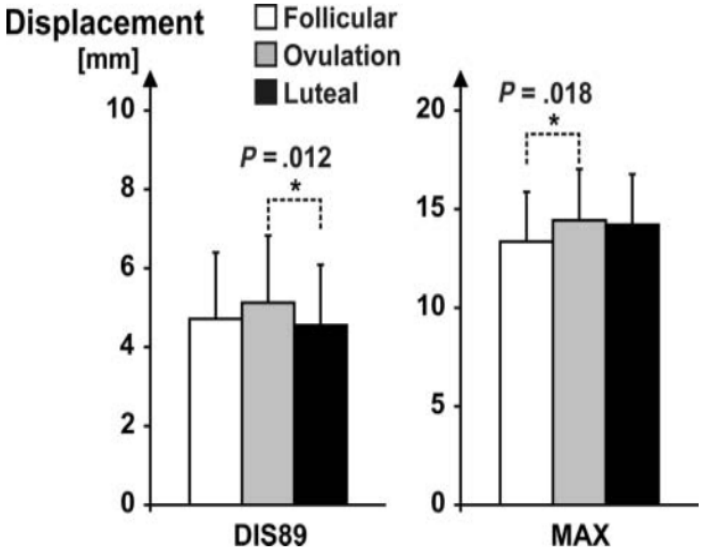

Figure 8. Mean (SD) laxity at the 3 test intervals (follicular, ovulation, luteal). DIS89, displacement 89 N of the load-displacement curve; MAX, displacement at manual maximum load. *$P < .05$. Taken from Park et al., 2009.

The data implies that there is a connection between the menstrual cycle and knee laxity, showing that as hormone levels fluctuate, so does laxity. Similarly, the distribution of where ACL injuries occur during the menstrual cycle follows the same trend. In addition to this, both sets of data share their peaks during the ovulatory phase, further suggesting a relationship

between laxity and injury. A majority of ACL injuries are a result of hyperextension, and, as mentioned earlier, this is likely to occur if the ligament were too loose and unable to restrain the knee from overextending. Therefore, an increase in knee laxity can easily result in the hyperextension of the knee joint that leads to ACL rupture. Once again, estrogen seems to play a factor in not only the occurrence of ACL injuries but also in knee laxity levels across the menstrual cycle.

Chapter 3: Estrogens Inhibition of LOX

Studies have revealed that there is an increase in susceptibility to ACL rupture and knee laxity in females during the ovulatory phase of the menstrual cycle where estrogen is at its highest. Wojtys et al., 2002 showed the distribution of female ACL injuries across the menstrual cycle and found that they followed the trend of estrogen fluctuations, as estrogen rose so did the number of injuries and vice versa, thus a majority of the injuries occurring during ovulation. Park et al., 2009 found a similar trend when looking at knee laxity across the menstrual cycle and concluded that in comparison to the follicular and luteal phase, there was significantly greater knee laxity during ovulation. These findings suggest that estrogen effects the female ACL in a way that results the knee to be more lax and thus prone to injury.

As mentioned earlier, estrogen is known to inhibit specific enzymes and the LOX enzyme is critical in the cross-linking of collagen. The stiffness, and therefore strength, of a ligament is reliant on collagen content and cross-linking density. If there were inhibition of LOX due to estrogen, then that would be detrimental to the ligament. Lee et al., 2015 had a purpose that was twofold: to observe if there were differences in the male and female donor's isolated ACL cells, as well as to determine the effect of estrogen on engineered ligament function and LOX activity. They hypothesized that an acute rise in estrogen would lead to a decrease in ligament mechanical properties through the inhibition of collagen synthesis (Lee et al., 2015).

Lee et al., 2015 used fibroblasts isolated from human ACLs from male and female donors to engineer three-dimensional ligament models. They first compared the ligaments to identify any sex differences in the constructs. There were no differences in the cross-sectional area (CSA) and collagen content (Figure 9A and B), however, the male derived ligaments had a collagen fraction by dry mass (Figure 9C) that was significantly lower (indicated by *) than that of the

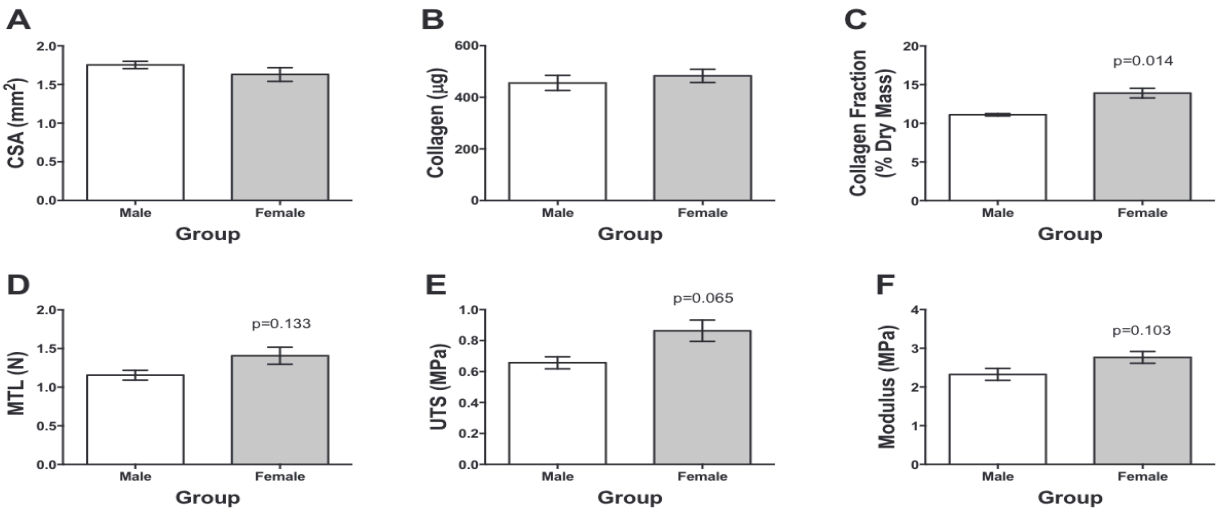

Figure 9. Engineered ligaments show little variation attributable to the sex of the donor. Engineered ligaments were formed using cells originating from male (n = 3) or female (n = 4) donors and the cross-sectional area (CSA; *A*), total collagen mass (*B*), collagen fraction (*C*), maximum tensile load (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. Taken from Lee et al., 2015.

female derived ligaments. No significant differences were observed in the mechanical properties of maximum tensile load (MTL) or modulus, but ultimate tensile strength (UTS) was lower in the males than it was in the female ligaments (Figure 9D, E, and F). Results showed that there was little difference in the engineered ligaments when compared to sex of donor, however, female derived ligaments were able to withstand a higher degree of stress.

To test the effects of physiological levels of estrogen on mechanical properties, the ligaments were supplemented with 5 pg/ml (low), 50 pg/ml (medium), and 500 pg/ml (high) of 17ß-estradiol (Lee et al., 2015). Estrogen levels were chosen based on the levels seen during the follicular (5 pg/ml) and ovulatory phase (500 pg/ml), the medium concentration was chosen to be a mid-point and give a 10-fold scale (Lee et al., 2015). There were no observed CSA differences in the ligaments treated with the different estrogen levels (Figure 10A). The low and medium doses of estrogen significantly increased (indicated by *) collagen content (Figure 10B), and collagen fraction by dry mass significantly increased (indicated by *) under all estrogen dosage

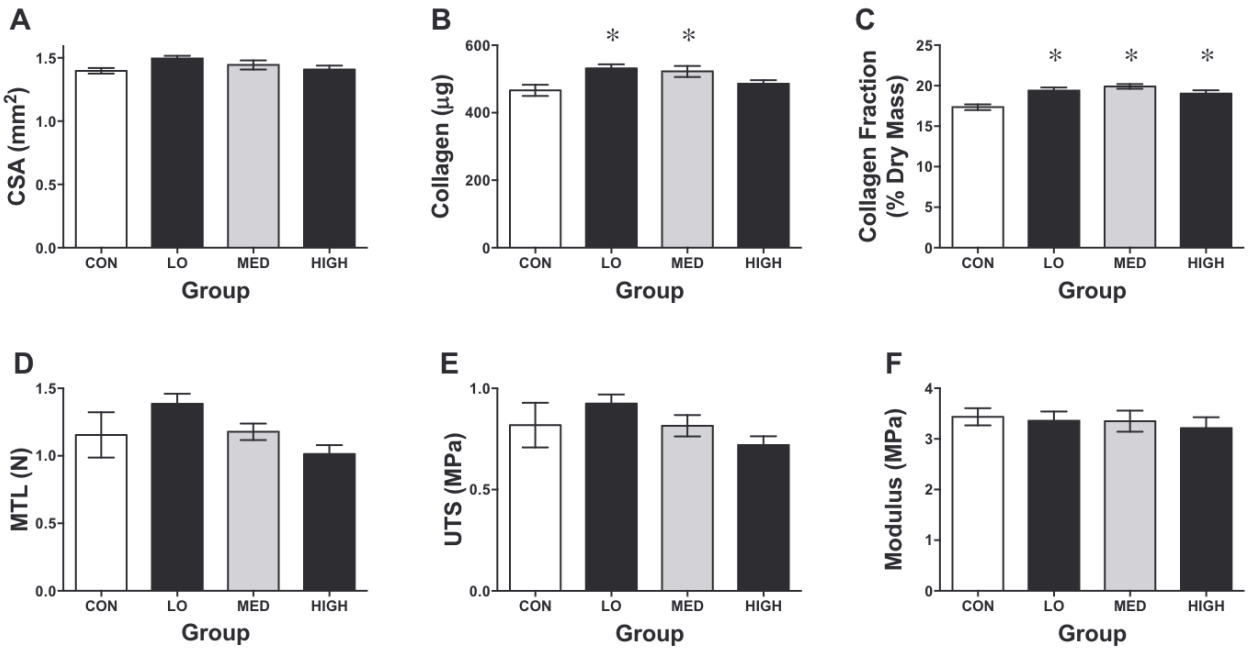

Figure 10. Low estrogen environment improves collagen content of engineered ligaments. Ligaments were engineered using female cells and treated with 0 pg/ml [control (CON)], 5 pg/ml (low), 50 pg/ml (medium), and 500 pg/ml (high) of 17ß-estradiol. The CSA (*A*), total collagen mass (*B*), collagen fraction (*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. Taken from Lee et al., 2015.

levels (Figure 10C). There were no observed significant differences in the mechanical properties of MTL, UTS, and modulus (Figure 10D, E, and F). The lack of change in mechanical properties despite there being an increase in collagen content suggests that there may be no relationship between the two when treated with estrogen.

Subsequently, short-term exposure with the high dose of estrogen was also tested for its effects on mechanical properties. The engineered ligaments were treated with 500 pg/ml of estrogen for the last 24 or 48 hours of a 14-day culture, this mimicked the estrogen levels seen during ovulation (Lee et al., 2015). There was no significant difference seen in CSA, collagen content, collagen fraction by dry mass, or MTL of the ligaments (Figure 11A, B, C and D), however, following the 48-hour exposure to the high dose of estrogen there was a significant decrease (indicated by *) in UTS and modulus (Figure 11E and F). Despite there being no

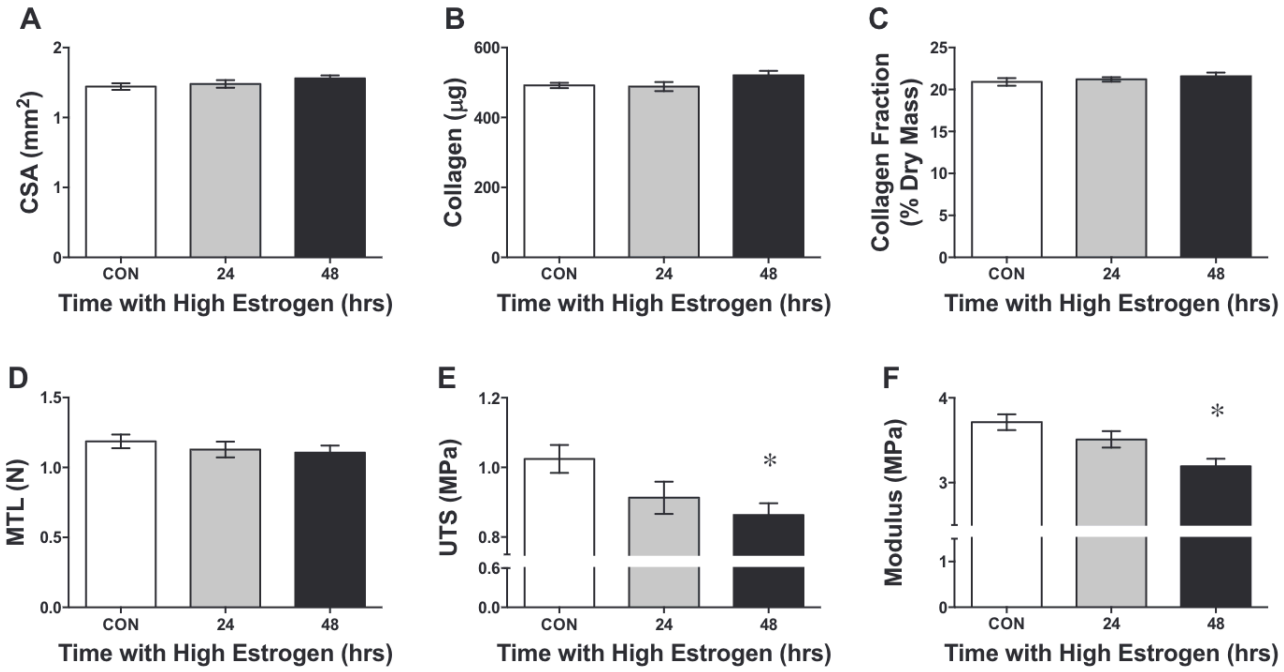

Figure 11. Transiently increasing estrogen levels in culture decreases the material properties but not the collagen content of engineered ligaments. Engineered ligaments were cultured in 5 pg/ml (low) estrogen for 14 days as a control while additional ligaments were subjected to an increase to 500 pg/ml (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. Taken from Lee et al., 2015.

change to collagen content and production, there was a significant decrease in the mechanical properties, tensile strength, and stiffness. To try and explain this, they treated the ligaments the same way but instead tested for LOX activity. Results showed significant decreases (indicated by * and †)

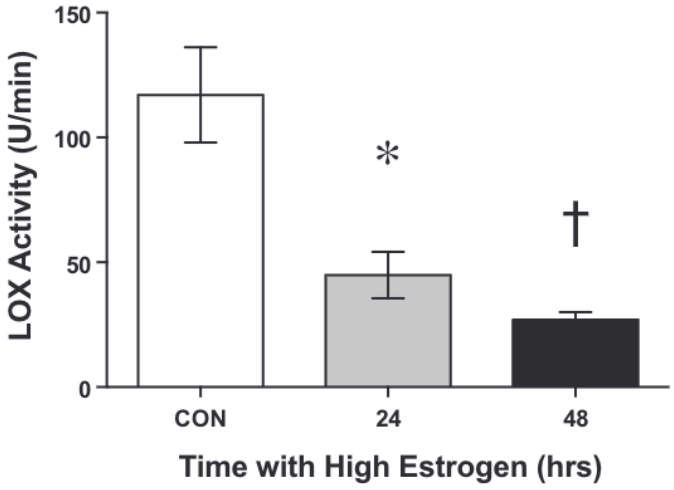

Figure 12. Lysyl oxidase (LOX) activity decreases in a time-dependent manner with exposure to high estrogen. Engineered ligaments were cultured in low (5 pg/ml) estrogen for 14 days as a control (CON) while additional ligaments were subjected to an increase to high (500 pg/ml) estrogen in the last 24 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). Taken from Lee et al., 2015.

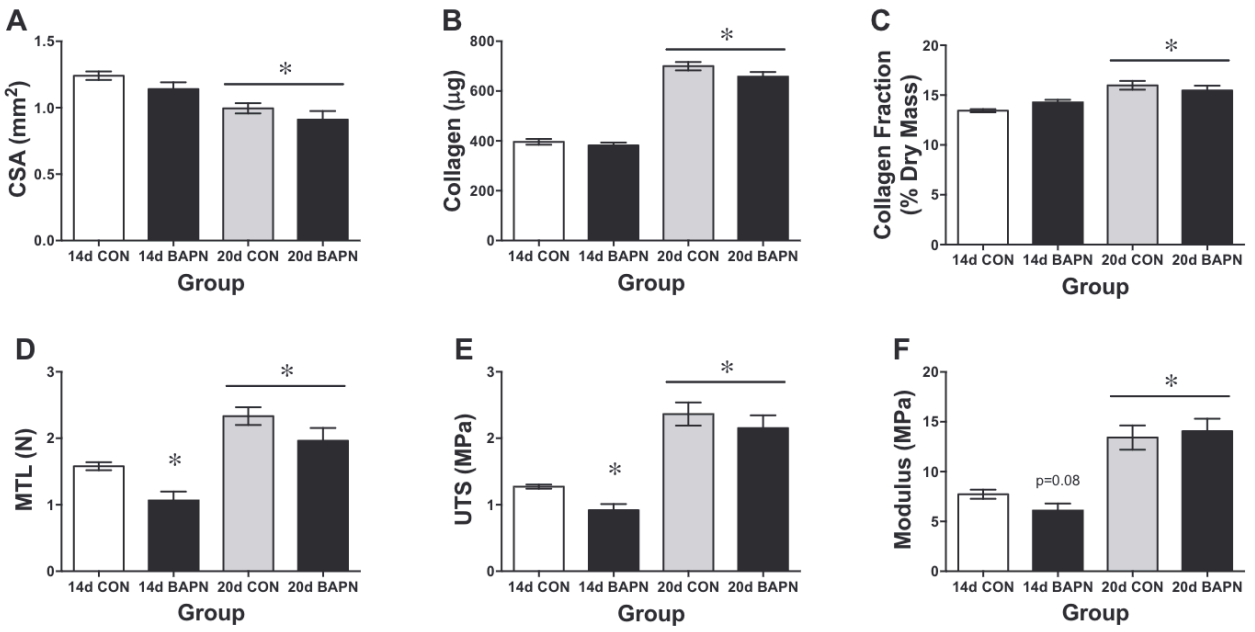

Figure 13. Inhibition of LOX using 1 mM of ß-aminoproprionitrile (BAPN) on the mechanical properties of engineered ligaments. Constructs were left untreated for 14 days (14d CON), treated for 24 h with 1 mM of BAPN (14d BAPN), left untreated for 20 days (20d CON), or treated with 1 mM BAPN for 24 h on 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. Taken from Lee et al., 2015.

in activity after 24-hours and 48-hours (Figure 12). LOX activity decreased by 62% after 24-hours and 77% after 48-hours of high estrogen exposure (Lee et al., 2015). This implies that high estrogen levels may significantly affect the enzyme, altering its cross-linking capability of collagen. To determine if the decrease in mechanical properties seen at high estrogen exposure was a result of inhibition of the LOX enzyme, the ligaments were treated with the LOX inhibitor ß-Aminoproprionitrile (BAPN) for the last 24 hours of a 14-day culture (Lee et al., 2015). Results of the BAPN treatment showed no effect on CSA, collagen content, or collagen fraction, but showed differences in all the mechanical properties of the ligaments, significantly decreasing (indicated by *) MTL and UTS (Figure 13A, B, C, D, E, and F).

The results from this study further support the idea that estrogen has an impact on female ACL injuries. At high dosages of estrogen, there were significant decreases in LOX activity and.

as mentioned earlier, LOX is important in the cross-linking of collagen and the density of those cross-links can affect ligament stiffness. Estrogen's inhibition of the enzyme at high levels explains the seen increase in knee laxity during the ovulatory phase of the menstrual cycle and thus the increase of ACL injury occurrence at that time.

Conclusion

Estrogen plays a critical role in the widespread presence of ACL injuries in female athletes. At high levels, the hormone inhibits the LOX enzyme, effecting the density of collagen crosslinks and therefore decreasing the density of those cross-links. This then affects the stiffness of the ligament, increasing laxity, resulting in a ligament that is more susceptible to injury because it is unable to prevent hyperextension. Estrogen's ability to inhibit LOX only occurs when levels of the hormone are high, thus why laxity and therefore most ACL injuries are sustained during ovulation. The structure of the female endocrine system is the reason the incidence rate for ACL injuries in females is 2- to 6- times higher than it is in males. Unlike females, males do not experience fluctuations in their hormones and maintain fairly stable levels at all times, hence a reduced risk for ACL injury.

Injuries to the ACL are detrimental to athletes, as these injuries often impact athletic involvement, both recreationally and competitively, and mental health (Voskanian, 2013). An ACL injury is generally season ending and requires surgical repair. Recovery entails extensive physical therapy for 6-9 months post-operation, during which the athlete is unable to compete (Voskanian, 2013). The recovery process can only begin after the injury is repaired, so after diagnosis, consultations, surgery, and physical therapy, the athlete is looking at a year of time away from competition. Unfortunately, this is not always enough. In some cases, the athlete is unable to return to their previous skill set and therefore returns to play with diminished abilities (Voskanian, 2013). For athletes whose identity is intertwined with competition and the hard work of countless years of honing their skills, scenarios such as this are devasting. The mental health of these athletes is a concern, as resentment and depression can develop from their inability to compete as they once did. For females, it is hard for them to not blame their own

body because it is the fault of their own biology that causes them to be predisposed to injuries such as one to the ACL. Therefore, it is important that females do what they can to supplement their already predisposed bodies.

In recent studies, researchers have discovered that oral contraceptives are an effective method that diminishes the risk for ACL injuries in females (Chidi-Ogbulo and Baar, 2019). This makes sense when thinking about the role of oral contraceptives and the reason for increased estrogen levels. Because the body is not preparing for pregnancy when a female takes oral contraceptives, there are no fluctuations in hormones; moreover, there are no high levels of estrogen (Chidi-Ogbulo and Baar, 2019). Despite this being a viable way to cheat the body into diminishing one of its predisposed effects on the female ACL, it is not sustainable or ethical. Situations could arise where a female athlete is pressured into taking oral contraceptives because she "cannot" run the risk of injuring her ACL because she has been made to think the team will fall apart without her on the field. No matter her reasoning, a female athlete should not be made to feel her only option is to take a medication to fit in or appease a coach. Despite oral contraceptives' ability to combat estrogens effects, they are not always an option and thus other prevention strategies must be taken to reduce the risk of ACL injuries in females. Luckily enough, estrogen is not the only predisposing factor for ACL injuries in females, but unlike estrogen, the other factors do not require drugs to reduce the effects.

One of these other factors that predisposes females to ACL injury is that they tend to be quadricep dominant. This means that in the upper region of the leg, the quad, is preferred in muscle recruitment and thus is stronger than the other muscles seen in the thigh (Voskanian, 2013). Unfortunately for females, the hamstring is the better equipped muscle for preventing ACL injuries as it is the main knee flexor because it combats hyperextension (Voskanian, 2013).

However, preventative training can be done that improves the hamstrings in females and thus reduces predisposition to ACL injury. The strength and recruitment, specifically of the hamstring, are important factors in knee stability, so programs that are focused on strengthening and improving recruitment patterns in the muscle could dramatically reduce ACL injury risk in females (Voskanian, 2013).

It is important to note that it is not only up to the athletes to know their own predisposing factors, as they are only one piece of the puzzle, but also up to the coaches, trainers, and parents to know that the female biology leaves them more at risk of sustaining certain injuries, like ones to the ACL. Through my own experiences in athletics, I have noticed that athletes are often at the mercy of others and not just themselves; because of this, it is important that the people in authoritative positions know the effects the female biology has on its athletes. In my opinion, the athletic world is still heavily based off standards set by males, without taking into account the biological sex differences between the sexes. For example, I have been a part of several strength programs that train males and females the same way despite differences in muscle dominance. Often, the lack of knowledge concerning the differences in the sexes and their effects on athletic performance is out of naivety rather than ignorance. However, this is not an excuse, it is time that these differences are considered that way the prevalence of predisposed injuries in females is reduced. Little can be done regarding estrogen's effects, but being aware of its impacts on the ACL encourages the importance of implementing preventative methods on factors that can be improved upon. If preventative care methods were begun from the time the athlete starts menstruation to when she is done competing, ACL injuries in females would decrease dramatically. With that said, implementation at any level is sure to help females. In the end, estrogen is going to play its role but being aware and improving what can be improved upon is

sure to help athletes tremendously. It is not the fault of the female athlete that her biology effects certain aspects that make her predisposed to certain injuries, but there is fault in the system that fails her by not being aware of her predisposing factors and not helping her reduce her risk to those injuries.

Acknowledgements

I would not have been able to complete my thesis without the help and encouragement of my professors, friends, and family— you all helped me see this paper through when often times I didn't. To my thesis advisor, Dr. Wanat, I'd like to express my sincere gratitude. Through this entire process, you read my every draft, answered my every question, and encouraged me on the weeks I became overtaken by this paper. A special thank you goes to Dr. Krochmal—I was able to write a thesis I was passionate about because of you and your introduction of the topic to me. Additionally, you never hesitated to help me, even though it was not your responsibility to and for that I am appreciative. With that, I would like to extend my appreciation to the entire Washington College Biology Department for not only preparing me to write a thesis but also for all my future endeavors, academically and personally. You all taught me lessons that were beyond science, you taught me about hard work and determination, and it is because of you all I am receiving a degree I am both passionate about but also proud of. This major requires a lot of hard work and determination, and despite that, you all never managed to diminish my love for science when that would've been so easy to do. You all are a tough group, each with your own challenging curriculum that is sure to send most running, but because of who you all are, you managed to keep some of us past Gen Bio. It would be foolish of me not to mention the group that received the other half of my time while at WAC: my friends and teammates. On and off the field you were by my side, and you were responsible for making college what it was. To my best friend, you know who you are, I owe you everything. Your love and support have never wavered, and I couldn't have done the past few years without you. Finally, to my family, without you I would not be where I am today. There aren't enough words to express my gratitude for the life you have provided for me, but I hope I can continue to make you all proud.

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