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The Effects of Hormonal Manipulation on Ligament Function

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The Effects of Hormonal Manipulation on Ligament Function

By

NKECHINYERE CHIDI-OGBOLU
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

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of the

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DAVIS

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2022

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DEDICATION

I'd like to dedicate this dissertation to **Koinonia** without whose prayers, support, and encouragement, I would have never made it through the years of research and months of writing. My Koinonia is large and extends to my parents, siblings, and friends. I wouldn't be here without you all. God bless you!

Koinonia

koi·no·nia | \ ˌkȯinəˈnēə, ˌkēnə-\

Definition of *koinonia*

1: the Christian fellowship or body of believers

2: intimate spiritual communion and participative sharing in a common religious commitment and spiritual community

ACKNOWLEDGEMENTS

Where to begin but at the beginning: I'd like to give the biggest thank you to my advisor, Dr. Keith Baar. If he wasn't so great, I probably would have walked right on out of this Ph.D. a long time ago. My blood, sweat, and buckets of tears (in a totally sterile way) went into this dissertation, and his brilliance, mentorship, and guidance breathed life into it and made it all make sense.

Next, I would like to thank the rest of the committee dream team without whose guidance and encouragement this dissertation would not see the light of day: Dr. Blaine Christiansen, Dr. Cassandra Lee, Dr. Michael Mienaltowski, and Dr. Alyssa Panitch!

Coming right up next is every single member of the Baar lab who made coming into the lab worth it. You all have really "raised the Baar" for my expectations of future colleagues and I really appreciate your friendship throughout the years.

I would like to thank my family who did not once hesitate to drop everything to lend their support in the best way they could including flying over to take care of me when I was on the edge of sanity.

My friends. I don't know how to even begin to describe how much you all mean to me. I am just so glad we had each other to go through this journey with and couldn't ask for better rant partners.

Lastly (but certainly not the least), it wouldn't be an Nkechi acknowledgment section if I didn't thank Kim Namjoon, Kim Seokjin, Min Yoongi, Jung Hoseok, Park Jimin, Kim Taehyung, and Jeon Jungkook, for being my Magic Shop. ARMY Forever, Bangtan Forever!

THE EFFECTS OF HORMONAL MANIPULATION ON LIGAMENT FUNCTION

ABSTRACT

Estrogen has a dramatic effect on musculoskeletal function. In the past, much of the research focus has been on the strong connection between estrogen and bone. However, recently the effect of estrogen on other musculoskeletal tissues such as muscle, tendon, and ligament has become the focus of more research. These studies make it clear that estrogen improves muscle proteostasis and increases bone density; however, the benefits to bone and muscle come at the cost of decreased connective tissue stiffness. Evolutionarily, this makes sense since laxer joints and better repair following injury would facilitate healthy childbirth and recovery. However, as more women participate in sports, these physiological effects of estrogen contribute to decreases in power and performance and make women more prone to catastrophic ligament injury. To promote female participation in an active lifestyle throughout their life span, more research is needed to determine how nutrition, training, and hormonal manipulation can be used to promote optimal performance at any age. Another hormone that affects sinew is Testosterone (T). In rats, supplementation with T results in stiffer tendons that absorb less energy before failure. In humans, the ratio of E2 to T is negatively correlated with ACL stiffness, suggesting that E2 overrides the effects of T in female ligaments. Further, women treated with aromatase inhibitors, that prevent the conversion of T to E2 commonly report musculoskeletal pain and experience tendinopathies, suggesting that the interplay between T and E2 is essential to sinew function. **The overall goal of my dissertation is to define the effects of hormonal manipulation on sinew function, with a specific focus on collagen synthesis, incorporation, and degradation using a 3-D engineered human ligament model.**

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CHAPTER 1: THE EFFECT OF ESTROGEN MANIPULATION ON TENDON AND LIGAMENT FUNCTION AND INJURY RISK

Abstract

There is a sex difference in the incidence of connective tissue injury. As estrogen (E2) and androgen receptors have been found on tendon/ligament cells, the sex difference may be due in part to the different post-pubertal sex hormone profiles in men versus women. E2 is known to have a dramatic effect on musculoskeletal function. In bone, the ability of E2 to improve tissue mass and function and slow the progression of osteoporosis is widely appreciated. E2 also improves muscle mass and strength, decreases injury, and improves recovery. However, unlike bone and muscle where estrogen improves function, in tendons and ligaments, E2 decreases stiffness and directly impacts performance and injury rates. The mechanism through which E2 influences tendon/ligament function is poorly understood and the field is filled with conflicting data. The effect of the androgen testosterone (T) on female ligaments is similarly poorly understood. While T is associated with higher connective tissue stiffness and higher collagen content, E2 seems to override that function in women despite T increasing with ovulation in the menstrual cycle. The goal of this project is to provide a better understanding of the effects of sex hormones on tendons and ligaments -- particularly their effects on collagen synthesis versus incorporation and how nutrition and pharmaceuticals could alter those effects.

Introduction

Estrogen has a dramatic effect on musculoskeletal function. Beyond its role as a sex hormone, estrogen has important roles in the development, maturation, and aging of extragonadal tissues such as bone (Cui et al., 2013; Hansen, Miller, et al., 2008; Ling-Ling et al., 2016), muscle (Dieli-Conwright et al., 2010; Enns & Tiidus, 2010) and connective tissues (Hansen, 2018; Hansen et al., 2009a; Hansen, Miller, et al., 2008; Hansen & Kjaer, 2014).

In young women, estrogen is produced from cholesterol in a series of reactions within the ovaries. The final reaction in the process is the conversion of testosterone to estradiol by the enzyme aromatase. In men and postmenopausal women, this reaction commonly occurs in adipose tissue which is high in aromatase activity (Nelson & Bulun, 2001). The most prevalent estrogen is 17β-estradiol with smaller amounts of estrone and estriol circulating as well (Heldring et al., 2007). As a steroidal hormone, estrogen can freely pass through the plasma membrane and move into the nucleus where it can bind to its nuclear receptors, the estrogen receptors (ER)α and β, also known as *ESR1* and 2 respectively, and modify gene expression (Heldring et al., 2007). Beyond the nucleus, estrogen has a variety of post-transcriptional effects such as regulating the redox state of the cell (Kumar et al., 2010), altering mitochondrial function (Yao & Brinton, 2012), and directly inhibiting the activity of specific enzymes (C. A. Lee et al., 2015a).

Estrogen secretion naturally varies in young women, increasing 10- to 100-fold over the menstrual cycle. Beyond E2, the menstrual cycle is characterized by significant changes in other important plasma hormones such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), and progesterone (Figure 1.1). 17β-estradiol levels rise from 5 pg/mL at the early follicular phase, to a peak of 200-500 pg/mL just before ovulation. Ovulation is followed by a rapid decrease in estradiol, then estradiol and progesterone both increase in the luteal phase giving a broad secondary peak. To prevent pregnancy, or simply to regulate hormone levels, many women take oral contraceptives that provide a daily low level of estrogen and progesterone. These pills typically maintain estradiol levels at approximately 25pg/mL and decrease the ovulatory rise in estrogen (Mishell et al., 1972). This daily dose of estrogen and or progesterone also eliminates the cyclic rise in LH and FSH (Figure 1.1B). In the absence of oral contraceptives or a severe negative energy balance, the menstrual cycle will occur from puberty until menopause when menses stop, FSH and LH rise, and plasma estradiol and progesterone concentrations remain constantly low (Figure 1.1C).
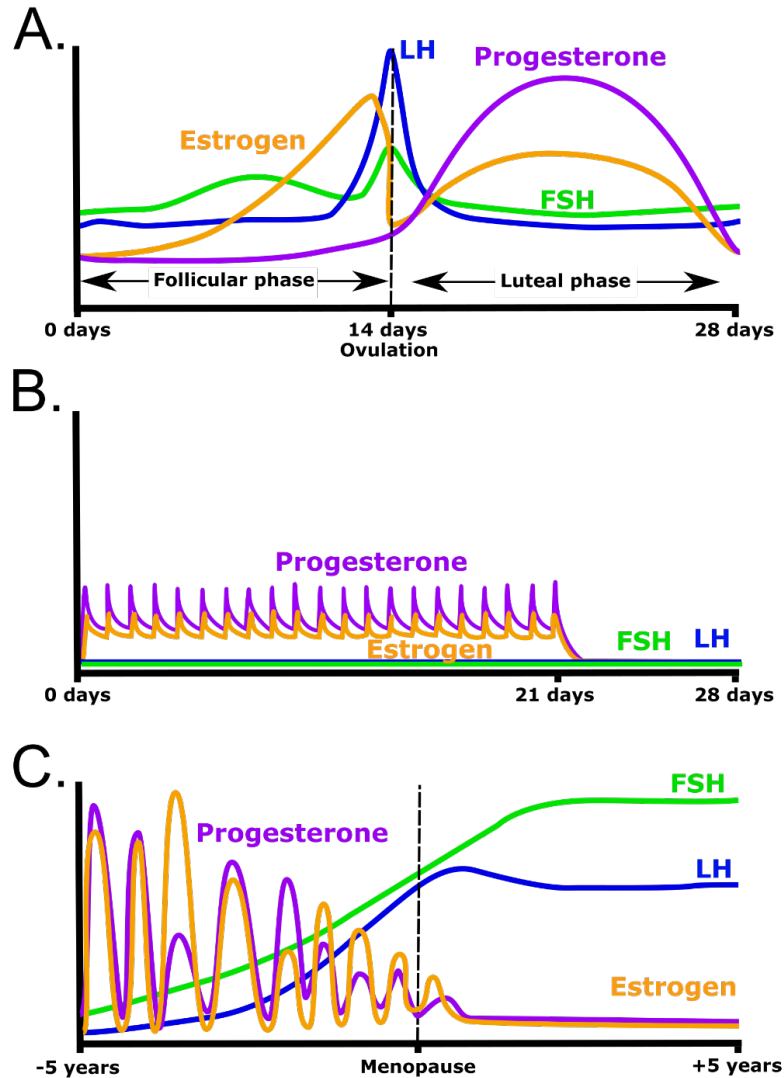
Figure 1.1. *Hormonal fluctuation during (A) a normal menstrual cycle, (B) while taking an oral contraceptive (OC) containing both estrogen and progesterone and (C) in the years before and after menopause.*

Estrogen receptors are present in all musculoskeletal tissues including muscle (Barros & Gustafsson, 2011; Luo & Kim, 2016), bone (Cui et al., 2013), ligament (Liu et al., 1996), and tendon (Bridgeman et al., 2010). Within these tissues, estrogen is known to regulate metabolism (Nelson & Bulun, 2001); however, it is still unclear whether these effects are beneficial or harmful. Consistent with a role

for estrogen in regulating musculoskeletal function, menstruating women suffer more ACL ruptures than men (Shultz et al., 2005, 2011), and menopause is characterized by an increased risk of musculoskeletal injury (Enns & Tiidus, 2010), accelerated bone and muscle wasting (Bassey et al., 1992; Frontera et al., 1991; Häkkinen & Pakarinen, 1993; Rice et al., 1989), and decreased sensitivity to anabolic stimuli (Bamman et al., 2011; PJ et al., 2003). To counteract many of the negative aspects of menopause, hormone replacement therapy (HRT) has been used to reduce muscle and bone loss and restore muscle protein balance (Hansen et al., 2012; Smith et al., 2014).

Given the sex differences in musculoskeletal injury risk and the growing number of active young women, the role of estrogen in musculoskeletal function is a burgeoning area of research. Here, I will review important developments, controversies, and unknowns in the relationship between estrogen and musculoskeletal function, with a specific focus on tendon and ligaments.

Sinew: Tendon and Ligament

Within the musculoskeletal system, tendons and ligaments (I will refer to these issues collectively as sinew in this dissertation) function as connective tissues between bone and muscle and between bone and bone, respectively. In both tissues, most of the dry weight is collagen: 60-85% for the tendon (Kjaer, 2004) and ~75% for ligament (Frank, 2004). Of this collagen, the majority is type I: 60% in tendon and up to 85% in a ligament. The mechanical properties of both tendon and ligament are dependent on collagen fiber density, diameter, orientation, and crosslinking. The fibers can be cross-linked in two ways: enzymatically and non-enzymatically. Enzymatic crosslinks are mediated largely by lysyl oxidase (*LOX1*) (Siegel+ & Fu, 1976; Siegel, 1976). Crosslinks can also be formed without a specific enzyme through a Maillard reaction between a sugar and an amino acid. These crosslinks are called advanced glycation end-products (AGE), and as would be expected are higher in diabetics with poorly controlled blood glucose levels (Dyer et al., 1993). Both enzymatic cross-linking, through *LOX1*, and non-enzymatic cross-linking

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through AGEs increase the stiffness of collagen-rich tissues (Marturano et al., 2014b; Reddy et al., 2002; Svensson et al., 2013). Two main differences between enzymatic and non-enzymatic cross-links are their locations and turnover rates. *LOX1* crosslinks lysines at the head and tail of collagen molecules while AGEs crosslink cysteine or tryptophan at any location within the collagen molecule (Münch et al., 1999; Reiser et al., 1992). AGEs have also been shown to decrease collagen turnover which impairs sinew function over time (Corman et al., 1998; Hammes et al., 1991).

Since a ligament, such as the ACL within the knee, shows a direct relationship between laxity and rupture (Myer et al., 2008), a stiffer ligament is preferred to maintain joint stability and prevent injuries. Due to its role in connecting a compliant muscle to a stiff bone, a stiffer tendon is not always beneficial. In terms of performance, a stiff tendon transmits the force produced by a muscle to the bone faster and this can improve speed and power. However, when a tendon becomes too stiff this produces a strain concentration in muscle. What this means is that more of the strain (stretch) produced in each movement is concentrated in the muscle that is connected to a stiff tendon than a muscle attached to a compliant tendon. In other words, instead of the tendon stretching while the muscle contracts isometrically (Griffiths, 1991), a stiff tendon does not stretch, and the muscle is forced to lengthen while contracting. The result is that a muscle attached to a stiff tendon will experience a more eccentric load for a given movement. Since eccentric movements produce more muscle injury than concentric or isometric movements (Brockett et al., 2004; Clarkson & Hubal, 2002; Griffiths, 1991; LaStayo et al., 2003) this means that muscles attached to stiff tendons will suffer more injury for a given movement than those attached to compliant tendons. Therefore, stiff ligaments are always better, stiff tendons can improve performance, but if the tendon becomes too stiff the associated muscle will suffer more injuries.

Interestingly, women suffer fewer muscle injuries and more ligament ruptures than men (Arendt & Dick, 1995; Edouard et al., 2016; Hägglund et al., 2009; Leblanc et al., 2017; Sewright et al., 2008). These observations are consistent with lower sinew stiffness in women than men. Since knee laxity changes with

estrogen levels through the menstrual cycle (Shultz et al., 2005), estrogen is believed to decrease sinew stiffness. Therefore, in the sections below, we will address how estrogen affects sinew mechanics and adaptation to loading.

Estrogen and Ligament

One of the best characterized musculoskeletal differences between men and women is the rupture rate of the anterior cruciate ligament (ACL). ACL ruptures occur 2-8 times more often among female athletes than their male counterparts (Adachi et al., 2008; Arendt & Dick, 1995). Given that there is a correlation between ACL injuries and knee laxity, (Myer et al., 2008; Ramesh et al., 2005) and an association between knee laxity and the menstrual cycle (Deie et al., 2002; Shultz et al., 2005, 2010, 2011; Shultz, Schmitz, et al., 2012; Shultz, Wideman, et al., 2012) several groups have investigated the relationship between ACL injuries and phase of the menstrual cycle (Adachi et al., 2008; Carcia et al., 2004; Heitz et al., 1999; Herzberg et al., 2017; H. Lee et al., 2013; Lefevre et al., 2013; Wojtys et al., 1998). The resulting studies in general find a higher risk of ACL injury during the pre-ovulatory and ovulatory phases than post-ovulatory or follicular phases of the menstrual cycle (Beynnon et al., 2006; Lefevre et al., 2013; Ruedl et al., 2009). For example, Wojtys *et al.* (Wojtys et al., 1998, 2002) found higher risk (Wojtys et al., 1998) and occurrence of ACL injury in the ovulatory phase (Wojtys et al., 2002) where E2 and LH are high and progesterone is low (Figure 1.2). To attempt to explain the increased ACL rupture in the pre-ovulatory phases, researchers have measured knee laxity throughout the cycle. In men and women with no history of knee injury, the men showed no statistical difference in knee laxity over time; however, in women laxity increased from 4.7±0.8 mm in the follicular phase, to 5.3±0.7 mm in the ovulatory phase (Deie et al., 2002). These authors concluded that knee laxity is dependent on the cyclic variation of female hormones (Deie et al., 2002). Similarly, Shultz *et al.* (Shultz et al., 2005) found that knee laxity increased in direct relation to elevations in plasma estradiol levels. The variations in laxity were also found to be cyclic in nature. When estrogen concentration increased during the menstrual cycle, knee laxity increased as well (Shultz

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et al., 2010, 2011; Shultz, Schmitz, et al., 2012). These authors found that knee laxity increased between 1 and 5 mm from the first day of menstruation to the day following ovulation, depending on estrogen levels. Lastly, Park and colleagues found a 17% decrease in knee stiffness during the ovulatory phase resulting in a change in knee laxity from 13.35 +/- 2.53 mm during the follicular phase to 14.43 +/- 2.60 mm during ovulation (Park et al., 2009) By contrast, Carcia *et al.* (Carcia et al., 2004) found no change in knee displacement in relation to cycle; however, it is important to note that these authors used self-reported cycle length to estimate menstrual phase, whereas the other studies directly measured estrogen levels in concert with knee laxity. Since Myer *et al.* (Myer et al., 2008) showed that for every 1.3 mm increase in knee displacement, the risk of ACL injury goes up 4-fold, the rise in knee laxity reported by Deie, Park, and Shultz could explain the 2- to 8-fold higher rate of ACL rupture in women (Adachi et al., 2008; Arendt & Dick, 1995).
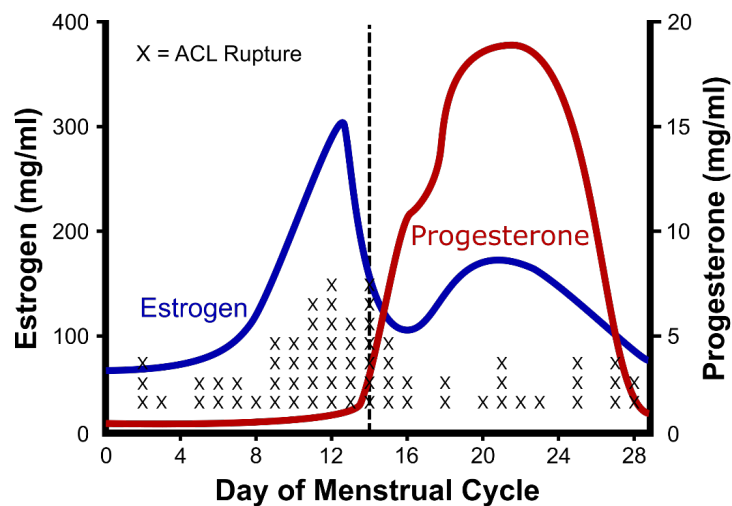

Figure 1.2. Relationship between estrogen and ACL rupture in a normal cycle. *The rate of anterior cruciate ligament (ACL) rupture in relation to female hormones throughout a standard menstrual cycle. Note that with the ovulatory rise in estrogen there is a concomitant rise in ACL ruptures. Adapted from (*Wojtys et al., 2002)*.

Since knee laxity changes with cycle phase, many active women want to know whether OCs could prevent the change in laxity and lower injury risk. In support of this idea, Gray *et al.* (Gray et al., 2016) found that young women (aged 15-19) who undergo surgical repair of the ACL are 18% less likely to have used oral contraceptives than matched controls (Gray et al., 2016). Further, Rahr-Wagner *et al.* (Rahr-Wagner et al., 2014) found a higher relative risk (RR) value of ACL injury in women who had never used OCs than in women who were long-term users (Rahr-Wagner et al., 2014). Together, these data suggest that ACL laxity changes through the cycle and eliminating the regular changes in estrogen (and other hormones) using oral contraceptives decreases the risk of ACL rupture likely by eliminating the periodic change in laxity.

With evidence pointing to hormonal fluctuations of the menstrual cycle influencing ligament injury risk, the question of how sex hormones, and estrogen, in particular, increase the risk of injury has been a focus of study for more than 20 years. In cell culture, Yu *et al.* (Yu et al., 1999) found an early decrease in proliferation and procollagen synthesis in freshly isolated ACL cells that becomes less apparent with time in culture (Yu et al., 1999). In contrast, Chen *et al.* (M. H. Chen et al., 2014) found a dose-dependent increase in proliferation of cells from the *ligamentum flavum* that lasted only 24 hours in culture. (M. H. Chen et al., 2014). Although the expression of collagen mRNA didn't change significantly, there was a decrease in the ratio of collagen to elastin at the protein level after the cells were treated with 17β-estradiol. The authors attributed this shift in protein to the upregulation of matrix metalloproteinase 13 (*MMP13*) which degrades collagen but not elastin (M. H. Chen et al., 2014). This suggests that estrogen could decrease collagen protein by increasing its breakdown and in the case of lumbar stenosis, prevent hypertrophy of the ligamentum flavum and reduce the risk of the disease (M. H. Chen et al., 2014). However, the effect of estrogen on collagen synthesis in ligaments has yielded conflicting results in other systems. Some studies suggest that estradiol has a negative effect on collagen synthesis (Hama et al., 1976; Liu et al., 1997), whereas others saw positive effects (C. A. Lee et al., 2015b;

C.-Y. Lee, Liu, et al., 2004; C.-Y. Lee, Smith, et al., 2004) and still others saw no effect (Mamalis et al., 2011; Seneviratne et al., 2004). Hama *et al.* (Hama et al., 1976) found decreased collagen content in the capsular ligament with estrogen administration in ovariectomized rats (Hama et al., 1976); however, Lee *et al.* (C.-Y. Lee, Liu, et al., 2004; C.-Y. Lee, Smith, et al., 2004) found increased collagen synthesis (C.-Y. Lee, Smith, et al., 2004) with a corresponding increase in Type I collagen mRNA (C.-Y. Lee, Liu, et al., 2004). Liu *et al.* (Liu et al., 1997) also found decreased Type I collagen synthesis at physiological estradiol levels in monolayer culture of fibroblasts derived from rabbit ACL (Liu et al., 1997). By contrast, in 3D ligaments engineered from human ACL cells, high estrogen results in increased collagen accumulation within the grafts (C. A. Lee et al., 2015b). Despite conflicting results on fibroblast proliferation and collagen synthesis, there is a consensus that the mechanical strength of the tissue decreases. In the human engineered ligaments, despite increased collagen content, the material properties of the tissue (UTS and modulus) decreased due to the inhibition of lysyl oxidase activity by estrogen (C. A. Lee et al., 2015b). In these experiments, treating engineered ligaments with physiologically high estrogen for 48 hours resulted in an 80% decrease in lysyl oxidase activity without changing *LOX1* expression (Figure 1.3). These data suggest that estrogen increases collagen accretion but decreases sinew stiffness by directly inhibiting lysyl oxidase and decreasing crosslinking.
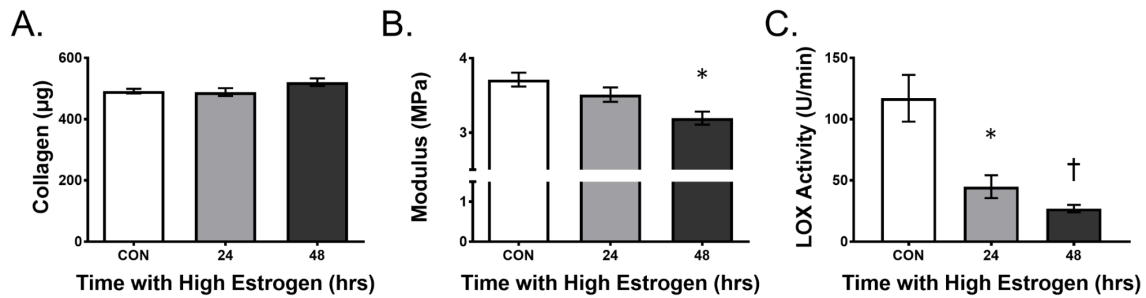
Figure 1.3. High estrogen decreases engineered ligament stiffness due to inhibition of lysyl oxidase. *(A) Collagen content, (B) tangent modulus, and (C) lysyl oxidase (LOX1) activity in ligaments engineered from human ACL cells isolated from women following 24 or 48 hours of treatment with physiologically high (500 pg/mL) of estrogen. Note that even though there is a slight rise in collagen, the stiffness of the grafts decreases concomitant with an increase in estrogen in the media. Adapted from* (C. A. Lee et al., 2015b)

Estrogen and Tendon

If estrogen decreases lysyl oxidase activity in sinews, this would be expected to decrease tendon stiffness and therefore decrease the incidence of injury to the associated muscles. As mentioned above women suffer fewer muscle injuries than men (Edouard et al., 2016; Hägglund et al., 2009) In professional soccer, women suffer 54% fewer muscle strains than their male counterparts (Hägglund et al., 2009). Most of the benefit results from decreases in groin (83% fewer) and hamstring (36% fewer) pulls. A decrease in tendon stiffness could also leave the tendon less prone to injury. In fact, women are at a lower risk of sustaining an Achilles' tendon rupture than men until menopause, after which the risk becomes similar in both sexes (Hansen & Kjaer, 2014, 2016). The use of OCs (which maintain moderate estrogen levels) has been linked with an increased risk of Achilles tendinopathy (Holmes & Lin, 2006), indicating that the periodic rise in estrogen to physiologically high levels may be needed to decrease Achilles' injury. Similarly, OCs have been linked with greater muscle damage and delayed onset muscle soreness after exercise (H. Lee et al., 2015; Minahan et al., 2015; Savage & Clarkson, 2002). As discussed above, an increase in muscle

damage is consistent with an increase in tendon stiffness, which decreases the shielding of the muscle from strain injury. Therefore, periodic rises in estrogen levels are necessary for the protective effect on tendon and muscle health.

There have been several elegant studies performed in women that have tried to establish the mechanism underlying the effect of estrogen on tendon health. Many of these studies have focused on collagen synthesis and the interactions between estrogen and exercise. Interestingly, the studies have contrasting results depending on age - premenopausal women compared to postmenopausal women and measurement modality - even when they come from the same research group. In premenopausal women, holding estrogen levels constant with oral contraceptives resulted in decreased exercise stimulated collagen synthesis (Hansen, Koskinen, et al., 2008; Hansen, Miller, et al., 2008; Kjær et al., 2009; C.-Y. Lee, Smith, et al., 2004; Miller, Hansen, Olesen, Flyvbjerg, et al., 2006; Peter Magnusson et al., 2007; Westh et al., 2008). In the first of these studies, a group taking oral contraceptives containing moderate estradiol was compared to non-OC users in the follicular phase, when estrogen levels are naturally low, both at rest and following 1 hour of kicking exercise. Patellar tendon collagen synthesis, measured 24 hours after exercise using microdialysis to capture the N-terminal peptide of procollagen I (PINP), was not significantly different at rest; however, following exercise, the women taking OC showed no change in collagen synthesis whereas the control women doubled PINP production (Hansen, Koskinen, et al., 2008). The same group repeated the study using stable isotope labeled proline and patellar tendon biopsies to detect the incorporation of newly synthesized collagen into the tendon (Hansen, Miller, et al., 2008). In contrast to the microdialysis experiment, OC use decreased resting collagen synthesis and neither group saw an increase in collagen incorporation into the patellar tendon after exercise (Hansen, Miller, et al., 2008). This contrasts with men where the same 1-hour kicking exercise increased new collagen incorporation 70% by 24 hours (Miller et al., 2005). Consistent with the stable isotope data from Hansen (Hansen, Miller, et al., 2008), when the same group compared the data in men to an equivalent cohort of women, tendon

collagen synthesis was 46% lower in the women at rest and was unaffected by exercise (Miller, Hansen, Olesen, Schwarz, et al., 2006). Together, these data suggest that in young active women, the incorporation of new collagen into the patellar tendon is lower and does not increase following exercise. It is important to note that this does not mean that women are synthesizing less collagen. The PINP data suggests that women synthesize more collagen in response to exercise; however, this collagen may not be incorporated into the tendon to the same degree in women. In support of this hypothesis, Laurent (Laurent, 1987) showed in muscle that 49% of newly produced collagen is degraded rapidly before it is incorporated. This raises the possibility that estrogen differentially regulates the synthesis, degradation, and/or incorporation of collagen into the matrix of the sinew.

In premenopausal women, a consistent moderate level of estrogen from OC decreases collagen synthesis; however, in postmenopausal women, hormone replacement therapy (HRT), which provides a daily moderate rise in estrogen, is linked with increased tendon collagen synthesis (Hansen et al., 2009a). In postmenopausal women, collagen incorporation into the patellar tendon was 47% higher in HRT users compared with control (Hansen et al., 2009a). Even though HRT boosted collagen incorporation at rest, exercise did not increase collagen incorporation further (Finni et al., 2009; Hansen et al., 2009a). Interestingly though, using PINP to measure collagen synthesis within the peritendinous space at the same time, Hansen and her colleagues saw more collagen synthesis in the tendons of the control women than the HRT users (Figure 1.4), again suggesting that estrogen affects collagen incorporation into tendons differently than collagen synthesis itself. As to which measure is the best indicator of long-term tendon structure/function, in a monozygotic twin study, the twins on HRT had a smaller Achilles tendon CSA when compared to the twins who did not take HRT (Finni et al., 2009) and a similar decrease in Achilles CSA was found in a separate study in a larger group of active women taking HRT (Cook et al., 2007). Together, these data suggest that the decrease in PINP in the microdialysate of a tendon may better represent the long-term changes in tendon structure/function than the increased short-term incorporation of stable isotopes.

One reason that the PINP measure may better reflect long-term changes is that the current stable isotope techniques are highly dependent on the delivery of the isotope to the tissue in a limited amount of time. Since tendon is relatively avascular, this may not be the best way to measure tendon turnover. However, with the ability to measure deuterated water incorporation into tissues over a much longer time frame, new isotope techniques could vastly improve our understanding of the dynamics of these tissues.
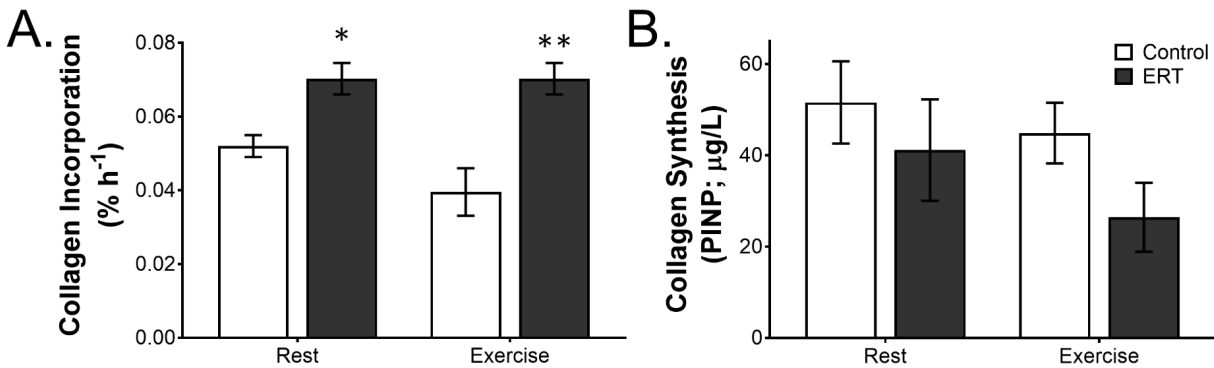
Figure 1.4. Differential measures of collagen incorporation and synthesis with estrogen replacement and exercise. *The rate of (A) collagen incorporation of proline into the patellar tendon or (B) the appearance of the N-terminal propeptide of collagen I in post-menopausal women +/- estrogen replacement therapy (ERT) and exercise. Note that with ERT collagen incorporation is higher in the same women where collagen synthesis is repressed. Further, exercise tends to decrease collagen incorporation and synthesis in controls, whereas ERT users show no effect on incorporation or a large drop in collagen synthesis. These data suggest that there is a large methodological discrepancy between the two measures. Adapted from* (Hansen et al., 2009a). ** indicates significantly different than control p < 0.05 while ** indicates p < 0.005*

To gain a better mechanistic understanding of how estrogen can increase collagen content while decreasing tendon mechanics in young women, researchers have turned to animal and cell culture models. Ovariectomized rats see a 28% decrease in collagen content in Achilles' tendon when compared with

intact mice (Ramos et al., 2012). When the rats are treated with genistein, a soy phytoestrogen, collagen content within the Achilles is returned to intact levels (Ramos et al., 2012). Interestingly, unlike native estrogen that decreases tendon stiffness, genistein showed no negative effect on the mechanical properties of the Achilles (Ramos et al., 2012), suggesting that phytoestrogens produce the increase in collagen without the negative effect on stiffness. In 2D cultured Achilles' tendon cells, Irie *et al.* (Irie et al., 2010) found that estrogen or a selective estrogen receptor modulator (SERM) increases the expression of *MMP13*, suggesting that estrogen could increase the rate of collagen turnover. As mentioned above, in our engineered sinew model that allows us to determine both collagen content and mechanics, collagen content increased significantly with increasing estrogen in the media; however, as with *in vivo* sinew the tissue stiffness decreased (C. A. Lee et al., 2015b). We have yet to determine whether the increase in collagen content was the result of a change in collagen synthesis, degradation, or incorporation (C. A. Lee et al., 2015b); however, the decrease in stiffness correlated with a decrease in *LOX1* activity.

One interesting possible explanation for how estrogen could increase collagen content is related to an indirect effect on insulin-like growth factor (IGF)-1. Both in humans (Hansen et al., 2013a) and in engineered ligaments (West et al., 2015) the administration of IGF-1 increases tendon collagen accumulation. In humans, both the incorporation of collagen into the patellar tendon and the local production of PINP were significantly increased with local IGF-1 administration (Hansen et al., 2013b), suggesting that IGF-1 can enhance tendon collagen synthesis and incorporation. Estrogen directly modulates both IGF-1 and IGF binding proteins (Hansen et al., 2009b) and may therefore mediate its positive effects through an increase in IGF-1 signaling. IGF-1 in turn can affect collagen content through an increase in protein synthesis as a byproduct of the production of the La-related protein (LARP) 6 (Blackstock et al., 2014). *LARP6* is a binding protein that is increased by IGF-1, directly binds to type I collagen mRNA, and specifically increases the translation of this isoform of collagen.

Given the sometimes-confusing data on the role of estrogen in musculoskeletal function, the question many active women have is: "Based on our current knowledge, what recommendations can be made for how to maximize musculoskeletal function?" From the data discussed above, it appears that like many other performance strategies, in young women hormonal cycling is something that needs to be handled differently depending on the phase of training. For young women who are not competing in anything at a high level, normal menstrual cycling is beneficial for musculoskeletal health and performance. In this population, the benefits of high estrogen on the anabolic response to exercise in muscle and tendon and improved muscle repair means that over time these women will have stronger muscles, tendons, and bones if they allow for the periodic rise of estrogen that occurs before ovulation. In competitive athletes, the benefits of normal cycling can be seen by contrasting them with those athletes who experience relative energy deficiency in sport (RED-S), formerly known as the female athlete triad (Heikura et al., 2018). With a chronic energy deficiency, women stop normal cycling, and estrogen levels drop to very low levels, resulting in amenorrhea, loss of bone mass, and increased risk of musculoskeletal injury (Heikura et al., 2018). Again, because of the beneficial effects on muscle, tendon, and bone, competitive athletes should look to maintain their normal cycling when they are looking to maximize their adaptation to training during the offseason or in the base phase of their training. As they begin to shift into the season, or during the specific preparation phase of training, they should consider taking an oral contraceptive that contains low levels of progesterone. The low level of estrogen in the OC would decrease the negative effects of the ovulatory rise in estrogen on tendon and ligament mechanics (C. A. Lee et al., 2015b), whereas an intriguing study by Hansen and colleagues (Hansen et al., 2011) showed that only high progesterone OCs decrease muscle protein synthesis. In this way, training would be performed in the absence of OCs and therefore lower tendon stiffness, higher anabolic responses to training, and better muscle repair on hard days. This would result in fewer muscle pulls and a greater metabolic cost of training, increasing the stimulus for adaptation and the likelihood of a healthy build-up

phase. Shifting to the low progesterone OC in the specific preparation phase, or season would help increase stiffness within tendon and ligament while not preventing muscle repair following quality sessions or games. The result would be a higher rate of force development resulting in better performance and a lower risk of catastrophic musculoskeletal injuries during the competitive season. However, it should be noted that this strategy would leave the athlete at a greater risk for catastrophic injury for ~5 days a month during training. Therefore, novel strategies to prevent the negative effects of estrogen on joint laxity are desperately needed to decrease the risk of catastrophic injuries in active women.

In postmenopausal women, the strategy is less clear. In this population, hormone replacement improves muscle mass and function by improving muscle repair and the response to feeding and exercise. Bone mass and function are also improved by HRT (Zhao et al., 2015). The problem is that long-term HRT use is associated with decreased tendon cross-sectional area, especially in an active population (Cook et al., 2007). The result may be a bigger, stronger muscle pulling on a small brittle tendon that is in turn connected to a stiffer bone. This would result in increased strain mismatch, differences in stiffness between connected tissues, that can produce strain concentrations and promote injury. However, not taking HRT would accelerate sarcopenia and osteoporosis. Therefore, to date, the data suggest that HRT is beneficial for musculoskeletal function in postmenopausal women, but extra care should be taken to maximize tendon function. What is really lacking for these women is a way to get the positive effects of estrogen on muscle and bone repair and anabolic responses to loading and nutrition without the negative long-term effects on tendon. Phytoestrogens may provide some hope, but much further work is needed to establish the efficacy of these natural products.

CHAPTER 2: EFFECTS OF ESTROGEN ON COLLAGEN SYNTHESIS AND DEGRADATION IN HMAN ENGINEERED LIGAMENTS

Abstract

Estrogen (E2) has been shown to influence musculoskeletal tissue function and injury through effects on mechanics and protein turnover. In bone and muscle, E2 tends to have overall positive effects, increasing bone mass through suppression of osteoclasts, and decreasing muscle strains/injury in women. However, in tendons and ligaments, E2 decreases stiffness, directly impacting performance and injury rates. As collagen makes up over 80% of the mass of a sinew, it greatly impacts the material and mechanic properties of the tissue. However, the effect of E2 on collagen synthesis and degradation rates remains unclear. The goal of this chapter was to examine how E2 affects collagen synthesis and degradation using two- and three-dimensional tissue culture models. Ligaments engineered from human ACL fibroblasts isolated from female donors were randomly assigned to two treatment groups: 1) Low Estrogen (LE2; 5 pg/mL 17β-estradiol), and 2) High Estrogen (HE2; 500 pg/mL 17β-estradiol). Mechanical (MTL: LE2: 0.5911 N, HE2: 0.39 N) and material (Modulus: LE2: 13.67±2.38 MPa, HE2: 13.22±3.81 MPa) properties of the ligaments tended to decrease with increasing estrogen. Estrogen slightly increased collagen content and this was attributed to a decrease in collagen degradation (LE2 (5 pg/mL): 43.35 ± 12.59 µg/mL, HE2 (500 pg/mL): 22.61 ± 12.17 µg/mL). These data suggest that estrogen tends to increase collagen content by inhibiting degradation, but this does not translate into improved mechanics likely due to the simultaneous inhibition of lysyl oxidase (*LOX1*) crosslinking.

Introduction

With the discovery of estrogen and androgen receptor (ERs and ARs) on tendon/ligament cells, a lot of work has been done to establish the role of hormones in sex differences in sinew function and injury

rates. Female athletes are 2-8 times more likely to rupture an ACL than their male counterparts (Adachi et al., 2008; Arendt & Dick, 1995) and high estrogen peaks in the menstrual cycle have been linked to increased laxity and ACL injury occurrence (Beynnon et al., 2006; Herzberg et al., 2017; Shultz et al., 2005; Shultz, Wideman, et al., 2012; Wojtys et al., 2002). This has different implications for ligaments versus tendons. While a stiffer ligament is always preferred, to provide joint stability, a tendon that is too stiff can cause the attached muscle to undergo more eccentric load and be more prone to injury. Therefore, a decrease in sinew stiffness could explain why women suffer fewer muscle injuries and more ligament ruptures than men (Adachi et al., 2008; Arendt & Dick, 1995; Edouard et al., 2016; Hägglund et al., 2009)

The mechanism through which E2 influences the function of sinew is not fully understood and the literature is filled with conflicting conclusions, specifically on its effects on collagen synthesis. Both tendons and ligaments are collagenous tissues with collagen constituting 60-85% of their dry weight (Frank, 2004; Kjaer, 2004) The stiffness of sinew is, therefore, dependent on collagen fiber density, orientation, and crosslinking, and as such E2's effect on collagen is particularly important to characterize. Collagen fibril density (collagen content) of any tissue should reflect the arithmetic sum of collagen synthesis and degradation. To date, whether E2 alters collagen synthesis or degradation has not been established.

When Hansen et al. measured incorporation of stable isotope-labeled proline into the patellar tendon, postmenopausal on HRT saw a 47% more incorporation than control women (Hansen et al., 2009a). When the same group measured synthesis of procollagen I intact N-terminal (PINP) within the peritendinous space, there was no statistical difference between groups but HRT women tended to synthesize less collagen (Hansen et al., 2009a). To explain this discrepancy, the working hypothesis was that E2 increases collagen incorporation into sinew without changing collagen synthesis rates. **To test this hypothesis, 3D engineered ligaments were**

treated with either low (LE2; 5 pg/mL) or high (HE2; 500 pg/mL) E2 and collagen synthesis (*LARP6* and procollagen protein/mRNA) and degradation (hydroxyproline in the media, MMP levels, and activity) were measured and total collagen content was determined by measuring hydroxyproline in the tissue.

Materials and Methods

Cell isolation

The University of California Davis Institutional Review Board approved all procedures and protocols. Remnants of ruptured human anterior cruciate ligaments were collected during standard ACL reconstruction surgeries and digested as previously described (Paxton et al., 2010). Before surgery, both subjects (2 females) signed informed consent forms. The donors were 20 and 18 years old. Ligaments were washed five times in sterile PBS and then placed in a 5% antibiotic/antimycotic (ABAM) solution for 2 h. The ACL remnants were digested in 0.1% collagenase type II dissolved in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin overnight at 37°C to free fibroblasts which were then collected by centrifugation (1,500 *g* for 5 min), washed three times with growth media (DMEM containing 10% FBS and 0.1% penicillin), and then plated and cultured as normal. All experiments were performed on cells at passage 4 or 5.

Ligament Formation

Ligament constructs were engineered as previously described (Paxton et al., 2010). ACL fibroblasts were expanded in culture using regular growth media. The cells were collected and resuspended in phenol red free growth media containing 10 U/mL thrombin, 2 μL/mL aprotinin, and 2 μL/mL aminohexanoic (AH) acid. Seven hundred fourteen microliters of this cell-thrombin solution were then plated onto sylgard-coated 35mm plates containing pinned brushite cement anchors (representing bone) at a concentration of two hundred fifty-thousand cells per plate. Two hundred eighty-six microliters of 20 mg/ml fibrinogen were then added to each plate to form a fibrin gel matrix. The cells proliferate within the gel matrix and,

over 7 days contract the fibrin around the anchors into a single tissue. Once contracted, the tissues continue to develop like an embryonic tendon/ligament. Half-size constructs (average length of 6 mm compared to the 12 mm of full-sized constructs) were also made for mRNA analysis. The constructs were fed every other day with growth media supplemented with 50 µM Proline, 200 µM Ascorbic Acid, and 5 ng/mL TGF-β (Peprotech, Rocky Hill, NJ, USA).

Estrogen Treatments

In early experiments, the feed media was supplemented with 5 (low) and 500 (high) pg /mL 17β-estradiol at each feeding for 14 days. These estrogen levels were selected to mimic the estrogen concentration seen in the follicular (5 pg/mL) and ovulatory (500 pg/mL) phases of the menstrual cycle. The experimental plan was later changed such that the treatment groups were assigned to different E2 levels after 8 days of normal feed media, thus treatment lasted 6 days.

Mechanical Testing

On day 14 of culture, the width and length of each construct were measured using a caliper, after which the constructs were tested using an Instron 68SC-1 mechanical testing system. Samples were submerged in phosphate-buffered saline and loaded and preconditioned using 10 loads of 0.2N before testing to failure at an elongation rate of 0.3 mm/s. The resulting load-deformation curve was used to determine maximal tensile load (MTL), and then normalized to cross-sectional area and initial length to determine stress and strain. The slope of the linear region of the stress-strain curve was used to obtain Young's modulus and the maximal stress recorded was used for the ultimate tensile strength (UTS).

Collagen Content

The collagen content of the ligaments was determined using a hydroxyproline assay (Woessner, 1961). After mechanical testing to failure, tissues were removed from their anchors and dried in an oven for 15 minutes at 120°C. The dry mass of each sample was measured and then samples were hydrolyzed

in 200 μL of 6 N HCl at 120°C for 2 hrs and dried for 1-1.5 hrs. The resulting dried pellet was then resuspended in 200 μL of hydroxyproline buffer and then further diluted 1:20 in the same buffer. 150 μL of Chloramine-T solution was added to each sample, vortexed, and allowed to sit at room temperature for 20 minutes, after which aldehyde-perchloric acid was added to each sample. The samples were well mixed and then incubated at 60°C for 15 minutes. The tubes were then cooled for 10 minutes and read at 550 nm on an Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, VT). Hydroxyproline content was used to determine collagen mass on the assumption that 13.8% of collagen is hydroxyproline. The collagen fraction was determined by dividing the collagen content by the dry mass of the tissue.

Collagen Degradation

To estimate the amount of collagen degradation, 1 ml of media was collected from each tissue before replacing the media on days 8, 10, 12, and 14. The media was dried using a Speed Vac SC110 and the dried pellet was hydrolyzed for the hydroxyproline assay as described above.

Collagen Synthesis

The synthesis of new procollagen was determined by western blotting. Briefly, human ACL fibroblasts isolated from female donors were expanded and plated into 24-well plates. They were allowed to proliferate until 100% confluent before treatment. Cells were treated with ascorbic acid free feed media (phenol-red free DMEM + 50 μM Proline + 5 ng/mL TGF-β) so that the procollagen could not be processed and then exported from the cells and the following estrogen conditions: control (0 pg/mL 17β-estradiol), low (5 pg/mL 17β-estradiol), medium (50 pg/mL 17β-estradiol), and high (500 pg/mL 17β-estradiol). The test media was added at different time points (0h, 6h, 12h, 24h, 42h, 48h) to determine how procollagen production changed over time or at a single 48-hour time point before collection. After completion of the experiment, the cells were placed on ice, media was aspirated, and the cells washed with ice cold phosphate-buffered saline (PBS). 75 μL of Laemmli sample buffer (LSB) was added to each

well and, then the lysates were collected, sonicated, and denatured at 100℃ for 5 minutes. Protein (15 μl protein per lane) was loaded on 4%-20% Criterion TGX Stain-free gels (Bio-Rad), run for 45 minutes at 200V and visualized after a UV-induced 1-minute reaction to produce fluorescence. Following quantification of all of the protein per lane, proteins were transferred from the gel to nitrocellulose or polyvinylidene difluoride (PVDF) membrane at 100V for 45 minutes. Membranes were Ponceau stained to confirm proper transfer and then washed and blocked in 1% fish skin gelatin dissolved in tris-buffered saline with 0.1% tween-20 (TBST) for 30 minutes, rinsed, and then incubated with the procol1a1 primary antibody (the SP1.D8 mouse monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank where it was deposited by H. Furthmayr) at a concentration of 1:250 overnight at 4℃. The next day, membranes were washed and incubated with HRP conjugated secondary antibodies at 1:5000 (goat) to 1:10 000 (mouse, rabbit) in 1% skim milk-TBST for 1 hour at room temperature. Immobilon Western chemiluminescent HRP substrate (Millipore, Hayward, CA, USA) was then applied to the membranes for protein visualization by chemiluminescence. Image acquisition and band quantification was performed using the ChemiDoc MP System and Image Lab 5.0 software (Bio-Rad). Protein levels of each sample were calculated as band intensities relative to total protein as described previously (Gilda & Gomes, 2013). Each group was loaded onto the gel in random order to avoid edge effects and lane bias. All samples at all time points were run on the same gel for each protein probed. Images of the bands in the figures display representatives for each time point.

Gene Expression

Half-size constructs made from the same donors were used for mRNA analysis. Following treatment with low or high estrogen for 6 days, the constructs were washed twice with PBS, flash-frozen in liquid nitrogen, and stored at -80°C until analyzed. Total cellular RNA was extracted from the engineered ligaments using TRIzol reagent (Invitrogen, Carlsbad, CA). Briefly, tissues were placed in a 1.7-mL Eppendorf tube with 250 μL of TRIzol. The samples were then homogenized by shaking for 30 minutes

after which they 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 the following primers: **LOX11** (forward:

CTGGGAGACCGTACTGGAAG; reverse: ACATCTAGAGCCCGCGAAG), *COL1A1* (forward:

GTCGAGGGCCAAGACGAAG; reverse: CAGATCACGTCATCGCACAAC), *COL3A1* (forward:

CACGGAAACACTGGTGGACAGATT; reverse: ATGCCAGATGCAACATCAAGGAC), *ESR1* (forward:

TGGAGTCTGGTCCTGTGAGG; reverse: GGTCTTTTCGTATCCCACCTTTC), *ESR2* (forward:

CATGCGAGTAACAAGGGCAT; reverse: TGGGAGCCCTCTTTGCTTTT), *LARP6* (forward:

TTGGAGGACGAGGAGGAGG; reverse: TGGTGCCACTGTGCCCG), and *MMP13* (forward:

AAGATGCGGGGTTCCTGATG; reverse: AGAAGTCGCCATGCTCCTTA). Gene expression analysis was

calculated using the method described in Pfaffl, 2001, using *GAPDH* (forward: ATGGGGAAGGTGAAGGTCG;

reverse: GGGGTCATTGATGGCAACAATA) as a reference. The absolute C_T values of *GAPDH* were not

different in any of the experimental groups.

Statistical analysis

All data are shown as means ± SD. All statistical analyses were performed using GraphPad Prism

9 software. Differences in means were compared between groups by non-parametric t-Test. A *P* value of

<0.05 was considered significant. The data presented in this dissertation are representative data selected

out of multiple experiments using multiple donors. Each individual point represents a technical replicate

(a single construct) from one donor, in one experiment.

Results

Effects of Estrogen on the mechanical and material properties of engineered ligaments

The engineered ligaments treated with low estrogen tended to have better material (Modulus:

LE2: 13.67 ± 2.38 MPa, HE2: 13.22 ± 3.81 MPa; fig 2.1B) and mechanical properties (MTL: LE2: 0.5911 ±

0.25 N, HE2: 0.39 ± 0.15 N; fig 2.1A) than those treated with high estrogen. There was no statistical

difference in cross-sectional area (LE2 (5 pg/mL): 0.6267 ± 0.07 mm^2; HE2 (500 pg/mL): 0.5850 ± 0.12 mm^2;

fig 2.1D).
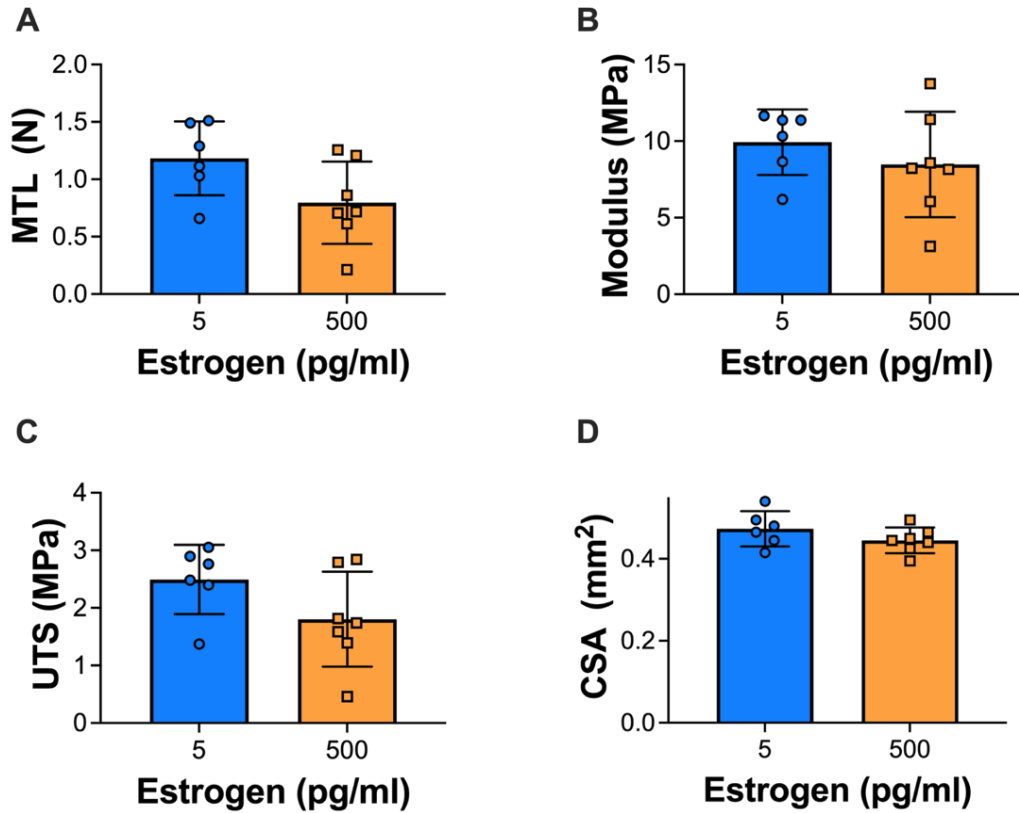
Fig 2.1. High estrogen decreases stiffness of engineered ligaments. *High E2 treatment for 6 days leads to a decrease in the mechanical properties of engineered ligaments as show by measuring(A) Maximum Tensile Load (MTL). The material properties (load and deformation normalized to size of tissues) are similarly decreased as seen with the (B) Young's Modulus, and (C) Ultimate Tensile Strength (UTS). There is no change to the cross-section area (D). Data are representative of 8 trials using 2 donors and 6 technical replicates. Donor: 062321*

Effects of Estrogen on collagen content, synthesis, and degradation.

   Collagen Content tends to increase with estrogen treatment. Although it did not reach statistical significance, there was a consistent trend towards an 8-10% increase in collagen content from low to high estrogen when the engineered ligaments were treated with estrogen for the entire 14-day culture time (LE2 (5 pg/mL): 273.2 ± 2.41 μg, HE2 (500 pg/mL): 299.5 ± 1.49 μg; fig 2.2). When engineered ligaments were treated with estrogen only from days 8-14 (fig 3.2A) the tendency towards an increase in collagen is no longer observed. There was no significant difference between the mass (LE2 (5 pg/mL): 1.525 ± 0.19 mg, HE2 (500 pg/mL): 1.620 ± 0.18 mg; fig 3.2B) or collagen concentration (LE2 (5 pg/mL): 1.525 ± 0.19 mg, HE2 (500 pg/mL): 1.620 ± 0.18 mg; fig 3.2C) of the constructs.
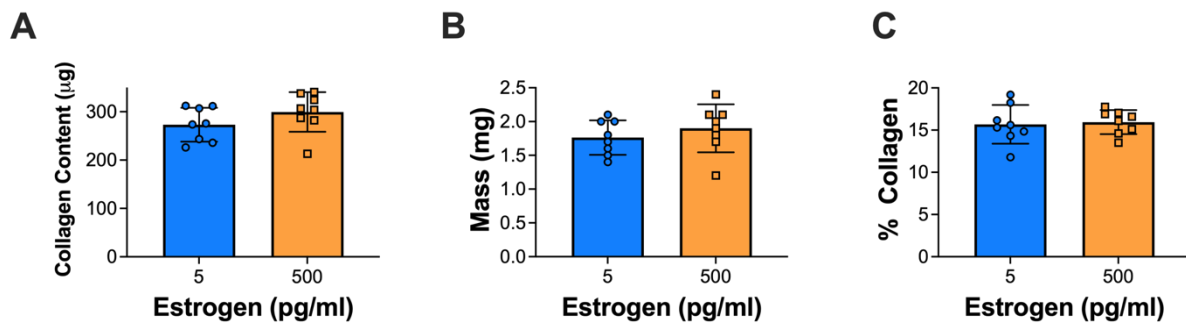
Fig 2.2. **Treating engineered ligaments for 14 days with high estrogen (E2) slightly increases the collagen content of engineered ligaments.** *(A) Collagen content, (B) Mass, and (C) collagen concentration of engineered ligaments after 14 days of treatment with low (5 pg/mL) or high (500 pg/mL) E2 concentration. Data are representative of 3 trials using 2 donors and 8 technical replicates. Donor #: 022318*
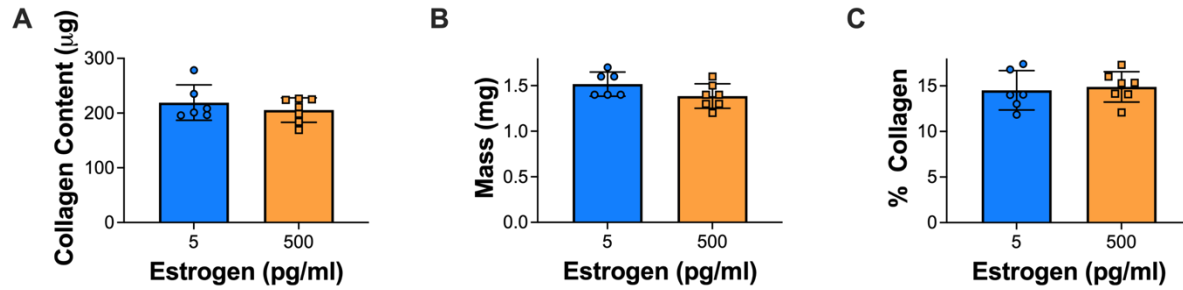
Fig 3.2. **With only 6 days of high estrogen (E2) treatment, there is no change in collagen content.** *(A) Collagen content, (B) Mass, and (C) collagen concentration of engineered ligaments after 6 days of treatment with low (5 pg/mL) or high (500 pg/mL) E2 concentration. Note that the collagen content no longer shows a slight increase with high estrogen treatment. Data are representative of 8 trials using 2 donors and 6 technical replicates. Donor #: 062321*

Collagen Synthesis is unaffected by estrogen treatment. Procollagen protein in the human ACL fibroblasts showed an 8-to-10-fold increase over 48h (Fig 2.4A). This increase was not statistically different between cells treated with estrogen (CTRL (0 pg/mL): 1.000 ± 0.50 mg, LE2 (5 pg/mL): 0.9358 ± 0.234 mg, ME2 (50 pg/mL): 0.9092 ± 0.30 mg, HE2 (500 pg/mL): 1.114 ± 0.23 mg; Fig 2.4B)
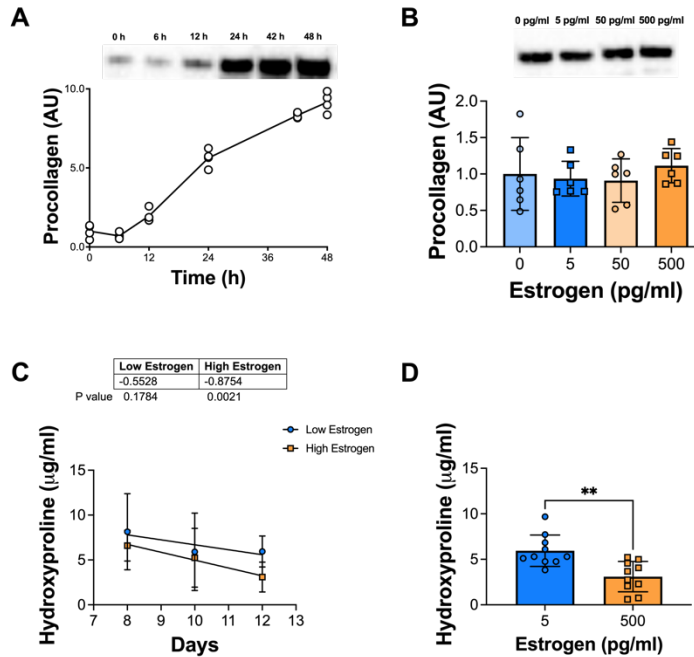
Fig 2.4. E2 does not seem to have any effect on collagen synthesis but there is moderate inhibition of collagen degradation. *Time course of procollagen protein measured by western blot (A) shows that procollagen protein expression increases over 48 h. Measuring procollagen after 48h of treatment with 0 pg/ml, 5 pg/ml, 50 pg/ml, and 500 pg/ml (B) showed no effect of E2 on procollagen synthesis. Hydroxyproline content in the media (C) decreases with time and high estrogen treatment (D). Data are representative of 4 trials using 2 donors and 10 technical replicates. ** indicates significantly different than 5 pg/ml Estrogen p < 0.005. Donor #: 062321*

Collagen degradation is lowered by estrogen. The level of hydroxyproline in the media decreased with time in culture, suggesting development differences in collagen degradation as a function of ligament age. Collagen breakdown in constructs treated with low estrogen tended to be greater than those treated with high estrogen (Days 8, 10, and 12 respectively: LE2: 8.150 ± 4.28 µg/mL, 5.903 ± 4.31 µg/mL, 5.938 ± 1.72 µg/mL; HE2: 6.600 ± 1.72 µg/mL, 5.242 ± 3.28 µg/mL, 3.098 ± 1.67 µg/mL; Fig 2.4C) with a significant difference between low estrogen and high estrogen observed on day 12 (Fig 2.4D).

Effect of Estrogen on gene expression in engineered ligament.

The high estrogen group expressed significantly less estrogen receptor (ER) mRNA than the low estrogen group (*ESR1*: LE2 (5 pg/mL): 1.183 ± 0.39; HE2 (500 pg/mL): 0.4881 ± 0.29, Fig 2.5A) showing that the estrogen treatment had the expected effect on estrogen receptor mRNAs. On the synthesis side, *COL1A1* and *LARP6* mRNA tended to decrease with increased estrogen concentration (*COL1A1*: LE2 (5 pg/mL): 1009 ± 285.62, HE2 (500 pg/mL): 538 ± 805.14; *LARP6*: LE2 (5 pg/mL): 154.3 ± 51.14, HE2 (500 pg/mL): 121.2 ± 63.10, Fig 2.5B, 5.2C). *LOX1* mRNA also moderately decreased with high estrogen (LE2 (5 pg/mL): 2.318 ± 0.68, HE2 (500 pg/mL): 1.618 ± 1.46, Fig 2.5D).
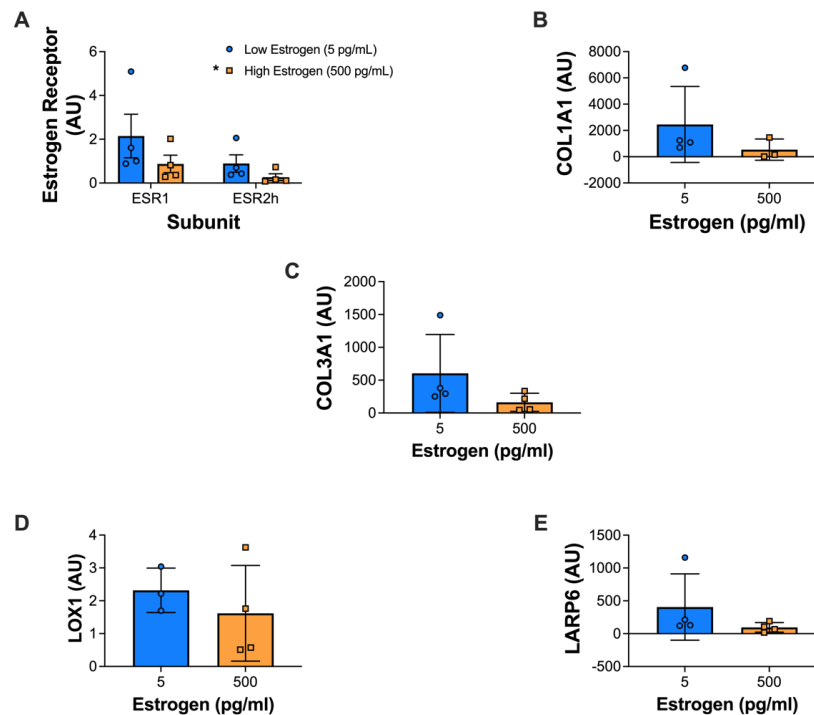
Fig 2.5. Estrogen receptors, *COL1A1*, *LARP6*, and *LOX1* mRNA tend to decrease with estrogen treatment. (A) Estrogen receptors 1 and 2 (*ESR1* and *ESR2*) mRNA expressions significantly decrease with high E2 treatment. Similarly (B) *COL1A1*, *COL2A1,* (C) *LOX1*, and (D) *LARP6* mRNA expression tend to decrease with high E2 treatment. *Data are representative of 8 trials using 2 donors and 6 technical replicates. * indicates significantly different than 5 pg/ml Estrogen p < 0.05. Donor: 062321*

Discussion

Women are 2-8 times more likely to rupture their ACL than their male counterparts (Arendt & Dick, 1995) and it is hypothesized that is due to structural differences in their ligaments because of different hormonal profiles. The incidence of ACL rupture increases with increasing estrogen along the menstrual cycle. As ligaments are mostly made up of collagen, their mechanical and material properties are largely dependent on collagen synthesis, degradation, incorporation, and crosslinking. However, there is conflicting data on the effects of estrogen on collagen synthesis, limited information on estrogen and collagen degradation, and nothing about estrogen and collagen incorporation. We have previously shown, and the data in this chapter tends to confirm, that estrogen negatively affects the mechanical properties of the ligament (C. A. Lee et al. 2015). We studied the effects of estrogen on collagen content and saw that consistently treating constructs with high estrogen (500 pg/mL) results in a slight increase in collagen content over 14 days; however, that difference does not reach statistical significance and is not observed during a shorter treatment time course of 6 days. Since collagen content is dependent on synthesis, degradation, and incorporation rates of collagen, we investigated the effects of estrogen on procollagen protein production, a marker of collagen synthesis, and hydroxyproline in the culture media, a marker of collagen degradation. We found no differences in procollagen protein with estrogen treatment, whereas degradation tended to decrease with estrogen treatment, reaching significance on day 12 of culture (day 4 of estrogen treatment). There was significantly less degraded collagen with the constructs treated with high estrogen suggesting that estrogen blocks degradation over time. Although moderate, the small protection against degradation is likely sufficient to explain the tendency for collagen content to increase within the engineered grafts. Together, these data suggest that while E2 has no effect on collagen synthesis, it decreases collagen degradation enough to have a limited impact on collagen content in the tissue. In order words, estrogen increases collagen content by slowing breakdown.

Estrogen tends to decrease the mechanical and material properties of sinew. Data from the current and past work in the laboratory suggests that this effect has little to do with collagen content and more to do with collagen crosslinking (C. A. Lee et al., 2015b). My data shows that mechanical and material properties tended to decrease with high estrogen treatment even with collagen tending to increase or stay the same.

To understand how collagen content in sinew is regulated by estrogen, I took advantage of our unique array of in vitro assays. To understand synthesis, I turned to a 2D assay of collagen synthesis using western blots to measure procollagen protein expression. First, I measured procollagen synthesis as a function of time to see the cycle through which the ligament cells synthesize collagen in response to feeding. Procollagen synthesis increased with time after 6 hours of growth media treatment. When procollagen was measured after 48h of treatment with growth media supplemented with increasing concentration of estrogen, there was no difference in procollagen production, suggesting that E2 did not affect procollagen synthesis. Previous 2-D studies found dose-dependent decreases in collagen synthesis with estrogen treatment (M. H. Chen et al., 2014; Yu et al., 1999) but this is likely because the estrogen concentrations used in those studies to show differences were supraphysiological. With the concentrations at physiological level, not much difference was observed between groups (M. H. Chen et al., 2014; Yu et al., 1999).

To understand how collagen breakdown was regulated by estrogen, I developed a novel collagen degradation assay using our engineered ligament model. Here, I cultured the engineered ligaments as normal, supplementing with either low or high E2. The media was collected instead of aspirated at each feeding for hydroxyproline analysis. Hydroxyproline forms during the post-translation modification of the polyproline type II (PP-II) propeptide chains in collagen synthesis and is therefore only present in synthesized collagen (Gaar et al., 2020). The rationale, therefore, was that any hydroxyproline found in the media should be collagen that had been synthesized and degraded either before being incorporated

into the grafts or as part of the turnover process after incorporation. Using this new assay, I demonstrated for the first time that hydroxyproline content in the media could serve as a non-specific indicator of collagen degradation. I found that there is a moderate protective effect against degradation over time which could explain the slight increase in collagen seen with 14 days of high estrogen treatment. This aligns with findings in other studies that have found estrogen to moderately inhibit collagenase activity while progesterone has an even greater effect (Marbaix et al., 1992; Wahl et al., 1977; Zhou et al., 2011).

Even though I have made a great deal of progress towards understanding how estrogen affects sinew collagen turnover and mechanics, to complete this work we would need specific measures of degradation such as zymography to measure matrix metalloproteinase (MMP) activity and western blots to measure MMP protein. We would also need to measure collagen crosslinking for differences between low and high estrogen conditions. Collagen crosslinks within our grafts could be measured using digital scanning calorimetry since more heavily crosslinked collagen denatures at a higher temperature than less crosslinked collagen. It is also worth noting that the studies of tensile and collagen properties in this chapter did not have a perfect control of 0 pg/mL estradiol and so they should be repeated to include that control group. An additional limitation is that my growth media contained fetal bovine serum which has varying concentrations of hormones, including estrogen. This introduced additional variability in the experiments which may contribute to the lack of statistically significant changes observed. However, as the patterns observed were consistent across several experimental iterations and multiple donors, E2's effects on collagen and tensile properties are still apparent.

Conclusions

From the data collected for this chapter, we can speculate that estradiol differentially modulates the structural and tensile properties of ligaments. We can infer that collagen content is minimally increased with E2 due to inhibited collagen degradation; however, there is likely a much more pronounced

effect of E2 on collagen crosslinking which causes decreased mechanical and material properties of sinew. This may provide an explanation for the increased incidence of ACL injury in women and would suggest that preventative interventions would need to focus on increasing collagen crosslinking more than content.

CHAPTER 3: PHYTOESTROGENS AND THE MECHANICAL PROPERTIES OF ENGINEERED LIGAMENTS

Abstract

High β-estradiol (E2) exposure, such as occurs around ovulation, tends to increase sinew collagen content but can decrease modulus through the inhibition of lysyl oxidase. Previous experiments in rats have shown that the phytoestrogen genistein (GE) increases the collagen content of tendons in a way that can replace endogenous E2, but without the decrease in mechanical properties. To determine whether this is a direct effect of phytoestrogens on sinew structure and function, we determined the relationship between phytoestrogen levels, mechanics, and collagen content. Three-dimensional (3D) ligaments engineered from human ACL fibroblasts isolated from female donors, were randomly assigned to four treatment groups: 1) control (CTRL; 0 µM GE), 2) low GE (LGE; 0.02 µM GE), 3) medium GE (MGE; 0.2 µM GE), and 4.) high GE (HGE; 2 µM GE). The ligaments underwent mechanical testing followed by analysis of collagen. There was a positive effect of GE on collagen content (CTRL: 158.4 ± 62.22 µg, LGE: 222.7 ± 60.62 µg, MGE: 259.1 ± 12.31 µg, HGE: 239 ± 26.90 µg), and this was reflected in collagen concentration (CTRL: 8.66 ± 0.24 %, LGE: 11.09 ± 0.29 %, MGE: 12.01 ± 0.45 %, HGE: 12.14 ± 0.24 %) as well. Further, the mechanical (MTL: CTRL: 0.58 ± 0.24 N, LGE: 1.202 ± 0.29 N, MGE: 1.090 ± 0.45 N, HGE: 0.9033 ± 0.24 N) and material (Modulus: CTRL: 21.03 ± 2.72 MPa, LGE: 22.99 ± 4.89 MPa, MGE: 18.94 ± 4.78 MPa, HGE: 21.03 ± 5.38 MPa) properties of the engineered ligaments treated with GE were higher than controls, suggesting that GE can increase sinew collagen and mechanical properties.

Introduction

Estrogen loss/deficiency, as seen in post-menopausal women is associated with increased risk of cardiovascular disease, accelerated bone and muscle wasting (EJ et al., 1992; K & A, 1993; Rice et al., n.d.; WR et al., 1991), and decreased sensitivity to anabolic stimuli (Bamman et al., 2011; PJ et al., 2003).

Estrogen replacement therapy (ERT) has been employed to reduce muscle and bone loss and restore muscle protein balance (Hansen et al., 2012; Smith et al., 2014). However, ERT comes with its own slew of adverse effects including higher risk of stroke (Brass, 2004; Henderson & Lobo, 2012), breast and endometrial cancer (W. Y. Chen, 2011; Furness et al., 2009), and sinew disfunction (Cook et al., 2007).

Genistein is a phytoestrogen found in soy products and could offer an alternative to ERT. It is structurally similar to 17β-estradiol and has been shown to bind to estrogen receptors (Paterni et al., 2014; Rietjens et al., 2013). Genistein is also associated with many beneficial health effects such as reduced cancer risk and alleviation of some of the disorders associated with estrogen loss such as osteoporosis and cardiovascular disease (Cassidy & Hooper, 2006; Cederroth & Nef, 2009; Patisaul & Jefferson, 2010). Interestingly, unlike endogenous E2, Ramos and colleagues (2012) found that genistein can increase the collagen content of the Achilles tendon without negatively affecting mechanics. In their study, female Sprague-Dawley rats with or without ovariectomy (OVX) were randomized into vehicle or GE treated groups, and then subdivided further into sedentary or exercise groups (Ramos et al., 2012). The GE rats were given 300 mg genistein/kg body weight/day (Ramos et al., 2012). When OVX rats were treated with genistein, collagen content within the Achilles returned to intact levels (Ramos et al., 2012) and unlike exogenous E2 that inhibits collagen crosslinking (C. A. Lee et al., 2015b), genistein increased hydroxylyslpyridinoline (HP) mediated collagen crosslinking resulting in normal mechanical properties (Ramos et al., 2012).

With this background, I hypothesized that the isoflavone genistein increases collagen content of sinew without impacting crosslinking. **To test this hypothesis, we conducted a dose response experiment using 3D engineered ligaments treated with increasing doses of GE. The constructs were then tested for mechanical and material properties and collected to measure collagen content.**

Materials and Methods

Cell isolation

Refer to Chapter 2 for a full description of cell isolation methods.

Ligament Formation

Refer to Chapter 2 for a full description of ligament formation methods. The ACL fibroblasts used were from the same two female donors that were subjects of the studies in Chapter 2 and all following chapters.

Genistein Treatments

Ligaments engineered from human ACL fibroblasts isolated from female donors, were initially randomly assigned to four treatment groups: 1) control (CTRL; 0 μM GE), 2) low GE (LG; 2 μM GE), 3) medium GE (MG; 20 μM GE), and 4.) high GE (HG; 200 μM GE). After it was observed that these concentrations of GE were cytotoxic and diminished mechanical properties, the concentrations of GE were decreased by two orders of magnitude so that the final treatment groups contained: 1) 0 μM GE, 2) 0.02 μM GE, 3) 0.2 μM GE, and 4.) 2 μM GE. These concentrations did not cause cell death and therefore were used for further analysis of protein and gene expression.

Mechanical Testing

Refer to Chapter 2 for Mechanical Testing methods.

Collagen

Refer to Chapter 2 for Collagen Methods.

DNA Isolation

Half-size constructs made from the same cell donors were used for DNA analysis. Following treatment with GE for 6 days, the constructs were washed twice with PBS, flash-frozen in liquid nitrogen and stored at -30°C until analyzed. Total cellular DNA was extracted from the engineered ligaments by

adding 500 mL of DNAzol reagent (Invitrogen, Carlsbad, CA) to each sample. 5 µL of 40 mg/mL Proteinase K was then added to each sample and the samples incubated overnight at 25°C after which they were centrifuged, and DNA was isolated by ethanol precipitation. DNA was rinsed with ethanol and resuspended in 30 µL of milli-Q water and DNA content read at 260 and 280 nm on an Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, VT).

Statistical Analysis

Refer to Chapter 2 for a full description of <u>Statistical Analysis methods</u>.

Results

Effects of phytoestrogen on the mechanical and material properties of engineered ligaments

Initial experiments with genistein concentrations at control (CTRL; 0 µM GE), low GE (LG; 2 µM GE), medium GE (MG; 20 µM GE), and high GE (HG; 200 µM GE), we saw that the genistein significantly diminished mechanical and material properties of the tissues with MTL, modulus and collagen content decreasing by 80-90% from CTRL to HGE (fig 3.1A, B). Measuring DNA content of the constructs showed that genistein at high concentration was cytotoxic. As there was no significant decrease in mechanics, collagen content, or cell mass at low GE (LGE; 2 µM GE), subsequent experiments used lower GE concentrations with 2 µM as the upper limit.
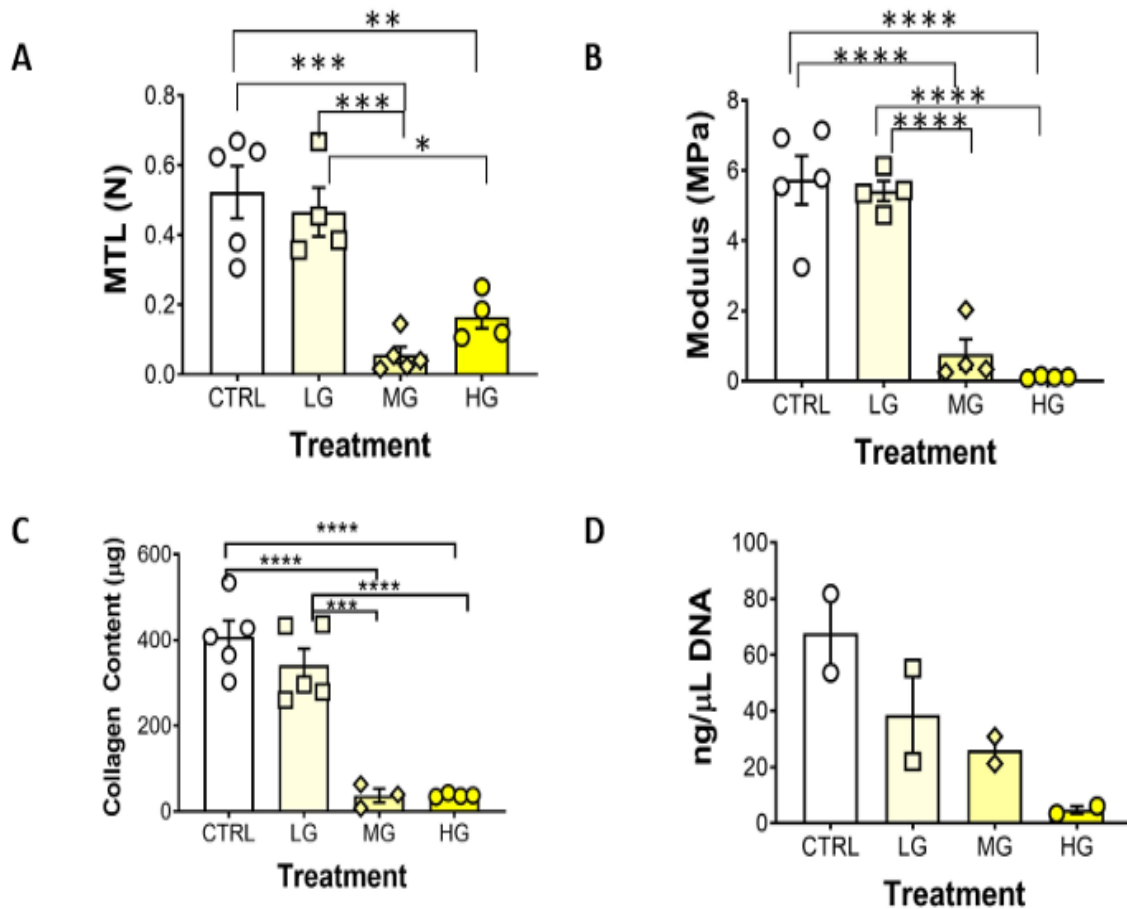
Fig 3.1. High concentration of Genistein results in diminished tensile properties and is cytotoxic. *(A) Maximum Tensile Load (MTL), (B) Young's Modulus, and (C) Collagen content of 3-D human-engineered ligaments treated with 0 µM GE (CTRL), 2 µM GE (LG), 20 µM GE (MG), and 200 µM GE (HG). Measuring DNA content of the treated constructs (D) shows that DNA is inversely proportion to Genistein concentration suggesting that Genistein is cytotoxic in high concentrations. Data are representative of 4 trials using 2 donors and at least 2 technical replicates. * p < 0.05 significant difference.*

At lower genistein concentrations (0 µM GE, 0.02 µM GE, 0.2 µM GE, and 2 µM GE) we saw a more positive effect. There tended to be improved engineered ligament mechanical properties, with significantly higher MTL in the 0.02 µM GE group compared with the 0 µM GE group (MTL: 0 µM: 0.58 ± 0.24 N, 0.02 µM: 1.202 ± 0.29 N, 0.2 µM: 1.090 ± 0.45 N, 2 µM: 0.9033 ± 0.24 N; fig 3.2A). The material

properties displayed as similar trend (Modulus: 0 μM: 21.03 ± 2.72 MPa, 0.02 μM: 22.99 ± 4.89 MPa, 0.2

μM: 18.94 ± 4.78 MPa, 2 μM: 21.03 ± 5.38 MPa; fig 3.2B) but did not reach statistical significance. Similarly,

there was no statistical significance between the cross-sectional area (CSA) of the constructs, however

there was a tendency for CSA to increase with GE concentration (0 μM: 0.4840 ± 0.04 mm^2, 0.02 μM:

0.5067 ± 0.09 mm^2, 0.2 μM: 0.5550 ± 0.09 mm^2, 2 μM: 0.5633 ± 0.06 mm^2; fig 3.2D).
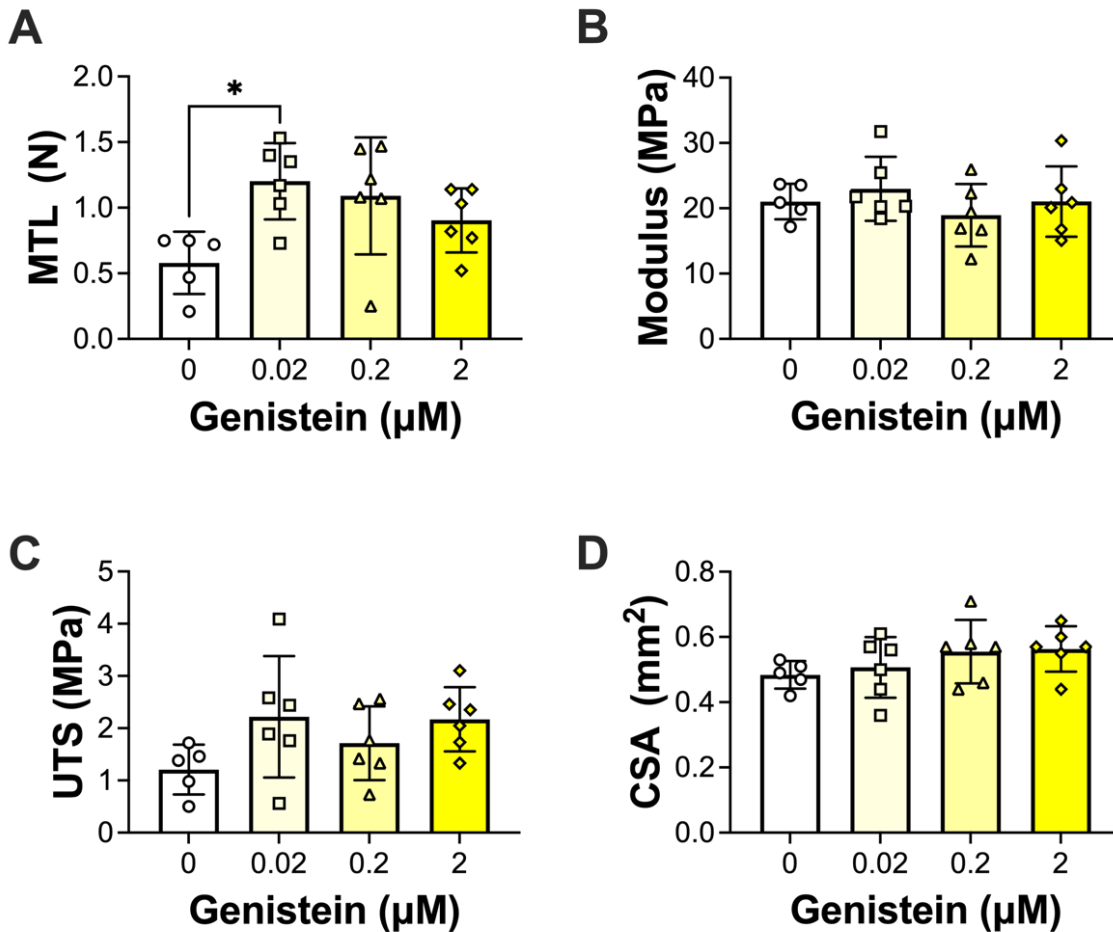
Fig 3.2. Mechanical Data from constructs treated with lower concentrations of Genistein. *(A) Maximum*

Tensile Load (MTL) of Genistein treated constructs peaks at 0.02 μM. *Young's Modulus (B) and Ultimate*

Tensile Strength (C) the graft construct does not change significantly with increasing doses of genistein

and the is no statistical difference in the cross-sectional area (CSA) of the grafts (D). Data are

*representative of 4 trials using 2 donors and at least 5 technical replicates. *p < 0.05 significant difference*

Effects of phytoestrogen on collagen content.

Treatment with GE also tended to have a positive effect on collagen content with this effect

peaking at 0.2μM GE (0 μM: 158.4 ± 62.22 μg, 0.02 μM: 222.7 ± 60.62 μg, 0.2 μM: 259.1 ± 12.31 μg, 2 μM:

239 ± 26.90 μg; fig 3.3A). The low and medium GE groups demonstrated statistically greater collagen

content than the control. This tendency was also reflected in collagen concentration (0 μM: 8.66 ± 0.24 %,

0.02 μM: 11.09 ± 0.29 %, 0.2 μM: 12.01 ± 0.45 %, 2 μM: 12.14 ± 0.24 %; fig 3.3C) since there was no

significant difference between the mass of the constructs (0 μM: 1.850 ± 0.71 mg, 0.02 μM: 2.000 ± 0.18

mg, 0.2 μM: 2.183 ± 0.29 mg, 2 μM: 2.000 ± 0.34 mg; fig 3.3B).
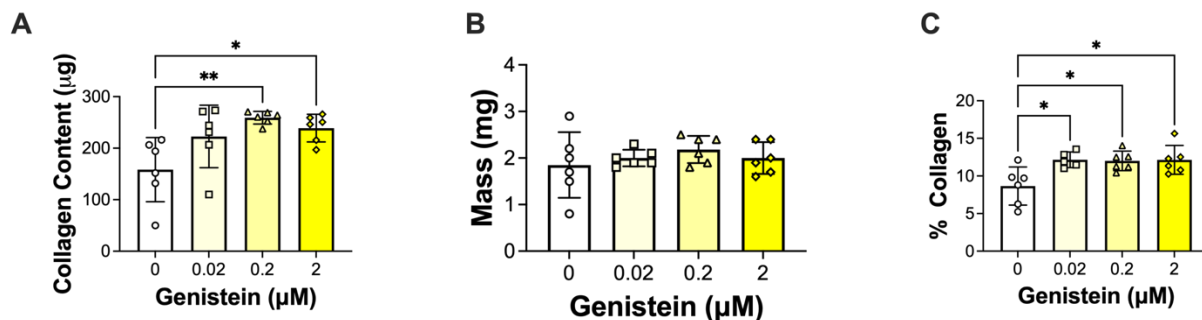
Fig 3.3. Collagen content and concentration increase with Genistein. *Collagen Content (A) and Collagen*

Concentration (C) from constructs treated with low concentrations of Genistein (0 μM GE, 0.02 μM GE, 0.2

μM GE, and 2 μM GE). There was no significant difference in the mass of the constructs with genistein

*treatment (B). Data are representative of 4 trials using 2 donors and at least 5 technical replicates. *p <*

0.05 significant difference

Discussion

Estradiol is known to negatively affect the mechanical and material properties of ligaments with

minimal effects on collagen content (Chidi-Ogbolu & Baar, 2019; Hansen & Kjaer, 2016; C. A. Lee et al.,

2015b). Using engineered ligaments, we have suggested that this effect on mechanical properties is due

to modulations to lysyl oxidase (*LOX1*) mediated collagen crosslinking (C. A. Lee et al., 2015b). The soy

phytoestrogen genistein was shown to restore collagen lost as a result of estrogen deficiency while simultaneously increasing hydroxylyslpyridinoline (HP) crosslinking in rats (Ramos et al., 2012). We, therefore, hypothesized that GE induces an increase in collagen content without the negative effects on mechanics due to inhibited crosslinking.

To test this hypothesis, we conducted an initial dose-response to determine the effects of GE on our engineered ligament model. These levels of GE were selected after looking at the blood concentration of GE following treatment *in vivo*. Our data show that at these high concentrations (greater than 2 µM), GE has cytotoxic properties on ACL fibroblasts. This is in line with previous data showing GE and its derivatives induce apoptotic cell death or cell cycle arrest at supraphysiological serum levels (Antosiak et al., 2017; Guo et al., 2001; Kikuta, 2020; Tsuboy et al., 2014). We, therefore, repeated the dose-response experiment using GE at concentrations two orders of magnitude lower. At the lower concentrations, we saw no cell death and could therefore determine the effect of GE on sinew collagen content and mechanics.

Genistein increased collagen content and concentration as seen in the rat studies done by Ramos(2012). My data also show that the lowest levels of GE increased collagen content 40%, and moderate GE increased collagen by 64%. By contrast, high E2 (500pg/ml) resulted in only an 8-10% increase in collagen that was not statistically significant. This suggests that, unlike E2 which decreases collagen degradation but does not affect collagen synthesis, GE increases collagen synthesis rates to maximize collagen incorporation into the grafts. Insulin-like growth factor (IGF)-1 has shown a similar pattern of effects to what we see with GE in our engineered ligaments (West et al., 2015. In humans, IGF-1 significantly increased both collagen synthesis and incorporation (Hansen et al., 2013b). Estrogen can directly modulate both IGF-1 and IGF binding proteins (Hansen et al., 2009b) and since GE displays estrogen mimetic properties, the positive effects of GE may be mediated through an increase in IGF-1

signaling. IGF-1 increases the production of the La-related protein (LARP) 6 (Blackstock et al., 2014) which in turn directly binds to type I collagen mRNA and increases the translation of this isoform of collagen.

We also see a positive effect of GE on the mechanical properties of our engineered ligaments suggesting that, unlike E2, GE does not inhibit collagen crosslinking. To confirm this, we would need to directly measure crosslinking using digital scanning calorimetry (Chapter 2). The differences in the effects of GE and E2 could be mediated by the preferential binding of GE to ER-β (*ESR2*) over ER-α (*ESR1*) (Kuiper et al., 1998). ER-β is associated with tendon healing (Bian et al., 2020) and increased collagen synthesis where ER-α has been linked to changes in collagen degradation (Markiewicz et al., 2013).

Even though it is clear that GE has a strong positive effect on sinew collagen content and mechanics, to complete this study, I would want to get a better mechanistic understanding of how GE is having these positive effects. Specifically, I would want to determine whether GE positively affects procollagen production or like E2 does it affect collagen degradation? If differences in the underlying mechanism can be found, are these the result of differential activation of estrogen receptors? Does binding to *ESR2* improve collagen dynamics, whereas *ESR1* diminishes this effect? If so, can we identify other *ESR2* agonists that could drive collagen content even higher? It would also be interesting to see whether genistein could counteract some of the negative effects E2 has on sinew mechanics.

Conclusions

The isoflavone genistein improves engineered ligament collagen content and tensile properties. However, at high concentrations, it is cytotoxic to ligament cells and can weaken ligaments leading to increase injury rates. These data suggest that intake of genistein should be controlled to benefit from the positive effects of genistein on sinew.

CHAPTER 4: INHIBITING CONVERSION OF TESTOSTERONE TO ESTROGEN IMPAIRS MECHANICAL AND MATERIAL PROPERTIES OF ENGINEERED LIGAMENTS

Abstract

Treatment of Estrogen Receptor (ER)-positive breast cancer in postmenopausal women with aromatase inhibitors (AI) has been associated with musculoskeletal disorders, which reverse with discontinuation of the drug. Cases of severe tendinopathies have also been described with the use of the AI letrozole, suggesting that AIs may cause systematic or local changes that negatively impact tendons. A dose-response experiment of 3D engineered ligaments with the AI letrozole on its own did not affect either mechanics or collagen. To better mimic the hormonal milieu, we next treated ligaments with both testosterone (T) and letrozole (AI) to determine the effect on mechanical and material properties and collagen content. Constructs engineered from cells from female donors were divided into four groups: 1) control (CON: 0 ng/mL AI; 0 ng/mL T); 2) letrozole (AI: 100 ng/mL AI; 0 ng/mL T); 3) testosterone (CON + T: 0 ng/mL AI; 100 ng/mL T); and 4) letrozole + testosterone (AI + T: 100 ng/mL AI; 100 ng/mL T). Following 6 days of treatment, the ligaments underwent mechanical testing, as well as analysis of collagen and mRNA. Testosterone improved mechanical (MTL: C: 0.5091 ± 0.25 N, AI: 0.7534 ± 0.17 N, T: 0.8566 ± 0.14 N, AI + T: 0.4147 ± 0.25 N) and material (Modulus: C: 2.535 ± 1.14 MPa, AI: 4.248 ± 1.21 MPa, T: 5.071 ± 1.47 MPa, AI + T: 2.575 ± 1.31 MPa) properties of the engineered ligaments but had no significant effect on collagen content (CON: 292.0 ± 34.69 μg, AI: 295.6 ± 62.17 μg, CON + T: $251.5.1 \pm 42.57$ μg, AI + T: 229.6 ± 44.67 μg). Letrozole prevented the beneficial effects of testosterone on mechanical and material properties while further decreasing collagen. AI significantly increased the expression of *COL3A1* and *LOX1*. These data suggest that testosterone opposes the effects of estradiol in that it has no effect on collagen content but improves sinew mechanics, possibly by increasing crosslinking.

Introduction

Estrogen is associated with the risk and progression of some cancers (Berry, 2005; Chumsri et al., 2011; Wood et al., 2009) and so drugs that inhibit the production of estrogen in postmenopausal women, such as aromatase inhibitors (AIs), are the first choice for therapeutic intervention (Chumsri et al., 2011; Gaillard & Stearns, 2011; Howell & Dowsett, 1997; Hyder et al., 2021; Wood et al., 2009). Aromatase (also called estrogen synthase) is a cytochrome p450 enzyme that is highly expressed in the ovaries of premenopausal women. It is expressed in lower levels in subcutaneous fat, liver, muscle, brain, and breast tissue (Hyder et al., 2021; Wood et al., 2009). There is also some indication that aromatase is expressed in ligaments (Rossi et al., 2010). The aromatase enzyme catalyzes the conversion of testosterone (T) into estradiol and is therefore a key enzyme in the biosynthesis of estrogens. Following menopause, when the ovaries no longer produce estrogen and aromatase activity in the ovaries drops, the conversion of testosterone to estrogen outside of the ovary becomes the primary synthetic pathway for E2 within the body. AIs, therefore, decrease plasma E2 levels (Knobloch, 2016) and are used as an adjunct therapy for estrogen receptor-positive invasive breast cancers (Goss et al., 2005; Winer et al., 2005).

While AIs have produced great therapeutic benefit in the treatment of cancers, studies on women treated with AIs report musculoskeletal symptoms as one of the most common adverse effects of the therapy (Henry et al., 2008; Morales et al., 2007). The most common recorded cases of musculoskeletal issues pertain to bone and muscle injuries/diseases such as bone fracture, myalgias, and arthralgias, and increased risk of osteoporosis (Mitsimponas et al., 2018). There have also been reports of tendon-related issues that were reversed with discontinuation of the AI therapies (Henry et al., 2008; Knobloch, 2016; Morales et al., 2007) A more recent study highlighted three cases of severe tendinopathies and muscle-tendon rupture in patients treated with the aromatase inhibitor letrozole (Mitsimponas et al., 2018). In all three cases, there were no signs of any underlying systemic disease (other than breast cancer), no abnormal physical activity, and no use of any drugs besides letrozole (Mitsimponas et al., 2018). In two of

the three patients, muscle-tendon ruptures were reported which, before this study had not been recorded in the literature. Taken together, these studies suggest aromatase inhibitors produce a functional deficit in collagen-containing tissues likely by inhibiting the conversion of testosterone to estrogen.

To determine whether the AI letrozole had a direct effect on sinew, I first conducted a dose-response experiment of 3D engineered ligaments treated with AI. The experiment consisted of four groups: 0 ng/mL (CTRL), 1 ng/mL (Low AI), 10 ng/mL (Med AI), 100 ng/mL (High AI) of letrozole. The tissues were cultured for 6 days with AI before mechanical testing and collagen quantification. There was no difference in the mechanical and material properties of the tissues, or their collagen content, suggesting that the drug had no direct negative effect on sinew structure-function. Having established that the drug had no direct effects, I next sought to determine whether inhibiting the conversion of testosterone to estradiol could alter sinew structure and function. Therefore, the experiments in this chapter compare the effects of AI and T alone with the combination of AI and T.

Materials and Methods

Cell isolation

Refer to Chapter 2 for a full description of cell isolation methods.

Ligament Formation

Refer to Chapter 2 for a full description of ligament formation methods.

Letrozole and Testosterone Treatments

The cytotoxicity experiment assigned the ligaments engineered from human ACL fibroblasts isolated from female donors into four groups: 0 ng/mL (CTRL), 1 ng/mL (Low AI), 10 ng/mL (Med AI), 100 ng/mL (High AI) of letrozole. The remaining experiments involved four different treatment groups: 1)

control (CON: 0 ng/mL AI; 0 ng/mL T); 2) letrozole (AI: 100 ng/mL AI; 0 ng/mL T); 3) testosterone (CON + T: 0 ng/mL AI; 100 ng/mL T); and 4) letrozole + testosterone (AI + T: 100 ng/mL AI; 100 ng/mL T).

Tensile Testing

Refer to Chapter 2 for a full description of Tensile testing methods.

Collagen

Refer to Chapter 2 for a full description of Collagen methods.

Gene Expression

Refer to Chapter 2 for a description of Gene Expression methods.

Western Blot

Human ACL fibroblasts isolated from female donors were expanded and plated into 24-well plates. They were allowed to proliferate until 100% confluent and then split into 4 groups to be treated with ascorbic acid free feed media (CON: 0 ng/mL AI; 0 ng/mL); letrozole (AI: 100 ng/mL AI; 0 ng/mL T); testosterone (CON + T: 0 ng/mL AI; 100 ng/mL T); or letrozole + testosterone (AI + T: 100 ng/mL AI; 100 ng/mL T). Refer to chapter 2 for further explanation of Western Blot methods.

Statistical analysis

Data comparing the effect of AI and T were grouped and analyzed using a 2-way ANOVA. The first factor was AI, and the second factor was T. Interactions between AI and T were also identified using GraphPad Prism. Where significant effects were noted, Tukeys HSD post-hoc testing was performed. For a complete description of the statistical analysis please see Chapter 2.

Results

Cytotoxicity Experiments

There were no differences observed in the mechanical and material properties of constructs treated

with increasing doses of letrozole. Similarly, no differences were seen in collagen content (fig 4.1).
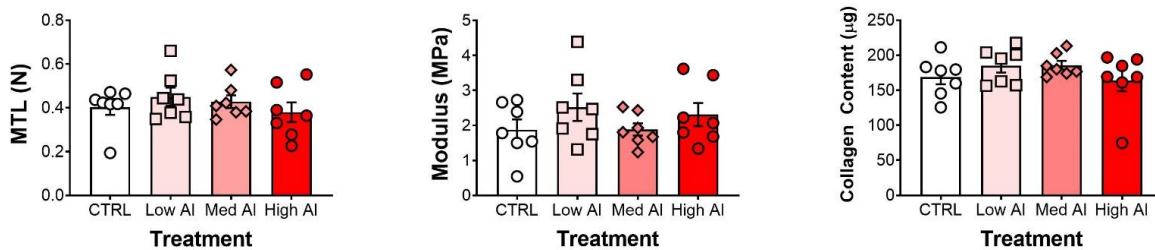
Fig 4.1. Letrozole does not affect human-engineered ligaments' tensile properties or collagen content.

(A) Maximum Tensile load (MTL), (B) Young's Modulus, and (C) Collagen content of 3-D engineered

ligaments treated with 0 ng/mL (CTRL), 1 ng/mL (Low AI), 10 ng/mL (Med AI), 100 ng/mL (High AI) of

letrozole. Data are representative of 2 trials using 2 donors and at least 7 technical replicates. *p < 0.05

significant difference

Mechanical and material properties of testosterone and letrozole treated engineered ligaments

Testosterone significantly increased both mechanical (MTL: CON: 0.5091 ± 0.25 N, AI: 0.7534 ±

0.17 N, CON + T: 0.8566 ± 0.14 N, AI + T: 0.4147 ± 0.25 N; fig 4.2A) and material (Modulus: CON: 2.535 ±

1.14 MPa, AI: 4.248 ± 1.21 MPa, CON + T: 5.071 ± 1.47 MPa, AI + T: 2.575 ± 1.31 MPa; UTS: CON: 2.535 ±

1.14 MPa, AI: 4.248 ± 1.21 MPa, CON + T: 5.071 ± 1.47 MPa, AI + T: 2.575 ± 1.31; fig 4.2B, 2.4C) properties

of the engineered ligaments. These increases were prevented by co-treatment with letrozole where MTL

and modulus were not different than control. There was also a statistical difference between the cross-

section areas (CSA) of the AI group (0.7133 ± 0.07 mm^2; fig 4.2D) and the AI + T group (0.8717 ± 0.05 mm^2;

fig 4.2D) but not between any other groups. The level of T (p = 0.0304) and the interaction between AI

and T (p = 0.0336) were significantly associated with the CSA of the constructs. The interaction between

AI and was significant for MTL (p =0.0007), UTS (p = 0.0002), and modulus (p = 0.0007).
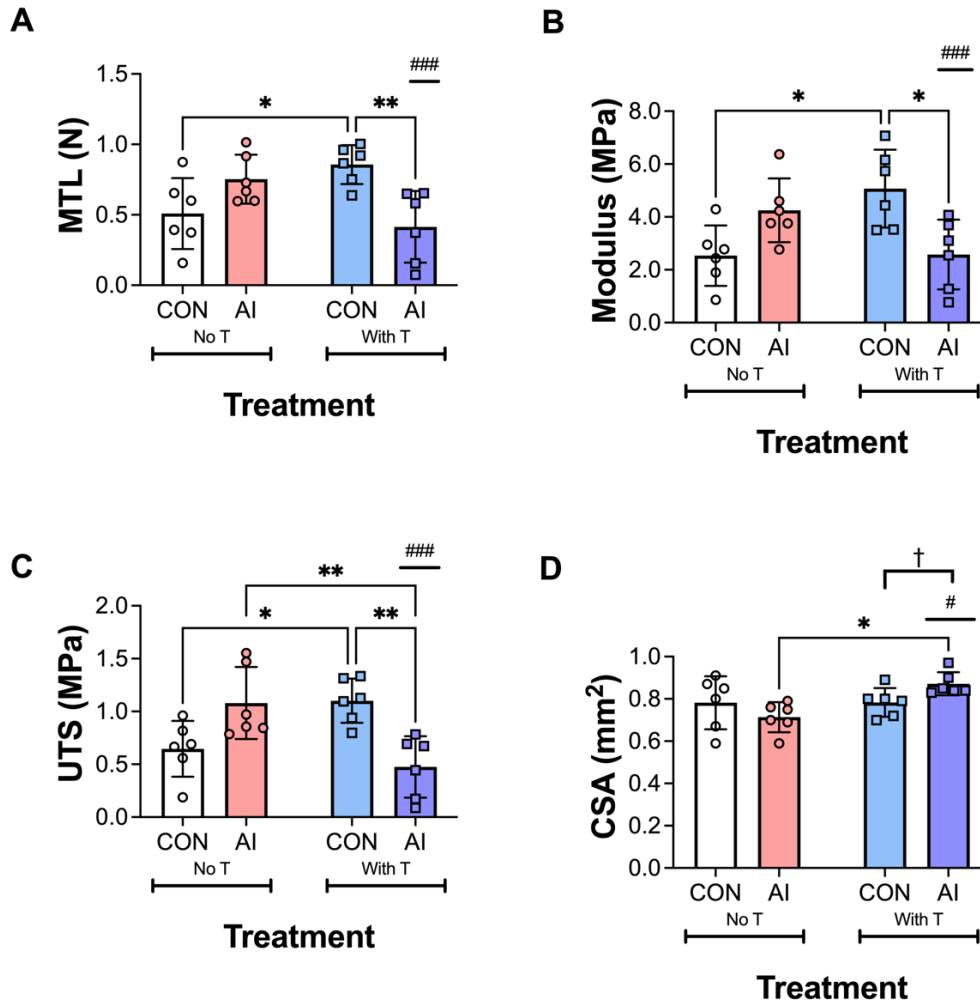
Fig 4.2. The increase in tensile properties with Testosterone is blocked by Letrozole. *(A) Maximum*

Tensile Load (MTL), (B) Young's Modulus, and (C) UTS of 3-D human-engineered ligaments increase with

Testosterone (T) and Letrozole (AI) treatment. This increase is blocked when treated with both T and AI.

There is no significant difference in the cross-section area (D) with either treatment. Data are

*representative of 5 experiments using 2 donors and 6 technical replicates per group. * indicates p ≤ 0.05,*

*whereas ** indicates p ≤ 0.01. ### indicates an interaction effect p ≤ 0.001. † indicates main effect of T p*

≤ 0.05.

Effects of Testosterone and Letrozole on collagen content and synthesis

Testosterone and collagen content and concentration. While there was no significant difference between the collagen content of the different treatment groups (CON: 292.0 ± 34.69 µg, AI: 295.6 ± 62.17 µg, CON + T: 251.5 ± 42.57 µg, AI + T: 229.6 ± 44.67 µg; fig 4.3A), the AI + T group demonstrated significantly lower collagen concentration than the AI group (CON: 10.19 ± 0.84 %, AI: 11.60 ± 1.16 %, T: 9.617 ± 1.06 %, AI + T: 8.825 ± 2.29 %; fig 4.3C) and T demonstrated a main effect of decreasing collagen content (p = 0.0144) and concentration (p = 0.0099).
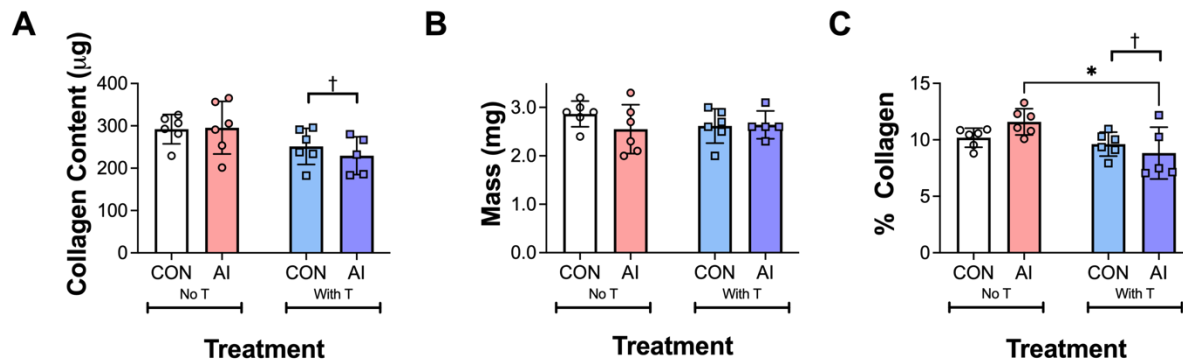
Fig 4.3. No difference in Collagen content between treatment groups. *Collagen Content (A) and collagen concentration (C) of the constructs showed a main effect of T even though the only group difference was observed in the collagen concentration between the AI and AI + T groups. Mass (B) was unchanged. Data are representative of 5 experiments using 2 donors and 6 technical replicates per group. * indicates p ≤ 0.05. † indicates main effect of T p ≤ 0.05, whereas †† indicates main effect of T p ≤ 0.01.*

No difference in collagen synthesis was observed with treatment. There was no statistical difference observed in procollagen synthesis with either T or AI treatment (CON: 292.0 ± 34.69 mg, AI: 295.6 ± 62.17 mg, CON + T: 251.5.1 ± 42.57 mg, AI + T: 229.6 ± 44.67 mg; fig 4.4)
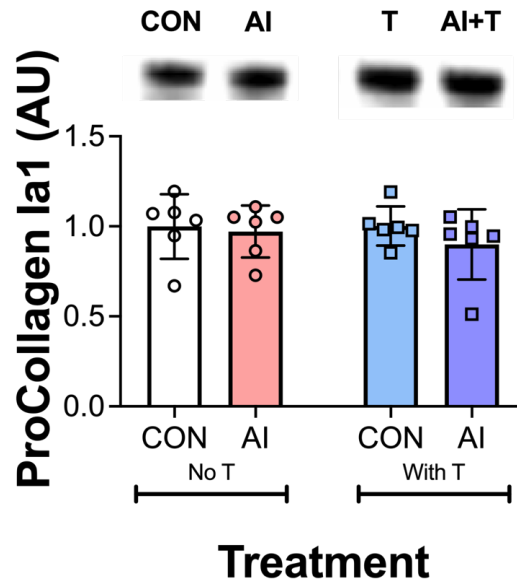
Fig 4.4. Procollagen protein synthesis is unaffected by Testosterone or Letrozole treatment. *Western Blot data from constructs shows that treating the engineered ligaments with either Testosterone (T) or Letrozole (AI) did not affect the synthesis of Procollagen 1a1. Data are representative of 2 experiments using 6 technical replicates per group.*

Effects of testosterone and letrozole on gene expression in ligaments

There was a main effect of T on *ESR1* (p = 0.0239) and *ESR2* (p = 0.0042) expression. Group differences were observed for *ESR2* between the control and T groups (*ESR1*: CON: 1.471 ± 2.54 , AI: 0.720 ± 0.30, CON + T: 0.224 ± 0.09, AI + T: 0.439 ± 0.34; *ESR2*: CON: 1.361 ± 0.72 , AI: 1.051 ± 0.50, CON + T: 0.343 ± 0.07, AI + T: 0.394 ± 0.36; fig 4.5A, 5.4B). T had a significant effect on *ESR1* (p = 0.0239) and *ESR2* (p = 0.0042) mRNA expression. There was tendency for *LARP6* mRNA expression to decrease with T treatment (*LARP6*: CON: 1.432 ± 0.80 , AI: 1.257 ± 0.60, CON + T: 0.551 ± 0.18, AI + T: 0.970 ± 0.76; fig 4.5C). *LOX1* showed no statistical differences between treatments groups (*LOX1*: CON: 1.009 ± 0.24 , AI: 1.888 ± 0.54, CON + T: 1.499 ± 0.76, AI + T: 2.016 ± 0.73; fig 4.5D), but AI had a main effect on *LOX1* expression (p = 0.0388). *COL1A1* and *COL3A1* mRNA were unaffected by treatment with T but there was a main effect of AI on *COL3A1* (*COL1A1*: CON: 1.083 ± 0.24 , AI: 1.645 ± 0.58, CON + T: 1.143 ± 0.43, AI +

T: 1.372 ± 0.45; *COL3A1*: CON: 1.050 ± 0.20 , AI: 1.504 ± 0.36, CON + T: 1.085 ± 0.36, AI + T: 1.595 ± 0.57;

fig 4.5E, 5.4F). *MMP13* was unaffected by treatment with either AI or T (CON: 1.109 ± 0.22 , AI: 1.706 ±

0.50, CON + T: 1.818 ± 0.47, AI + T: 1.678 ± 1.03; fig 4.5G)
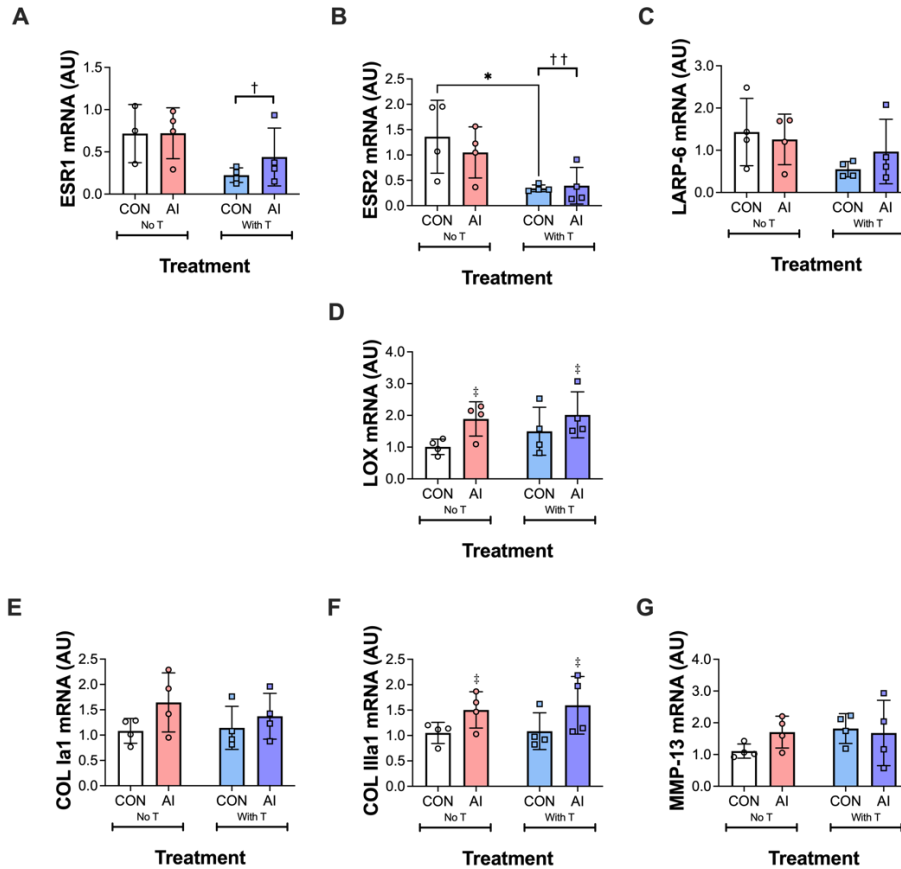
Fig 4.5. Letrozole increases Col1 and Col3 mRNA expression while Testosterone tends to decrease *LARP6*

mRNA expression. *(A) ESR1, (B) ESR2, (C) LARP6, (D) LOX1, (E) COL1A1, (F) COL3A1, and (G) MMP13 mRNA*

expression data from constructs treated with either Letrozole (AI), Testosterone (T), or a combination of

*both. Data are representative of 2 experiments using 4 technical replicates per group. * indicates p ≤ 0.05,*

† indicates main effect of T p ≤ 0.05, †† indicates main effect of T p ≤ 0.01, and ‡ indicates a main effect

of AI p ≤ 0.05.

Discussion

Post-menopausal women with estrogen receptor-positive breast cancers are often treated with the reversible aromatase inhibitor (AI) letrozole (Chumsri et al., 2011; Howell & Dowsett, 1997; Wood et al., 2009). However, letrozole is associated with a host of musculoskeletal effects including tendinopathies and in some cases muscle-tendon rupture (Gaillard & Stearns, 2011; Hyder et al., 2021; Mitsimponas et al., 2018). To investigate how AIs could affect tendon/ligament health, I first tested the cytotoxicity of the drug by subjecting our engineered ligaments to increasing doses of letrozole and found no differences in tensile properties or collagen content. Since the drug had no effect on its own, I next tried to mimic the hormonal milieu in a postmenopausal woman by adding testosterone (T) in the absence of exogenous estradiol (E2). The resulting experiments demonstrated that T increased the mechanical and material properties of sinews without increasing collagen content. Co-treatment with T and AI prevented the increase in sinew mechanics induced by T, suggesting that in postmenopausal women connective tissue stiffness may be maintained through the combined effects of higher T and an important, albeit lower E2 level. However, when the conversion of T to E2 is prevented, sinew mechanics decrease, and this decrease may make tendons and ligaments more prone to injury.

Despite positive effects on the tensile properties of the engineered ligaments, T had a main effect to decrease collagen content and concentration. As the mechanical and material properties of ligaments are functions of both collagen content and the density of the collagen cross-links (Curwin et al., 1994; C. A. Lee et al., 2015b; Marturano et al., 2014a), this data would suggest that the improvement in tensile properties is likely associated with collagen crosslinking. With estradiol, the decrease in mechanics at the same or greater collagen content with E2 was the result of the inhibition of lysyl oxidase (C. A. Lee et al., 2015b, Chapter 2). T may work counter to E2 and increase mechanical and material properties with less collagen through increasing crosslinking. Even though increasing crosslinking might improve power and decrease injury rates in males, excessive collagen crosslinking has been linked to adverse cardiovascular

effects (López et al., 2016). If T is making sinew stiffer by inducing more collagen crosslinking, this effect is not isolated to connective tissues, so stiffness in all tissues with collagen should be increasing. This would imply that collagen in blood vessels is stiff as well and cannot expand as easily leading to increased blood pressure. That could explain the association between T and cardiovascular diseases (CVDs) such as coronary artery disease and hypertension (Elagizi et al., 2018; Gandaglia et al., 2014; Kloner et al., 2016; Oskui et al., 2013; Webb & Collins, 2017). Supporting the hypothesis that estrogen and testosterone operate as direct opposites, estrogen protects against many CVDs with premenopausal women at lower risk for CVDs and the risk for coronary artery disease increases with menopause and/or estrogen deficiency (Kloner et al., 2016; Lagranha et al., 2010; Murphy, 2011).

Surprisingly, while testosterone and letrozole individually had positive effects on tensile properties of the engineered ligaments, the combination of both blocked their positive effects independently. For the combination of AI and T, where mechanics are decreased even though collagen remains unchanged, collagen crosslinking likely returns to baseline levels. Together, these data suggest that T is not only blocking the effects of E2 on lysyl oxidase activity, if this were the case mechanics would go up even more in the AI + T group where T is high and E2 is at its lowest. Instead, my data suggest that T requires a permissive level of E2 to increase the tensile properties of tissues and without any E2 testosterone is unable to alter crosslinking on its own.

Another interesting finding is that the main effect of T to decrease collagen content seemed unrelated to procollagen synthesis. Collagen content is the sum of collagen synthesis and collagen degradation (Mays et al., 1991) therefore the effect of T on the constructs is likely to increase collagen degradation. Once again, this would be opposite to what we have measured in response to E2 treatment (Chapter 2). Whereas E2 decreases degradation, T appears to increase breakdown. An increase in collagen degradation without a concomitant increase in synthesis would lead to a progressive decrease in collagen content, making the tendons progressively weaker but stiffer (brittle) and therefore more prone to

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injuries. This could explain the rate of tendon rupture with anabolic steroid use (Dunn et al., 2017) and the arthralgia, tendinopathies, and muscle-tendon rupture seen in postmenopausal women on AI therapy.

To show that testosterone treatment had biologically appropriate responses, I measured the expression of estrogen receptors 1 and 2 (*ESR1* and *ESR2*) as testosterone is known to interact with estrogen receptors (Asano et al., 2003; Dimitrakakis et al., 2003) and decrease their expression over time. Consistent with this past work, T's main effect was to decrease both *ESR1* and *2*. There was no change in collagen mRNA between treatment groups in line with the procollagen and collagen content data. However, it is worth noting that there was a main effect of AI, increasing expression of *COL3A1* and tending to increase *COL1A1* mRNA. This could mean that AI reduces the ratio of type 1 to 3 collagen in sinew. Over the short time of our experiments, this may not alter mechanics; however, extended changes in the ratio of 3 to 1 could lead to a decrease in mechanics over time. This modification could also be part of how AI attenuates the positive effects of T on tensile properties by increasing type 3 collagen. There was also a main effect of AI to increase *LOX1* mRNA, suggesting the increase in mechanics with AI alone could result from more *LOX1* production. However, why this would not be beneficial when combined with T remains a mystery.

Data from this chapter begin to explain how testosterone and estrogen combine to affect sinew function. However, there is still a lot of work to be done in this area. To complete these studies, I would want to directly measure collagen degradation using the hydroxyproline in the media assay as well as using zymography to measure matrix metalloproteinase (MMP) activity. We would also need to directly measure collagen crosslinking to confirm the effect of AI and/or T on collagen crosslinking. Studies have shown different crosslinks being modulated to maintain mechanical properties of ligament (Ramos et al., 2012) so an interesting possibility is that T and AI modulate different collagen crosslinks. To determine the exact crosslinks affected by AI and T, we could do liquid chromatography followed by mass spectrometry. This would be better than the rough estimate of crosslinking that we would get using digital

scanning calorimetry as explained in Chapter 2. An additional limitation could be due to our donor sources. Our donors are 18-20 years old but postmenopausal women are usually aged 50 and over. Any epigenetic modifications that could come with aging are therefore not considered in these studies. However, as we have not seen much epigenetic differences in our ACL cells in terms of their responses to treatments (C. A. Lee et al., 2015b), these results are likely still a validation indication of what occurs in postmenopausal women.

Conclusions

This data from this chapter demonstrated that testosterone works counter to E2 and increases sinew mechanics while decreasing collagen content. This improvement in mechanics is blocked with the use of the AI, Letrozole. As we see no difference in procollagen synthesis with AI or T treatment, we can infer that T most likely increases collagen degradation and crosslinking. Over time, this would be expected to result in smaller sinews that have higher stiffness. The addition of AI with T prevents the increase in stiffness without affecting the decrease in collagen content. Over time, this combination would result in small weak tissues that are more prone to injury. These data suggest that the tendon-related issues experienced by postmenopausal women on AI therapy may be due to a decrease in both collagen content and mechanics resulting in increased sinew laxity.

CHAPTER 5: DISCUSSION

Hormones have significant effects on musculoskeletal function. The different hormonal profiles in men and women underlie different levels of bone, muscle, and sinew mass as well as differences in tissue function, and/or injury rates. This dissertation focused on how hormones modulate sinew in particular- taking a deep dive into the effect of hormonal manipulation on the tensile properties and collagen given the importance of collagen content, orientation, and crosslinking on sinew function. The main findings of my dissertation are:

1. Estradiol tends to increase collagen content through inhibited collagen degradation, however, there is a much more pronounced inhibition of collagen crosslinking that causes decreased mechanical and material properties of sinew.

2. The phytoestrogen genistein has a similar but much more pronounced effect on collagen content leading to an increase in mechanical and material properties of the grafts. This suggests that genistein can improve collagen content without inducing an overriding decrease in collagen crosslinking the way the E2 does.

3. Testosterone has opposite effects on sinew that we see with E2 in that it increases the tensile properties of sinew without changing collagen content, suggesting stronger/more collagen crosslinks. However, letrozole blocks these effects suggesting that some estrogenic activity is necessary to preserve increases in tensile properties.

In vivo, estradiol levels are associated with knee laxity likely by decreasing ligament stiffness. Sinew stiffness is influenced by collagen content and crosslinking. Collagen content is in turn determined by the rates of collagen synthesis, degradation, and incorporation. My data clearly shows that E2 treatment results in a small (8-10%) increase in collagen content and concentration in our engineered ligament model. This effect on collagen content is consistent with a small, but reproducible decrease in

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collagen degradation. This, together with the fact that E2 does not increase procollagen synthesis, leads me to conclude that the slight increase in collagen content is the result of decreased collagen breakdown. Even with the increase in collagen content with E2 treatment, I did not see a commensurate improvement in mechanics. Instead, I consistently observed lower mechanics in the high collagen group. This finding is consistent with previous work from our lab showing that E2 inhibits crosslinking. The result is that sinews treated with high levels of E2 have more collagen but poorer mechanical and material properties. These results are consistent with regularly menstruating women having ligaments with much more laxity making them prone to ligament injuries. These data could explain the higher incidence of ACL injuries in women.

Surprisingly, the soy phytoestrogen, genistein, showed a much greater increase in collagen content and concentration than E2 (~60% versus 10%). At the lowest concentration tested, the increase in collagen content translated into an increase in mechanical properties. These data suggest that phytoestrogens like GE may be useful in increasing collagen content and sinew function. However, it is clear that too much GE has the potential to eliminate any benefit derived from this isoflavone. Therefore, to determine the optimal dose of GE we would need data on digestibility as well as the rate of delivery of GE to the tissue. Therefore, even though high levels of GE are cytotoxic, we hypothesize that GE might be useful in replacing estradiol in postmenopausal women. To estimate an appropriate dose, previous work in rats saw plasma levels of GE between 0.39 and 3.36 µM after feeding with 125-300 µg dietary genistein/g AIN-93G growing rodent diet (Ju et al., 2001). Together with a recent analysis of peritendinous amino acid concentration around the Achilles tendon of people after administration of an oral amino acid supplement saw leucine concentrations reach upwards of 150 µM two hours after supplement intake (Carroll et al., 2021). Since leucine content in the blood after a similar supplement would be approximately the same (Burke et al., 2012), this suggests that for tendons like the Achilles, it is possible to consume too much GE; however, how much would reach synovial ligaments like the ACL remains to be determined.

The male sex hormone testosterone (T) produced a diametrically opposite effect on ligament mechanics compared with estradiol and phytoestrogens. With T, I observed increased tensile properties with no change in collagen content suggesting more crosslinking. Together with the E2 data above, these data likely explain a number of performance, injury, and disease differences between men and women. For example, stiffer tendons and ligaments in males would be expected to increase force transfer resulting in greater power. Stiffer ligaments would also be expected to decrease rates of ligament rupture, whereas stiffer tendons would increase the rate of muscle pulls, both of which are seen in athletes. On the downside, stiffer collagen within the vasculature would be expected to increase blood pressure and result in higher rates of cardiovascular disease. All of these predictions based on the change in sinew mechanics I measured in this dissertation are seen in the literature. Therefore, changes in collagen crosslinking have the capacity to dramatically affect multiple health and performance outcomes.

Even though there are clear differences in sinew function based on hormone levels, I did not directly measure collagen crosslinking to directly test my hypothesis to explain the mechanism underlying the observations I have made. This is a significant limitation of my dissertation. To properly understand how these hormones affect collagen crosslinking, it would be best to directly identify and quantify the number and type of collagen crosslinks using liquid chromatography followed by mass spectrometry. Another way to estimate crosslinking would be to use a scanning calorimeter to determine the temperature at which the collagen denatures. Tissues with greater crosslinking would denature at a higher temperature since the extra crosslinking stabilizes the collagen within the tissue. Other important limitations of my dissertation include the lack of isolation of the hormone effects. Phenol-red free media was used to remove the estrogen mimetic effect of phenol-red; however, the growth media used still contained fetal bovine serum (FBS) which may have resulted in differences in hormone levels on our engineered ligaments. Using dextran-coated charcoal-stripped FBS would have been more suited to this hormone-related research.

Even with these limitations, my data, together with the existing literature suggest that despite a small inhibition of collagen degradation, estrogen's influence on sinew function and injury rates is largely a result of the inhibition of lysyl oxidase mediated collagen crosslinking resulting in weaker ligaments that are therefore more prone to injury. This effect could be ameliorated by supplementing with low doses of genistein which appears to have a protective effect on collagen degradation, as does E2, without the overriding inhibition of collagen crosslinking. Testosterone likely increases collagen crosslinking leading to positive but opposite effects to E2 on sinew mechanics, however, seeing as AI therapy blocks these positive effects, some estrogenic activity is needed.

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