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Serum relaxin levels affect the in vivo properties of some but not all tendons in normally menstruating young women

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ABSTRACT

Relaxin (hRLX) is a hormone reported to affect collagen synthesis. Its effects are also thought to be modulated by other sex hormones, including oestrogen, which has previously been found to be associated with alterations of in vivo tendon properties. There is thus a potential for hRLX to impact on collagen, which could result in tendon structural and mechanical properties being modified. The present study therefore aimed to determine any interaction between hRLX and tendon stiffness, in normally menstruating women (n =12). Tendon properties were determined using a combination of dynamometry and B-mode ultrasound, whilst serum hRLX levels were established by ELISA. Serum hRLX level was seen to be negatively associated with patellar tendon stiffness ($r = -0.56$; $P < 0.001$), explaining 31% of the variance in this parameter. There was no association between hRLX and gastrocnemius tendon stiffness ($P > 0.05$), or with the cross-sectional area of either of the two tendons ($P > 0.05$). In young, normally menstruating women, hRLX appears to have a significant effect on the patellar but not the gastrocnemius tendon stiffness. Where it has an effect, this appears to be on the intrinsic properties rather than on the dimensions of said tendon. Future work to elucidate the physiological cause of this selectivity in the impact of relaxin will be key to mapping the impact of the endocrine system on the phenotype of tendinous tissue.

Relaxin is related to the insulin family and is described as a pleiotropic peptide hormone with a range of physiological targets and actions. It was first described by Frederick Hisaw in 1926 (Hisaw, 1926), who showed in animals that ligaments relaxed following injection with pregnant guinea-pig serum. In humans to date, three different isoforms of relaxin have been described, namely H1, H2 and H3 (Hudson *et al*. 1983, 1984; Bathgate *et al*. 2002, respectively). However, relaxin is not solely a pregnancy related hormone; in non-pregnant women, relaxin has been detected during both the follicular and the luteal phases of the menstrual cycle (Wreje *et al*. 1995).

The research to date indicates that, across several species, only in females, there are measurable serum levels of relaxin. We propose that it is this circulating relaxin that has the potential to affect peripheral tissues, such as tendon, via its known action on collagen content. Indeed, relaxin has been reported to reduce collagen turnover both in animals and in vitro, resulting in a reduced tissue collagen content (Unemori & Amento, 1990; Samuel et al. 1996). Through its effects on collagen and extracellular matrix components, relaxin therefore has the potential to affect the mechanical properties of connective tissues. Following the identification of relaxin receptors on the anterior

cruciate ligament in women (Dragoo et al. 2003; Galey et al. 2003), it has been shown in an animal model (the guinea pig), that following 21 days of treatment with relaxin, females' anterior cruciate ligament was significantly weaker during load to failure testing than untreated control ligaments (μ=40.4 *vs*. 64.1 N, P =0.001; Dragoo *et al*. 2009). As relaxin has been shown to affect the constituents of a variety of connective tissues, it is possible that it could affect the mechanical properties of tendon.

Reductions to the integrity of collagenous tissue, such as tendon, via changes to the extracellular matrix can not only increase the risk of injury (i.e. tendon ruptures and tears), but also alter the motor patterns and control, leading to muscular or other related injuries via changes to the tendon integrity and elasticity. Tendon elasticity is known to affect both the velocity of muscle shortening and the length of the contractile component, which in turn directly influence the force generated. Tendon properties also have the capacity to influence motor control and the ability to maintain postural balance/stability, with stiffer tendon structures being associated with increased ability to balance, potentially reducing the risk of falls (Onambele *et al*. 2006). Owing to the above-outlined importance of tendon properties in aspects of daily function, any alteration in its composition could have implications for performance, daily function and risk of injury. Thus, further knowledge of factors that could influence tendon properties (such as endocrine factors) is therefore of significant importance. Consequently, the aim of this study was to describe the relationship between naturally occurring serum relaxin levels and *in vivo* tendon mechanical properties in women.

Methods

Participants

Twelve healthy, recreationally active women (age 24±2.5 years, mass 63.8±2.9 kg, height 1.68± 0.02 m, physical activity frequency 4.8±0.9 h week−1), who were experiencing normal menstrual cycles (reported 28–32 day cycles for the last 6 months) and had not taken any form of hormonal contraceptive during this time (i.e. the 6months preceding the study), volunteered to participate in the study. The investigation was approved by the local Ethics Committee, and all subjects gave their written informed consent to participate. The study conformed to the principles of the World Medical Association's Declaration of Helsinki. Participants visited the laboratory prior to the test session to allow familiarization with the protocols. Participants attended three testing sessions at time points over the course of a menstrual cycle, once during days 1–4, once during days 12–14 and once during days 20–23 (day 1 was defined as the first day of menstruation). The order of testing was randomized. During each testing session, the participant's venous blood was sampled for later biochemical analyses, and patellar and medial gastrocnemius tendon mechanical properties were assessed.

Measurement of tendon forces

Torque output during isometric knee extension and plantar flexion was determined using a dynamometer (Kin Com, type 125 AP, Chattanooga, TN, USA), with the participant in a seated position. For the knee extension efforts, the knee was fixed at 90 deg flexion (full extension= 0 deg) and hip at 85 deg (supine= 0 deg), and a lever attachment cuff was placed on the lower leg at ~3 cm above the medial malleolus. During the plantar flexion efforts, the knee was fully extended and the hip flexed to 90 deg; the foot was fixed in a neutral anatomical position, where the sole of the foot was at 90 deg to the tibia. The centre of rotation of the dynamometer lever arm was aligned with the joint centre, and straps were fixed across the chest, hip, thigh of the test limb and around the foot during the plantar flexion efforts to prevent any extraneous movement. Three maximal isometric knee extension and plantar flexion efforts were carried out to ensure tendon pre-conditioning prior to the test. Participants were instructed to perform ramped isometric contractions from rest to maximum over 3–4 s. Three trials of the knee extension test and of the plantar flexion were performed with 180 s rest between contractions of the same muscle group. A 10 min rest between knee extension and plantar flexion efforts was required in order to allow time to change the dynamometer set-up. The starting contraction (i.e. knee extension or plantar flexion) was randomized between individuals but remained

constant within an individual over the three testing days. Tendon force was calculated as $F_{tend} = (P + P_{antag})/T_{arm}$, where F_{tend} is the force in the tendon, P the observed torque output, P_{antag} the antagonist (hamstring or tibialis anterior) co-contraction torque and T_{arm} the tendon moment arm. The moment arm length of the medial gastrocnemius tendon was obtained using the tendon travel method (An *et al*. 1984), and patellar tendon moment arm was determined from previous reports to be 44.7mm (Baltzopoulos 1995; Krevolin *et al*. 2004). Correction for relative muscle physiological cross sectional area of the medial gastrocnemius (Fukunaga *et al*. 1992) was applied to the calculation of medial gastrocnemius tendon force.

Estimation of co-contraction using EMG activity

The EMG of the long head of the biceps femoris muscle (BF) and tibialis anterior (TA) were measured in order to ascertain the level of antagonistic muscle co-contraction during the isometric knee extension and plantar flexion performances, respectively (Pearson & Onambele, 2006). Assumptions were that BF is representative of its constituent muscle group (Carolan & Cafarelli, 1992) and that BF and TA EMG relationships with knee flexors and ankle plantar flexion torque, respectively, are linear (Lippold, 1952). Two self-adhesive Ag–AgCl electrodes (type N10A; Medicotest, Huntingdon, UK) were placed in a bipolar configuration, with a constant inter electrode distance of ~20 mm, at a site corresponding to the distal one-third of the length, in the mid-line of the belly of the BF. For the TA, placement of the electrodes was in the mid-line of the TA muscle belly, halfway between the centre of the belly and the distal myotendinous junction of the TA. Prior to electrode attachment, the skin was prepared by shaving, abrading, and cleaning with an alcohol-based solution in order to minimize the resistance. The reference electrodes (type Q10A; Medicotest) were placed on the lateral tibial condyle for BF and the lateral malleolus of the ankle for TA. The electromyographic signals were high- and low-pass filtered between 10 and 500 Hz, respectively, (Neurolog filtersNL 144 and NL 134; Digitimer, UK), pre amplified (×1000; Neurolog remote AC preamplifier NL 824; Digitimer), amplified (×2; Neurolog isolation amplifier, NL 820; Digitimer) and A/D converted at 2000 Hz (KPCI 3101; Keithley Instruments, UK). A series of three maximal isometric knee flexion and dorsiflexion contractions were carried out to obtain the EMG at maximal flexion torque. The root mean square EMG activity corresponding to the peak torque period was analysed over 50 ms epochs and averaged for a 1 s period during the plateau of peak torque. This has previously been suggested to be acceptable in terms of signal-to noise ratio (Hermens *et al*. 2000). Electromyographic activity of the BF and TA during knee extension and plantarflexion, respectively, was divided by the maximal flexor EMG, and the maximal flexor torque was then multiplied by this value to determine co-contraction torque.

Measurement of tendon elongation

Elongation of the tendons of interest was assessed using a 7.5MHz, 40mm linear array, B-mode ultrasound probe (AU5; Esaote UK, Reading, UK) with a depth resolution of 49.3 mm, as previously described (Pearson & Onambele, 2005; Burgess *et al*. 2009). Briefly, during the graded isometric knee extensions, the probe was positioned in the sagittal plane over the patellar tendon and the apex of the patellar tendon (proximal end; see Fig. 1*A*). Elongations of the medial gastrocnemius were assessed during the graded isometric plantar flexions. The probe was placed in the sagittal plane over the myotendinous junction of the medial head of the gastrocnemius muscle (See Fig. 1*B*). Three efforts were recorded for each tendon, with sufficient rest (120–180 s) between efforts.

For both tendons, an echo-absorptive marker was placed between the probe and the skin to act as a fixed reference from which measures of elongation could be made. Ultrasound images were recorded in real time onto mini DV via S-video output and captured onto PC at 25 Hz using Quintic Biomechanics (9.03 version 11; Quintic Consultancy Ltd, Coventry, UK). The ultrasound output was synchronized (using an electronic square-wave signal generator) with the force and EMG records to allow temporal alignment. Tendon excursions were determined at intervals of 10% of the maximal force (from 0 to 100%) using ImageJ(National Institutes of Health, Bethesda, MD, USA).

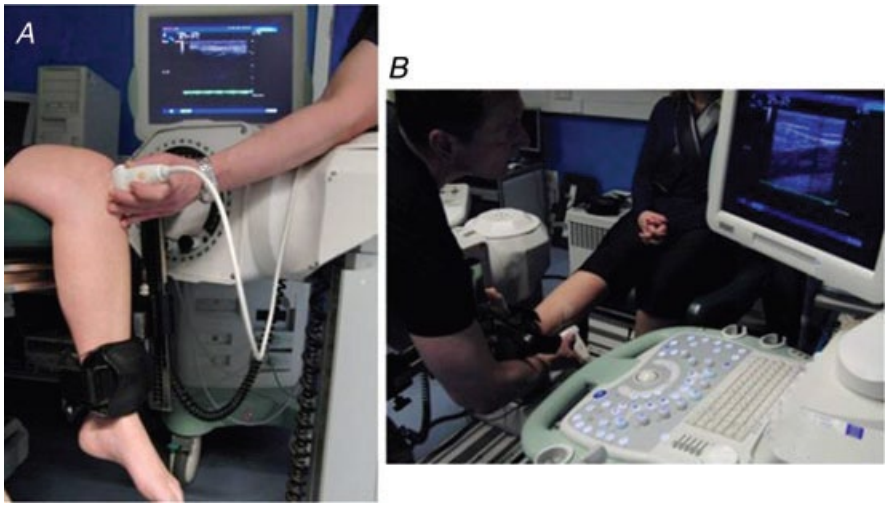

Figure 1. Experimental set-up during the assessment of patellar (*A*) and medial gastrocnemius (*B*) tendons
This figure is for illustrative purposes only; during experiments, EMG electrodes would also be in place on the antagonist muscle group.

Calculation of tendon properties

The tendon force–elongation relationships were fitted with second-order polynomial functions forced through zero. Tendon stiffness measures (in newtons per millimetre) were calculated from the slope of the following: (i) a tangent at 100% maximal force, i.e. maximal stiffness; (ii) a tangent at a specific force level represented by the lowest maximum from the group data (i.e. 1143 N for the quadriceps and 370 Nfor the plantar flexors), i.e. standard force stiffness; and (iii) the mean of stiffness values at several force levels (i.e. 10–100% maximal voluntary contraction, every 10% increment), i.e. average stiffness.

Measurement of tendon cross-sectional area

Patellar tendon cross-sectional area (PT_{CSA}) was also assessed with the knee joint at 90 deg. The PT_{CSA} was measured as the average from transverse-plane ultrasound images taken at 25, 50 and 75% of total patellar length (ie. PT_L, which was determined from sagittal-plane ultrasound images and measured form the inferior pole of the patella to the superior aspect of the tibial tuberosity). Medial gastrocnemius cross-sectional area (MGT_{CSA}) was assessed with the foot in the neutral position (sole of foot at 90 deg to the tibia) and lower limb straight. The MGT_{CSA} was measured as the average from transverse plane ultrasound images taken at 1, 2 and 3 cm above the tendon insertion point in the calcaneus and corrected based on the assumption that the medial gastrocnemius tendon cross-sectional area occupies a fraction of the average Achilles tendon cross-sectional area equivalent to the relative physiological cross-sectional area of the medial gastrocnemius muscle with respect to the entire triceps surae muscle (Fukunaga *et al*. 1992).

Hormonal measures

Ten-hours-fasted blood samples were taken from the median antecubital vein (5ml) by a trained phlebotomist at the beginning of each testing session. The blood sample was allowed to clot while refrigerated (5◦C), centrifuged at 3578 ***g*** for 6min, and then the serum was separated and stored at −20◦C until analysis. The samples were coded for each participant and testing session. Relaxin (hRLX;

Immundiagnostik AG, Bensheim, Germany; sensitivity <0.8 pgml-1; intra-assay variability of 3.3%) content was analysed using the standard ELISA procedures.

Statistical analyses

Acute fluctuations in relaxin levels and tendon properties over the course of the menstrual cycle were determined using a one-way repeated-measures ANOVA (or Friedman's test where appropriate, i.e. if data were not normally distributed as determined by the Shapiro–Wilk test). Where a significant main effect was present, *post hoc* Student's paired *t* tests were performed and *P*-values Bonferroni corrected to examine any differences highlighted. Spearman correlation coefficients were determined to examine the relationship between serum relaxin levels and tendon mechanical properties. Significance was set to $P < 0.05$. Intraclass correlation coefficients (ICC) were calculated to estimate reliability of the measures. All data are presented as means ± SEM.

Results

Reliability of measurements

Within-session ICCs were 0.91 for patellar tendon elongation, 0.945 for medial gastrocnemius tendon elongation, 0.923 for knee extension torque, 0.912 for plantarflexion torque, 0.98 for PT_{CSA}, 0.98 for MGT_{CSA}, 0.92 for P_{TL}, 0.98 for MG_{TL} and 0.98 for relaxin level.

Acute fluctuations in relaxin levels and tendon properties

There were no significant differences in the level of serum relaxin, the cross-sectional area of the tendons, or the mechanical properties of either the medial gastrocnemius or patellar tendons across the three phases of the menstrual cycle ($P > 0.05$; see Tables 1 and 2 for values).

Table 1. Mechanical properties of the medial gastrocnemius (MG) and patellar tendons (P) over the course of the menstrual cycle

Stiffness variable (N mm^{-1})	Days 1–4	Days 12–14	Days 20–23	Mean
MG maximal stiffness	70.8 ± 7.2	82.3 ± 17.3	72.8 ± 9.7	75.3 ± 41.4
MG average stiffness	55.4 ± 5.4	68.5 ± 13.8	62.3 ± 8.1	62.1 ± 33.3
MG standard force stiffness	58.1 ± 4.6	71.5 ± 12.2	68.2 ± 9.6	65.9 ± 31.9
P maximal stiffness	1238.0 ± 149.1	1493.4 ± 177.5	1163.9 ± 146.8	1298.6 ± 551.8
P average stiffness	935.1 ± 110.3	1125.3 ± 130.7	848.5 ± 102.4	969.6 ± 404.5
P standard force stiffness	910.2 ± 105.2	1073.9 ± 109.9	826.5 ± 90.7	936.9 ± 359.5

The maximal stiffness is taken at 100% maximal voluntary contraction. The average stiffness is the mean of stiffness values at force levels from 10 to 100% (with 10% force increments). The standard force stiffness is that taken at the same absolute force for all subjects (i.e. 370 N for the gastrocnemius and 1143 N for the patellar tendon).

Table 2. Cross-sectional area of the MG and P tendons and serum relaxin levels over the course of the menstrual cycle

Variable	Days 1–4	Days 12–14	Days 20–23	Mean
Medial gastrocnemius tendon cross-sectional area (mm^2)	18.0 ± 0.5	18.1 ± 0.5	17.9 ± 0.4	18.0 ± 0.3
Patellar tendon cross-sectional area (mm^2)	71.8 ± 2.0	71.7 ± 2.1	71.0 ± 2.2	71.5 ± 1.2
Relaxin (pg ml^{-1})	82.1 ± 23.8	86.2 ± 25.5	94.1 ± 27.1	87.5 ± 25.0

Chronic relationship between relaxin levels and tendon properties

Relaxin concentrations were seen to account for between 26 and 31% of the variation in patellar tendon stiffness. Significant correlations were found between serum relaxin levels and stiffness, for the patellar tendon only ($r = -0.561$, $P < 0.001$ for maximal stiffness; $r = -0.560$, $P < 0.001$ for average stiffness; $r = -0.510$, $P = 0.002$ for standard force stiffness; see Fig. 2). Relaxin levels accounted for between 0.4 and 2.2% ($P > 0.05$) of the variance in tendon stiffness in the medial gastrocnemius, but these associations were not significant. There were no significant associations between tendon cross-sectional area and relaxin levels ($r = -0.193$, $P > 0.05$ in the patellar and $r = -0.233$, $P > 0.05$ in the gastrocnemius tendons).

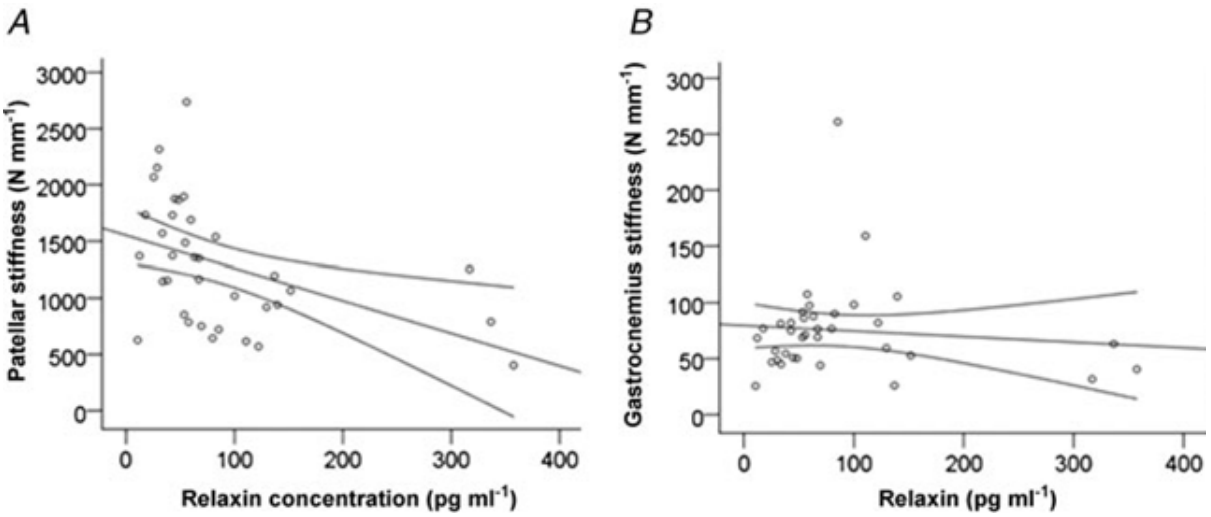

Figure 2. Bivariate associations between serum relaxin level and maximal tendon stiffness in the patellar (*A*; $r = -0.56$, $P < 0.001$) and medial gastrocnemius tendons (*B*; $r = -0.15$, $P = 0.4$, n.s.)
Data are shown with 95% confidence intervals.

Discussion

The present study shows that in a group of normally menstruating women (not taking any form of medical contraception), the menstrual cycle impacts neither on relaxin nor on the structural and mechanical properties of the medial gastrocnemius and patella tendons ($P > 0.05$). However, a significant relationship ($P < 0.002$–0.001) is seen between absolute levels of relaxin and patellar tendon stiffness, with relaxin explaining up to 31% of the variability in patellar tendon stiffness.

To our knowledge, this is the first study to examine the potential relationship of relaxin to *in vivo* tendon structural and mechanical properties in humans. Our data are somewhat congruent with those examining relaxin effects in animal models. Dragoo *et al*. (2009) reported that in female guinea-pigs, those administered relaxin showed increased anterior cruciate ligament laxity, though this was not seen to be significantly different from control animals. However, the failure loads were significantly reduced in relaxin-treated animals. Here we identify a significant pattern of decreased patellar tendon stiffness with increased levels of relaxin, but not for the medial gastrocnemius tendon. These different findings could be explained in part by the potential for relaxin receptor density differences or even sensitivity to the hormone. Indeed, previous work has alluded to differential expression and/or level of function in different relaxin receptors (Kern & Bryant-Greenwood, 2009; Kong *et al.* 2010). Of course, the mechanisms we propose here are mere speculations, but certainly they point towards the direction for future work. Whether the reduced stiffness we have found in the present study to be associated with high relaxin levels would increase the injury risk is difficult to ascertain with any certainty, but it is clear that strain for any given load seems to be increased in those women with the highest levels of hRLX. This

alone may indicate that with repeated strains, increased micro-ruptures would be present in tendons exposed to higher relaxin levels, ultimately leading to increased risk of rupture or major tears of the tendon structure.

Previous work has indicated that relaxin has the ability to affect the ligamentous tissues, for example, increasing compliance of the cervix and vagina to aid birth during pregnancy (Bradshaw et al. 1981). There exist no prior data that confirm the existence of relaxin receptors in human tendon. However, relaxin receptors have previously been identified in ligamentous tissues from women (Dragoo et al. 2003; Galey et al. 2003). In female athletes, there has been reported to be a greater incidence of anterior cruciate ligament injuries than in men, with reported injury rate being four to eight times that in men (Malone et al. 1993; Hewett et al. 2006). This increased injury rate has been associated with the levels of circulating sex hormones during the menstrual cycle; here the authors showed that injury rates increased during the ovulatory phase, with 72% of reported injuries occurring during this portion of the menstrual cycle (Adachi et al. 2008). Incidentally, the ovulatory phase also coincides with high oestrogen levels, hence an elevated modulation of oestrogen onto relaxin. Indeed, the effects of relaxin have been previously reported to be influenced by concurrent levels of other female sex hormones (notably oestrogen and progesterone), with oestrogen enhancing the effect of relaxin on collagen and progesterone antagonizing it (Samuel et al. 1996). We did not find any change in tendon stiffness associated with menstrual cycle variation. Perhaps tendon metabolism will not allow for such acute variation as with the menstrual cycle. However, our findings suggest that chronic levels of relaxin can be associated with tendon stiffness differences. However, these appear only in the patellar tendon in this case. A potential limitation of patellar tendon properties measured at the proximal site is that not accounting for any distal movement may underestimate the total elongation of this tissue under loading (Hansen et al. 2006; Onambél et al. 2007). However, the relative measurements are still valid, hence the description of changes is appropriate. Other potential limitations to the present study may include participant numbers, the specific tendons measured, as well as, potentially, the intervals between testing sessions, which arguably would benefit from a number of repeated measures across the cycle. What is more, in the synopsis here, we discuss mechanistic changes in ligamentous tissue (described in the literature); in fact, these may not necessarily be the same as changes seen in tendon tissue.

Regarding the particular point about ‘specific tendon limitation’ mentioned above, we propose that either relaxin receptors (number or density) or indeed the sensitivity of the said receptors varies between tendinous structures, thereby impacting on the overall potential for relaxin to modulate mechanical/structural properties. Another possibility is any differences in tendon metabolism between the sites, i.e. collagen turnover rates, independent of relaxin levels. This supposition is justified below.

Relaxin is a peptide hormone of approximately 6 kDa and belongs to the insulin family; of its two disulphide- linked chains, the B chain contains the receptor interaction site. In both human and animal models, relaxin appears to be associated mainly with females and has previously been reported predominantly in pregnant and non-pregnant female sera, whilst in males it appears only in the seminal fluid. It has been described as having the ability to exert its effects through receptors at the tissue surface; here oestrogen has been suggested to able to modulate the relaxin receptors, increasing the effectiveness of the response of a target organ to relaxin (Winn *et al*. 1994; Sherwood *et al*. 1998).

Amongst its potential biological effects are those on type I collagen, a major structural component of the tendon. Here previous work identifies relaxin as being collagenolytic, in that total collagen content is reduced with relaxin. A possible mechanism is via the increased production of collagenase (Granstr m *et al*. 1992) or matrix metalloproteinases (Qin *et al*. 1997). Here it is known that ligamentous tissues from different anatomical sites have different responses to injury, again congruent with the notion of differential relaxin receptor density by site and/or metabolic differences. Zhou *et al*. (2005) reported that injured cells from the anterior cruciate ligament had greater matrix metalloproteinase activity, in contrast to the medial cruciate ligament. Thus, if relaxin exerts an effect on matrix metalloproteinase in a similar manner in tendon, it could be reasoned that with injury or general overload, the tendon exposed to higher

relaxin levels may be compromised more than one with less relaxin present. Previous work has indicated that relaxin affects type I collagen synthesis in human fibroblasts, causing a reduction in collagen content (Unemori & Amento, 1990). With use, the tendon will undergo constant remodelling of micro-damaged cells, but if collagen synthesis and content are reduced via relaxin, it would cause a weakening of the structure; this could be seen as reduced stiffness, as reported in this study.

In conclusion, in young, normally menstruating females, hRLX appears to be significantly correlated with patellar tendon stiffness. Where it has an association (i.e. in the patellar tendon), this appears to be with the intrinsic properties rather than with the dimensions of said tendon. The reduced patellar stiffness that we have seen with high hRLX could lend itself to increased potential for extremes of strains, hence higher injury risk in women. Indeed, increased tendon compliance may influence the motor control and rates of force development as it relates to the knee, potentially adversely affecting risk of anterior cruciate ligament injury. Future work (with larger sample sizes and/or other tendons) to elucidate the physiological cause for this selectivity in the impact of relaxin will be key to mapping the impact of the endocrine system on the phenotype of tendinous tissue.

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