Published in final edited form as:

Scand J Med Sci Sports. 2012 October ; 22(5): e108–e114. doi:10.1111/j.1600-0838.2012.01516.x.

The soy isoflavone genistein inhibits the reduction in Achilles tendon collagen content induced by ovariectomy in rats

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Abstract

The objective of this study was to evaluate effects of genistein and moderate intensity exercise on Achilles tendon collagen and cross-linking in intact and ovariectomized (OVX) female Sprague-Dawley rats. Rats were separated into eight groups (n=9 per group): intact or OVX, treadmill exercised or sedentary, genistein-treated (300 mg•kg⁻¹•day⁻¹) or vehicle. After 6-weeks, tendons were assayed for the collagen-specific amino acid hydroxyproline and hydroxylslypyridinoline (HP). Collagen content was not influenced by exercise (p=0.40) but was lower (p<0.001) in OVX vehicle rats compared to intact vehicle rats (OVX: 894±35 µg collagen/mg dry weight, intact: 1185±72 µg collagen/mg dry weight). In contrast, collagen content in OVX rats treated with genistein was greater (p=0.010, 1198±121 µg collagen/mg dry weight) when compared to untreated rats and not different from intact rats (p=0.89). HP content was lower in OVX genistein-treated when compared to intact genistein-treated rats, but only within the sedentary animals (p=0.05, intact-treated: 232±39mmol/mol collagen, OVX-treated: 144±21mmol/mol collagen). Our findings suggest that ovariectomy leads to a reduction in tendon collagen, which is prevented by genistein. HP content, however, may not have increased in proportion to the addition of collagen. Genistein may be useful for improving tendon collagen content in conditions of estrogen deficiency.

Keywords

hydroxyproline; hydroxylslypyridinoline; ovariectomized; exercise; phytoestrogen

Introduction

It is well documented that the loss of estrogen associated with post menopause results in an increased risk of cardiovascular disease, osteoporosis, and sarcopenia in women (Dionne et al. 2000; Maltais et al. 2009; Subbiah 2002). There is also recent evidence demonstrating that estrogen can influence tendon extracellular matrix (ECM), specifically collagen synthesis in women (Cook et al. 2007; Hansen et al. 2009; Hansen et al. 2008; Hansen et al. 2009). Indeed, *in vitro* studies of tendon fibroblasts suggest that estrogen deficiency may decrease collagen turnover (Irie et al. 2010) and tendon collagen synthesis is lower in post-menopausal women not taking estrogen therapy (ET) (Hansen et al. 2009). In experimental studies using the ovariectomized (OVX) rat the activity of the ECM degrading protein matrix metalloproteinase (MMP)-2 is increased in tendon (Pereira et al. 2010), which has been associated with a decrease in collagen content in some tissues (Jackson et al. 2002).

Supporting this hypothesis, a significant decrease in type I collagen content has been documented in non-tendon connective tissue in post-menopausal women (Moalli et al. 2004). Estrogen also appears to activate lysyl oxidase (Sanada et al. 1978), the enzyme regulating the addition of lysine and hydroxylysine-based cross-links into collagen fibrils (Eyre et al. 1984). Therefore, a lack of estrogen could alter collagen and/or cross-linking content of tendon, both of which are important for maintaining the tendon tensile strength and stiffness (Chan et al. 1998; Franchi et al. 2007).

Although ET is used as a substitute therapy to counter the natural loss of circulating estrogen levels in postmenopausal women (Ronkainen et al. 2009), this form of therapy is often associated with significant adverse health effects including increased incidence of coronary artery disease (Hulley et al. 1998; Manson et al. 2003) and certain cancers (Chen 2011; Furness et al. 2009). Genistein, a naturally occurring isoflavone phytoestrogen, has structural similarities to estrogen, and has been shown to bind to estrogen receptors (Casanova et al. 1999). We have demonstrated that short-term genistein treatment reduces arterial blood pressure and heart rate (Al-Nakkash et al. 2010) and increases cardiac ischemic tolerance in the OVX rat (Al-Nakkash et al. 2009). Thus, genistein has been promoted as an alternative to ET due to its beneficial effects on cardiovascular and reproductive health without the unequivocal effects on the female reproductive system (Anthony et al. 1996) or cancer incidence (Sliva 2008). Whether genistein treatment produces favorable effects on tendon collagen or cross-linking in the OVX rat remains to be determined.

Moderate intensity exercise is generally recommended for postmenopausal women to help maintain bone mass, reduce the risk of metabolic diseases, and improve quality of life (Kohrt et al. 2004). Whether exercise stimulates tendon collagen synthesis in women is still debatable, as studies have shown that exercise may (Hansen et al. 2008) or may not (Hansen et al. 2009; Miller et al. 2007) increase tendon collagen synthesis. Additionally, in young women tendon adaptations to exercise appear to be blunted when compared to males (Magnusson et al. 2007). In contrast, the effects of chronic exercise on tendon adaptations have not been extensively explored in estrogen deficient females. Furthermore, no studies to date have evaluated the combined effects of genistein treatment and exercise on tendon structural properties in estrogen deficit females. Therefore, in this study, we have evaluated the effect of ovariectomy and genistein treatment with or without treadmill exercise on Achilles tendon collagen content and hydroxylslypyridinoline (HP) cross-linking in female Sprague-Dawley rats. We hypothesized that ovariectomy would lead to a reduction in tendon collagen content and HP cross-linking and that genistein treatment and/or treadmill exercise would prevent any changes in collagen or HP cross-linking induced by ovariectomy.

MATERIALS AND Methods

Study Protocol

Female Sprague-Dawley rats (n=72) were purchased (200–300 grams, Harlan Laboratories, Indianapolis, IN) as either OVX or intact. These rats were randomly placed into vehicle or genistein-treated groups, then subdivided further into sedentary or exercise groups (Table 1). Animals were surgically implanted with a subcutaneous constant day-release pellet (300 mg genistein•kg body weight⁻¹•day⁻¹) or placebo (vehicle) pellet. Rats in the exercise groups completed treadmill running (Columbus Instruments Exer 3/6 treadmill, Columbus, Ohio) five days per week for six weeks. Exercise was progressed from 10 minutes to 30 minutes by adding five minutes per week. Speed was maintained at 15 meters•minute⁻¹ (~60% of estimated maximal oxygen uptake) for the first three weeks then increased to 20 meters•minute⁻¹ (~80% of maximal oxygen uptake) for the remaining three

weeks (Hoydal et al. 2007; Wisloff et al. 2001). Rats were caged in pairs, allowed access to genistein-free food (a specially formulated casein-based diet (Al-Nakkash et al. 2006), DyetsInc, Bethlehem, PA), and water ad libitum, and maintained on a 12-hour light-dark cycle. This investigation was approved by the Midwestern University Institutional Animal Care and Use Committee and all animals were cared for in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (National Research Council 2011).

Tissue Analysis

After the completion of the 6-week treatment period animals were euthanized and Achilles tendons were carefully extracted and immediately frozen in liquid nitrogen and stored at -80°C . Prior to the analysis of collagen and HP crosslinks, a 5–10 mg portion of the tendon was freeze dried for 36 hours and then reweighed to obtain tendon dry weight. Samples were then hydrolyzed for 24 hours at 100°C in 6 N HCl (Carroll et al. 2012).

Collagen Analysis—Tendon collagen concentration was determined by quantification of the collagen specific amino acid hydroxyproline (HYP) by high-performance liquid chromatography (HPLC) and fluorometric detection, as we have previously described (Carroll et al. 2012). Briefly, derivatized samples were injected onto an HPLC (LC-20AB and SIL-20, Shimadzu Scientific Instruments, Columbia, MD, USA) and separation of HYP was achieved via an XTerra RP 18, 5 μm , 250 mm $\times$ 4.6 mm column (Waters, Milford, MA, USA) using an isocratic mobile phase of 35% acetonitrile and 65% acetic acid (3% glacial acetic acid, sodium acetate buffered to pH 4.3) at a $1.0\text{ mL}\cdot\text{min}^{-1}$ flow rate. Peaks were monitored at 260 nm excitation/316 nm emission (RF-10AXL, Shimadzu Scientific Instruments) and integrated with chromatography software (LC Solution Ver. 1.2, Shimadzu Scientific Instruments, Columbia, MD, USA).

Collagen Cross-linking Analysis—Hydroxylslypyridinoline concentrations were determined using HPLC, as we have previously described (Carroll et al. 2012). A 500 μL aliquot of the hydrolyzed sample (see *Collagen Analysis*) was evaporated to dryness overnight at ambient temperature (Thermo Fisher Scientific Savant SPD131DDA SpeedVac Concentrator, www.fishersci.com), reconstituted in cross-link buffer (0.5% (v/v) heptafluorobutyric acid (HFBA) in 10% acetonitrile), and injected into the HPLC system described above. Samples were compared to known HP standards (PYD/DPD HPLC Calibrator, 8004, Quidel Corp., San Diego, CA, USA). Separation was achieved with a Restek RP C18, 5 μm , 150 $\times$ 4.0 mm ID column (9174564, Restek Corporation, Bellefonte, PA, USA) using an isocratic method [mobile phase A (0.13% HFBA) and mobile phase B (0.13% HFBA, 75% acetonitrile)]. Samples were eluted using 17% mobile phase B from 0–17 minutes, followed by 100% mobile phase B for a 5-minute wash. The column was then re-equilibrated with 17% mobile phase B for 8 minutes prior to the next injection. A flow rate of 1.0 mL/min was used. Fluorescence was monitored at 295 nm excitation/395 nm emission and peaks were integrated with chromatography software (LC Solution Ver. 1.2).

Statistics

Animal body weight was evaluated with a balanced two-way repeated measures analysis of variance (ANOVA) while all other variables were evaluated with a balanced three-way ANOVA [exercise (sedentary vs. exercise), treatment (vehicle vs. genistein), and condition (intact vs. OVX)]. The Student-Newman-Keuls post-hoc test was used to explore differences when a significant interaction was detected. Values were considered significant at an alpha level of $p < 0.05$. All data are expressed as mean $\pm$ standard error. All data were analyzed using SigmaPlot Version 11 (Systat Software, Inc, Chicago, IL).

Results

Body Weight

The effects of ovariectomy, genistein treatment, and exercise training on body weight are shown in Table 2. Body weight increased in all groups ($p<0.001$) during the 6-week intervention. A significant main effect for condition (intact vs. OVX) was detected ($p<0.001$). Post hoc testing indicated that the OVX rats treated with vehicle weighed significantly more than intact animals independent of exercise training.

Collagen and Tissue Water Content

Tissue water content was not influenced by exercise, ovariectomy, or treatment with genistein ($p=0.61$; Figure 1), thus all collagen data are expressed relative to tissue dry weight. Tendon collagen content was not influenced by exercise (Figure 2) and no significant three-way interaction was detected ($p=0.40$). There was, however, a significant two-way interaction ($p=0.025$, Figure 2b) for condition (intact vs. OVX) $\times$ treatment (vehicle vs. genistein). The post-hoc analysis of this two-way interaction indicated that: 1) in OVX-vehicle rats, tendon collagen content was 28% lower compared to intact-vehicle animals (OVX: 894 ± 35 $\mu\text{g collagen}\cdot\text{mg dry weight}^{-1}$, intact: 1185 ± 72 $\mu\text{g collagen}\cdot\text{mg dry weight}^{-1}$, $p<0.001$, Figure 2b), 2) treatment with genistein did not alter collagen content in intact animals (intact-vehicle: 1185 ± 72 $\mu\text{g collagen}\cdot\text{mg dry weight}^{-1}$, intact-genistein: 1099 ± 42 $\mu\text{g collagen}\cdot\text{mg dry weight}^{-1}$, $p=0.55$, Figure 2b) and 3) OVX animals treated with genistein had greater collagen content (Figure 2b) than untreated OVX animals (OVX-genistein: 894 ± 35 $\mu\text{g collagen}\cdot\text{mg dry weight}^{-1}$, OVX-vehicle: 1198 ± 121 $\mu\text{g collagen}\cdot\text{mg dry weight}^{-1}$, $p=0.010$) weight.

Collagen HP Cross-linking

A significant ($p=0.014$) three-way interaction was detected for HP cross-linking with post-hoc testing indicating a condition (intact vs. OVX) $\times$ treatment (vehicle vs. genistein) interaction within the sedentary animals ($p=0.05$, Figure 3a). Specifically, HP cross-linking was lower in genistein-treated OVX rats when compared to intact rats (Figure 3a) but only when considering the sedentary animals. There was an opposite trend in the exercise animals, i.e. HP cross-linking was greater in genistein treated OVX animals when compared to untreated animals but lower in intact treated animals compared to untreated intact rats (Figure 3b). This difference, however, did not reach statistical significance ($p=0.12$).

Discussion

To our knowledge this is the first investigation to examine the interactive effect of ovariectomy, genistein treatment, and exercise training on collagen and HP cross-linking content of tendon. Neither moderate intensity physical training nor genistein treatment had an influence on Achilles tendon collagen content in intact animals. In contrast, our results show that ovariectomy resulted in a substantial decrease in Achilles tendon collagen content, which was prevented by genistein treatment. The lack of an effect of genistein in intact animals suggests that genistein supplementation in young females may not have the negative effects on tendon often associated with exogenous estrogen given to young females (Hansen et al. 2008; Hansen et al. 2009). These data are also consistent with *in vitro* studies demonstrating no effect of physiological estrogen levels on fibroblast collagen synthesis (Seneviratne et al. 2004). We also observed a trend ($p=0.05$, Figure 2a) for collagen HP cross-linking content to be lower in sedentary genistein-treated OVX rats, suggesting that the addition of HP cross-links may not have been proportional to the addition of collagen with genistein treatment in these animals. Exercise, however, seemed to normalize HP cross-link content in genistein-treated OVX rats (Figure 2b). The observed effect of genistein on

tendon collagen in OVX rats is considerable and suggests that the further study of genistein as a beneficial therapy for conditions of estrogen-deficiency is warranted.

The only known enzyme regulating the addition of HP cross-links into collagen fibrils, lysyl oxidase, has been shown to be activated by estrogen in skin and bone (Sanada et al. 1978). Our data, however, suggest that the presence of estrogen is not an absolute requirement to maintain the appropriate ratio of HP cross-links per collagen molecule, i.e. HP cross-linking likely decreased in proportion to the decrease in collagen content with ovariectomy. Although genistein treatment increased collagen content in OVX animals, cross-linking may not have increased in proportion to the addition of collagen, at least in sedentary animals. It is possible that the enhancement of collagen production by genistein also altered the fibril diameter distribution in favor of smaller diameter fibrils, as seen with estrogen therapy (Hansen et al. 2009), which could reduce the possibility for establishment of intra-molecular cross-links. A longer treatment regimen or the addition of exercise may be needed to increase HP cross-linking in proportion to collagen in OVX animals treated with genistein.

The lack of an observed effect of exercise training on collagen and HP cross-linking content in our intact animals could be due to the intensity of exercise training or the sex of the animals. Several studies (Magnusson et al. 2007; Miller et al. 2007) have demonstrated that the ability of tendon to adapt to exercise is blunted in females and our findings would seem to confirm these data. Additionally, the intensity and/or duration of exercise training may not have been adequate to induced changes in tendon structural properties. Using a treadmill protocol of higher intensity and longer duration (60 minutes per day for 8 weeks) we have recently demonstrated that chronic treadmill exercise increases Achilles tendon collagen HP cross-linking but not collagen content in male rats (Carroll et al. 2012).

In line with our hypothesis, ovariectomy resulted in a substantial decline in tendon collagen content and this effect was prevented by administration of genistein. The decline in tendon collagen is consistent with previous studies in non-tendon connective tissue where type I collagen content was reduced (Moalli et al. 2004) and MMP-2 activity increased (Pereira et al. 2010) in estrogen-deficient states. Tendon strain for a given stress is higher in females compared to males regardless of age (Carroll et al. 2008). A decline in collagen content in postmenopausal women could result in a weakened tendon (Davison 1989) and even greater strain at a given stress, which may increase the tendon's susceptibility to strain injury (Magnusson et al. 2007; Riley 2008; Wang et al. 2006).

The ability of genistein to enhance collagen content in the OVX rat is substantial given the fact that the rats were treated for a period of only 6 weeks. Although future studies are needed to define the mechanism(s) by which genistein influences tendon ECM, tendons do express estrogen receptors (Sciore et al. 1998) and genistein is known to bind estrogen receptors and have estrogenic effects on other tissues (So et al. 1997; Suetsugi et al. 2003; Tissier et al. 2007; Zava et al. 1997). In heart tissue, estrogen treatment has been shown to decrease MMP-9 activation and reduce collagen accumulation in volume-overload hearts of OVX rats (Voloshenyuk et al. 2010), suggesting that genistein may have decreased the increase in MMP activity associated with ovariectomy (Pereira et al. 2010). Genistein may also directly influence collagen synthesis. For example, in a model of oxidative stress where collagen synthesis is suppressed, genistein treatment prevented the oxidant induced decrease in collagen synthesis (Sienkiewicz et al. 2008). The effect of genistein on collagen synthesis may be via the IGF-1 signaling pathway, an important growth factor regulating collagen synthesis (Murphy et al. 1997). In studies of human fibroblasts, genistein has been shown to upregulate insulin-like growth receptor protein expression (Sienkiewicz et al. 2008). Additionally, in contrast to estrogen therapy (Hansen et al. 2009), which decreases IGF-1 bioavailability, genistein treatment increases serum IGF-1 in postmenopausal women (Marini

et al. 2007). These differences between genistein and estrogen highlight the need for additional studies evaluating the mechanism(s) by which genistein influences tendon collagen.

Interestingly, genistein appears to reduce collagen accumulation (i.e., fibrosis) in the heart (Mizushige et al. 2007; Voloshenyuk & Gardner 2010) and lung (Day et al. 2008) in pathological states. Furthermore, in contrast to tendon, collagen content is elevated in some connective tissues in post-menopausal women (Falconer et al. 1996), suggesting either a tissue specific effect of estrogen deficiency and genistein action and/or a different mechanisms of action on collagen metabolism in healthy versus diseased states.

PERSPECTIVE

In summary, we demonstrate that genistein treatment may be an effective means to prevent a decline in tendon collagen content in a rat model of the postmenopausal state. Moreover, our findings confirm that estrogen is an absolute requirement for maintaining normal levels of tendon collagen in females. As the most abundant protein in mammals, collagen is an important component of many tissues and is the predominant protein in tendon. Collagen is also an important constituent of bone, skeletal muscle, and heart, thus our findings may have implications for these tissues. The lower HP content in the OVX-treated sedentary rats suggests that the addition of HP cross-links may not have been proportional to the addition of collagen in these animals. Exercise, however, seemed to normalize HP cross-linking in genistein-treated OVX rats. The ability of genistein to prevent declines in tendon collagen may have important implications for post-menopausal women and our findings continue to support the potential health benefits of genistein consumption. Future studies are needed to evaluate the functional implications of genistein's effect on tendon and whether these effects extend to other tissues.

Acknowledgments

The authors would like to thank Jamie Tedeschi for assistance with animal training and Jason Kamilar, PhD for statistical guidance. Funding Support: Midwestern University Kenneth A. Suarez Summer Research Fellowship to J.E. Ramos and M.S. Moore, College of Health Sciences, Midwestern University Intramural Awards to C.C. Carroll and T.L. Broderick, and NIH 1R15DK071625-01A2 to L. Al-Nakkash.

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Figure 1. Achilles tendon water content (percentage of total tissue weight). Intact (I), Ovariectomized (O), Vehicle (V), Genistein (G), Sedentary (S), Exercise (E). Data presented as mean $\pm$ standard error.

Figure 2.

Achilles tendon collagen content normalized to tendon dry weight. a) All groups. Intact (I), Ovariectomized (O), Vehicle (V), Genistein (G), Sedentary (S), Exercise (E). Each bar represents $n=9$; b) Data highlighting two-way [condition (intact vs. ovariectomized) $\times$ treatment (vehicle vs. genistein)] interaction. Each bar represents $n=18$. * $p<0.001$, Intact-Vehicle vs. OVX-Vehicle. † $p=0.010$, OVX-Vehicle vs. OVX-Genistein. Ovariectomized (OVX). Data presented as mean $\pm$ standard error.

A: HP Cross-linking, Sedentary Rats Only **B: HP Cross-linking, Exercised Rats Only** **Figure 3.**

Achilles tendon hydroxylysypyridinoline (HP) normalized to collagen content. a) plot of the two-way [condition (intact vs. ovariectomized) $\times$ treatment (vehicle vs. genistein)] interaction (* $p=0.052$) within the sedentary rats. b) plot of the two-way [condition (intact vs. ovariectomized) $\times$ treatment (vehicle vs. genistein)] interaction ($^{\dagger}p=0.119$) within the exercise rats. Data presented as mean $\pm$ standard error.

Table 1**Groups Assignments**

Ovary Status	Physical Activity	Treatment
Intact	Sedentary	Vehicle
		Genistein
	Exercise	Vehicle
		Genistein
Ovariectomized	Sedentary	Vehicle
		Genistein
	Exercise	Vehicle
		Genistein

Female Sprague-Dawley rats were randomized into a total of 8 groups (n=9/group) according to presence/absence of ovaries, 6 week of exercise or sedentary lifestyle, and 6 weeks of $300 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ genistein constant regime pellet supplementation or empty control vehicle.

Table 2

Animal Body Weights

	IVS	IVE	IGS	IGE	OVS [†]	OVE [‡]	OGS	OGE
Initial Body Weight (g)	240±10	228±8	232±15	237±15	274±10	284±12	245±5	261±14
Final Body Weight (g)	269±12 *	268±8 *	263±16 *	264±11 *	318±9 *	327±6 *	307±8 *	300±10 *

* p<0.001, main effect increase with time in all groups.

[†] p<0.001, main effect for condition (intact vs. OVX), OVS and OVE greater than all intact groups. Intact (I), Ovariectomized (O), Vehicle (V), Genistein (G), Sedentary (S), Exercise (E). n=9 animals per group.