

Ethinyl oestradiol administration in women suppresses synthesis of collagen in tendon in response to exercise

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Women are at greater risk than men of sustaining certain kinds of injury and diseases of collagen-rich tissues. To determine whether a high level of oestradiol has an acute influence on collagen synthesis in tendons at rest and in response to exercise, one-legged kicking exercise was performed for 60 min at 67% of maximum power by healthy, young oral contraceptive (OC) users when circulating synthetic (ethinyl) oestradiol was high ($n = 11$, HE-OC) and compared to similar women who had never used OCs when circulating endogenous oestrogen was low ($n = 12$, LE-NOC). Interstitial fluid was collected 24 h post-exercise through microdialysis catheters placed anterior to the patellar tendon in both legs and subsequently analysed for the amino-terminal propeptide of type I collagen (PINP), a marker of tendon collagen synthesis. To determine the long-term effect of OC usage, patellar tendon cross-sectional area (CSA) was measured by magnetic resonance imaging (MRI). A lower exercise-induced increase in tendon collagen synthesis was observed in HE-OC than in LE-NOC (Δ PINP (mean \pm S.E.M.) 1.5 ± 5.3 versus 24.2 ± 9.4 ng ml⁻¹, $P < 0.05$). Furthermore, serum and the interstitial peritendinous tissue concentrations of insulin-like growth factor I (IGF-I) and IGF-binding proteins showed a reduced bioavailability in HE-OC compared with results in LE-NOC. No difference in patellar tendon CSA was observed between groups. In conclusion, the selective increase in tendon collagen synthesis in LE-NOC but not HE-OC 24 h post-exercise is consistent with the hypothesis that oestradiol inhibits exercise-induced collagen synthesis in human tendon. The mechanism behind this is either a direct effect of oestradiol, or an indirect effect via a reduction in levels of free IGF-I. However, the data did not indicate any long-term effect on tendon size associated with chronic OC use.

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Women are seemingly at a greater risk than men of sustaining certain kinds of sports injury (Engstrom *et al.* 1991; Arendt & Dick, 1995; Bijur *et al.* 1997; Bjordal *et al.* 1997; Bell *et al.* 2000) and disease (Larsson *et al.* 1993; Wolfe *et al.* 1995; Lahita, 1996) in collagen-rich tissues such as bone, ligament, muscle connective tissue and tendon. For instance, women are at a 2–8 times greater risk of anterior cruciate ligament (ACL) ruptures than men when performing similar sports activities (Huston *et al.* 2000). It has been suggested that part of this discrepancy in injury risk might be explained by differences in sex hormones (Hewett *et al.* 2006).

The strength and biomechanical properties of tendons play an important role in the force transmission of contractile energy from myofibrillar protein structure

to the skeletal bones during locomotion (Kjaer, 2004). Collagen constitutes approximately 90% of the total protein of tendons, or 65–75% of the dry weight of tendons. This collagen is predominantly type I (~60%) arranged in tensile-resistant fibres (Jozsa & Kannus, 1997). In men it has been shown that an acute bout of exercise increases type I collagen synthesis in tendon (Langberg *et al.* 1999, 2001; Miller *et al.* 2005) and resistance training results in patellar tendon hypertrophy (Kongsgaard *et al.* 2007). In contrast to the findings in men, recent findings from our laboratory have shown a similar tendon CSA (Achilles' and patellar tendon) in experienced female runners and untrained women, while tendon CSA adjusted for body size was larger in equally trained male runners (Westh *et al.* 2008). This might indicate sex-specific adaptation of tendons to training.

Oestrogen receptors have been localized in synoviocytes in the synovial lining, fibroblasts in the ACL stroma and cells in the blood vessel walls of the ligament (Liu *et al.* 1996), and animal data have shown that tendons express transcripts for oestrogen receptors (Hart *et al.* 1998). In relation to this a more than 40% reduction in collagen synthesis and a reduction in fibroblast proliferation have been observed *in vitro* in tissue samples from the ACL when oestradiol was administered in physiological doses (Liu *et al.* 1997; Yu *et al.* 2001). In addition, ovariectomized rabbits exposed to oestradiol for one month have reduced tensile strength of the ACL compared to a control group of ovariectomized rabbits not supplemented with oestradiol (Slauterbeck *et al.* 1999). *In vitro* studies and animal studies on bone and ACL tissue have shown an interaction between exercise, the level of oestradiol, and oestrogen receptors on collagen turnover (Chen *et al.* 2001; Tobias, 2003; Lee *et al.* 2004; Lee & Lanyon, 2004; Saxon & Turner, 2005). Expression of type I collagen was reduced in fibroblasts when cyclic loading and oestrogen were administered together, but increased when they were administered separately (Lee *et al.* 2004). A suppressive effect of oestrogen administration on fibroblast response to cyclic loading may be mediated by reduced bioavailability of IGF-I, since oral administration of oestrogens (E2 or ethinyl oestradiol) reduces serum (s-) IGF-I and raises concentrations of IGF-binding proteins (IGFBPs) and s-growth hormone (GH) (Ho & Weissberger, 1992; Campagnoli *et al.* 1993; Kelly *et al.* 1993; Bellantoni *et al.* 1996; Westwood *et al.* 1999). In cultured tenocytes, IGF-I exerts a potent proliferative and pro-survival effect (Scott *et al.* 2005). Furthermore, the observed proliferative response of cultured tenocytes to loading (Tsuzaki *et al.* 2000) and the increase in collagen synthesis in response to exercise *in vivo* has also been associated with the presence of IGF-I (Olesen *et al.* 2006b). Therefore, oral administration of oestrogens may reduce the responsiveness of tendons to mechanical loading indirectly by reducing the local level of free IGF-I. A local change in IGF-I in tendon tissue in response to oestrogen administration has to our knowledge not been reported in the literature.

Our primary aim was to investigate whether a high level of oestradiol has an acute influence on collagen synthesis in tendon at rest and in response to exercise. We hypothesized that the response of tendon collagen synthesis to exercise would be lower in women when they were exposed to a high level of oestradiol by OCs compared with women when they were exposed to a low level of endogenous oestradiol. This hypothesis was based on the assumption that synthetic oestradiol (ethinyl oestradiol), like endogenous secreted oestradiol (17- β oestradiol), has an inhibiting effect on collagen synthesis (Liu *et al.* 1997). In addition, GH, IGF-I and IGFBPs were measured in serum and in the interstitial peritendinous tissue to clarify the effect of oestradiol administration

on the level of these growth factors in the peritendinous tissue.

Tendon stress is inversely proportional to tendon CSA during mechanical loading. It has not been reported whether long-term use of OCs has an associated influence on tendon size and occurrence of tendon injury rates. An observed higher risk of lower back pain (Wreje *et al.* 1997), bone fractures (Cooper *et al.* 1993) and persistent pelvic pain and pelvic joint instability (Saugstad, 1991) in users of OCs than in controls has been observed. These findings indicate that OC usage could have a long-term negative effect on collagen-rich tissue quality or quantity. In contrast, a prospective study noted fewer non-specific traumatic soccer injuries in women using OCs compared to women who did not use OCs (Moller-Nielsen & Hammar, 1989). Therefore, the epidemiological data are too scarce and inconsistent to firmly establish an association between OC use and the risk of injuries related to the collagen-rich tissue in tendon, ligament and bone.

A secondary aim in the present study was to evaluate if long-term use of OCs has an influence on patellar tendon CSA and thereby indirectly an effect on the risk of sustaining an injury. This was examined by comparing tendon CSA by magnetic resonance imaging (MRI) in women who had not used OCs with chronic OC users. In OC users, ethinyl oestradiol is elevated for 3 weeks and low for 1 week in each menstrual cycle. In non-users of OCs, exposure to endogenous oestrogens is low in the follicular phase, but similar to OC users, the oestradiol concentration is enhanced during the other phases of the menstrual cycle. Therefore, total exposure to oestrogens might not be greatly different between groups. Based on this, no specific direction of an effect of OC usage on patellar tendon CSA could be assumed.

Methods

Subjects

Twenty-three young, healthy women were recruited for the study. All were non-smokers, nulliparous, non-users of medication (except OCs), and without orthopedic and metabolic disorders, as judged by history and routine medical examination. The subjects gave informed consent to the protocol adhering to the *Declaration of Helsinki* and the Ethics Committee of Copenhagen and Frederiksberg Communities (KF-01-032/04) approved the study.

Eleven women were long-term users of OCs (7.2 ± 2.1 treatment years (mean \pm s.d.)). The used types of OCs were Lindynette ($n = 7$) (30 μ g ethinyl oestradiol and gestoden 0.0075 mg per day) or Cilest ($n = 4$) (35 μ g ethinyl oestradiol and 0.25 mg norgestimate per day). The additional 12 women had a normal menstrual cycle and had never used OCs. A cycle length within the range of 21–35 days for at least one year was defined

Table 1. Subject characteristics

	LE-NOC	HE-OC
Age (years)	24 ± 4	24 ± 2
Weight (kg)	65.0 ± 8.4	63.7 ± 8.4
Height (m)	1.67 ± 0.07	1.70 ± 0.06
BMI (kg m ⁻²)	23.3 ± 2.4	22.0 ± 2.6
Body fat (%)	31.1 ± 8.6	27.7 ± 7.2
LBM (kg)	42.6 ± 4.1	43.9 ± 4.4
Workload (Watt _{submax})	44 ± 9	37 ± 7
Workload (%Watt _{max})	68 ± 6	67 ± 8
PT (times week ⁻¹)	2.2 ± 1.3	2.4 ± 1.6
PA (h week ⁻¹)	4.9 ± 2.1	5.4 ± 2.0
Bike, transport (% of PA)	61 ± 30	58 ± 23
Patellar tendon, mean CSA (mm ²)	75 ± 7	82 ± 10

Values are means ± s.d. No significant differences between LE-NOC (women who had never used oral contraceptives tested at low circulating levels of oestradiol) and HE-OC (oral contraceptive users tested in the hours after ingestion of oestradiol) for any of the parameters were observed. Body composition was measured by DEXA. LBM, lean body mass; BMI, body mass index; PT, physical training; PA, physical activity (bike transportation and PT).

as normal. The two groups were otherwise similar in the following characteristics: age, height, weight, body mass index (BMI), and body composition measured by Dual-Energy x-ray absorptiometry (DEXA). Additionally, the groups were similar with regard to training status as assessed by a questionnaire used to determine frequency and hours spent on regular planned physical training per week. Since cycling is a widely used form of transport for young women in Copenhagen, the participants were also asked to calculate the total time per week they spent cycling (Table 1).

Design and methods

Our primary aim was to elucidate whether a differential level of oestradiol had an influence on the response to exercise in tendon collagen synthesis in the *in vivo* situation in young women. To make the total difference in oestrogens (endogenous secreted and synthetic ethinyl oestradiol) between groups as large as possible, LE-NOC were tested in the early follicular phase of the menstrual cycle when the level of oestrogen is low, whereas HE-NOC were tested on days 18–21 of the pill phase. In the HE-OC group, measurements of tendon synthesis were performed in the hours after the last pill ingestion, when the ethinyl-oestradiol concentration peaks (Jung-Hoffmann *et al.* 1991).

The effect of exercise on tendon collagen synthesis was determined by using a one-legged kicking exercise model and by measuring tendon collagen synthesis 24 h later in the resting leg and the contralateral leg, which had performed strenuous exercise. The time-point for the measurement of tendon synthesis was based on earlier

findings in men showing a peak in tendon synthesis 24 h post-exercise when using a similar exercise protocol (Miller *et al.* 2005).

Synthesis of collagen was measured by the microdialysis technique (Lonnroth *et al.* 1987). The procedure is described in more detail below. Dialysate was collected from the peritendinous space anterior to the patellar tendon and analysed for the amino terminal propeptide of type I collagen (PINP) as a marker of collagen synthesis (Langberg *et al.* 2001). Dialysate from both legs was obtained simultaneously. The remaining dialysate was analysed for IGF-I and IGFBPs (1–4).

Two weeks prior to the study, subjects visited the laboratory for determination of the workload they would use during the experiment when exercising on a one-legged modified Krogh ergometer. After warming up for 5 min without resistance, the subjects began one-legged kicking (35 kicks per minute) for 3 min with a 0.5 kg load; the load was increased by 0.3 kg every 3 min until the subjects could no longer maintain the cadence and this workload was defined as maximum workload (W_{max}).

On day 1 of the experiment, the subjects performed the one-legged kicking exercise for 1 h at 67% of each subject's W_{max} . The following day subjects arrived at the laboratory at 08.00 h having fasted for the previous 12 h. After baseline urine and blood samples for analysis of sex hormones and growth factors, the subjects received a standardized commercial clinical nutrient drink (Semper, Frederiksberg, DK: 15% protein, 64% carbohydrate and 21% fat) in divided doses every 30 min until the end of the experiment. The drink provided the equivalent of 1.4 × basal metabolic rate per 30 min period, with a double dose at initiation of feeding. Basal metabolic rate was estimated from the subject's fat free mass determined by the skin-fold technique (Durnin & Womersley, 1974; Cunningham, 1982). The reason for the standardization of nutrient intake was that energy and macronutrient intake is known to have an influence on protein metabolism in general. In particular, an increase in synthesis of skeletal muscle proteins in response to provision of essential amino acids is well-documented (Tipton & Sharp, 2005). Knowledge about the effects of food intake on tendon metabolism is limited. New results indicate that collagen synthesis in tendons and muscle connective tissue is not acutely influenced by nutrition (Babraj *et al.* 2005), but we decided to standardize the nutrient intake to diminish any influence of a potential confounding factor. Furthermore, the subjects completed weighed food records for 3 days prior to the experiment. No difference in energy intake and macronutrient composition between groups was observed (Table 2). The subjects were instructed to avoid strenuous physical activity for at least 2 days prior to and during the experimental days. Isotope data from earlier studies indicate that the fractional patellar tendon collagen synthesis peaks around 24 h postexercise (Miller

Table 2. Energy intake and macronutrient intake

Habitual diet	LE-NOC	HE-OC
Energy intake (kJ day ⁻¹)	9033 ± 2032	9221 ± 1983
Energy intake RMR ⁻¹	1.45 ± 0.31	1.49 ± 0.35
Protein (g day ⁻¹)	82 ± 29	90 ± 17
Protein (g kg ⁻¹)	1.3 ± 0.4	1.3 ± 0.2
Protein (g (kg DEXA LBM) ⁻¹)	2.0 ± 0.6	2.0 ± 0.3
Protein (E%)	15 ± 4	17 ± 2
Fat (E%)	23 ± 7	24 ± 6
CHO (E%)	58 ± 6	57 ± 7
Alcohol (E%)	4 ± 6	2 ± 5

Values are means ± s.d. No significant differences between groups were observed for any of the parameters. Abbreviations: RMR, resting metabolic rate; LBM, lean body mass; CHO, carbohydrate; E%, percentage of daily energy intake.

et al. 2005) and is similar to resting values 72 h post-exercise in young women (Miller *et al.* 2006).

Microdialysis. Dialysate was sampled from each leg by microdialysis technique (Langberg *et al.* 1999) on the day following the one-legged exercise bout to detect changes in metabolites in the local tissue fluid surrounding the patellar tendon. After previous preparation of incision sites with local anaesthetic (lidocaine (lignocaine) 1%), ethylene oxide-sterilized catheters with high molecular mass cut-off (3000 kDa, membrane length 30 mm, i.d. 0.50 mm) were inserted under ultrasound guidance as previously described (Langberg *et al.* 1999) in the peritendinous spaces of patellar tendons. The inflow tube of the microdialysis catheter was connected to a high-precision syringe pump with an infusion rate of 2 µl min⁻¹. The catheters were perfused with a Ringer-acetate solution mixed with radioactive labelled glucose (D-[3-³H]glucose, specific activity 0.25 mCi; 0.25 m) in aqueous solution steri-pack, Perkin Elmer Life and Analytical Sciences, Boston, MA, USA). A sample vial was placed at the end of the outflow tube, and after 30 min perfusion of the catheter, dialysate was collected in three 1 h periods. The relative recovery over the membrane was determined for each dialysate sample (Scheller & Kolb, 1991). The dialysate (3 µl) was pipetted into a counting vial and mixed with 3 ml scintillation fluid (Ultima Gold, Perkin Elmer) and the samples were counted in a β-counter. The mean relative recoveries for the microdialysis catheters did not differ between groups (LE-NOC, 29 ± 12%; HE-OC users, 27 ± 10%).

Magnetic resonance imaging. Patellar tendon CSA was determined by MR axial plane images (General Electric Sigma Horizon LX 1.5 Tesla, T1 weighted S.E.) with the following MRI parameters: TR/TE 400/15 ms, FOV 16, matrix 256 × 256 ms, slice thickness 5.0 mm, spacing 0 mm and 12 slides in total from each tendon (bilateral)

starting from apex patellar and ending at the tibial attachment. CSA was measured at the tibial and patellar attachments. All measurements were obtained manually using the software program WEB 1000 in a blinded fashion. The CSA for each anatomical spot was measured three times for each subject. The average intra-individual coefficient of variation (CV%) was 4%, whereas the inter-individual CV was 11%.

Blood, dialysate and urine analysis. All blood samples were collected from a cubital vein into sealed vials. After separation by centrifugation at 4°C the blood samples were either immediately analysed for s-oestradiol and s-progesterone or stored at -80°C until analysis for s-testosterone, s-GH, s-IGF-I, s-IGFBP1 and s-IGFBP3.

Oestradiol was analysed by chemiluminescent competitive immunoassay (Immulite 2500) (NPU 1972, oestradiol; Diagnostic Product, Los Angeles, CA, USA). The detection level for the analysis is 10 nmol l⁻¹. Progesterone was analysed by architect microparticle enzyme immunoassay (Abbott Diagnostics, Wiesbaden, Germany). The analysis for oestradiol and progesterone were performed at Hvidovre Hospital, Denmark. The analysis for testosterone was performed at Statens Serum Institute, Denmark. Prior to analysis, internal standard solution containing deuterated androstenedione and testosterone was added to the sample. The samples were extracted with diethyl ether, evaporated and reconstituted in 50% methanol. Testosterone was measured in the serum extracts by liquid chromatography mass spectrometry (LC-MS) using an atmospheric pressure chemical ionization (APCI) interface (CV < 15%).

Dialysate fluid was analysed for PINP using a sandwich ELISA utilizing purified α1-chain specific rabbit antibodies donated by B. Teisner, Department of Medical Microbiology, University of Odense, Denmark (Jensen *et al.* 1998). The within- (double determination) and between-assay CVs were on average 2.2% and 4.9%, respectively.

Serum GH was measured using non-competitive time-resolved monoclonal immunofluorometric assay (TR-IFMA; Wallac Oy, Turku, Finland), as previously described (Frystyk *et al.* 1999). Serum IGF-I and dialysate IGF-I were determined at Aarhus University Hospital by TR-IFMA after acid-ethanol extraction, as previously described (Frystyk *et al.* 1995). The intra-assay CV for this IGF-I assay is below 5%. Serum IGFBP1 was determined by an in-house radioimmunoassay (RIA) performed as described by Westwood *et al.* (1994), with modifications as previously described (CV 5%) (Krassas *et al.* 2003). Serum IGFBP3 was measured by commercially available IRMA (BioSource Europe, Nivelles, Belgium) (CV < 5%). Small volumes of tendon dialysates were analysed for IGFBPs 1–4 by Western ligand blotting, as previously described (Flyvbjerg *et al.* 1992).

Statistics. To test if oestrogen has an effect on the response of tendon synthesis to exercise, unpaired Student's two-tailed *t* tests were performed to compare group differences in (A) resting PINP values and (B) Δ PINP values (rest – exercise). The effect of exercise within each group was tested by paired Student's two-tailed *t* tests. Unpaired Student's two-tailed *t* tests were used to test for between-group differences in s-testosterone and s-progesterone, tendon CSA, s-GH, s-IGF-I and s-IGFBPs. The results for IGFBPs were only tested for differences between HE-OC and LE-NOC by comparing the resting and post-exercise legs separately since initial paired *t* tests showed no effect of exercise. The MRI results were tested for a between-group effect in CSA of the patellar tendon both at the proximal and distal level of the tendon. The baseline characteristics of the subject groups are presented as mean \pm standard deviation (s.d.), whereas the results are presented as mean \pm standard error of the mean (s.e.m.). The level of significance was set at $P < 0.05$. The statistical analyses were performed using the statistical software package Prism version 4.01 (GraphPad, San Diego, CA, USA) 2004.

Results

The women performed the strenuous exercise bout at an average of $67 \pm 2\%$ of W_{\max} without any difference between the two groups (Table 1).

Sex hormones

On the day of the experiment, the concentration of s-17- β oestradiol was very low in both groups. In the LE-NOC tested in the early follicular phase s-17- β oestradiol was in the very low end of the range during a normal menstruation period (follicular phase < 0.60 nmol l⁻¹, reference values from Hvidovre Hospital, Denmark). One subject reached a concentration of 0.16 nmol l⁻¹, whereas the others were at or below the analytical detection level (≤ 10 nmol l⁻¹). Within the HE-OC-group s-17- β oestradiol was below analytical detection level in all except one (0.26 nmol l⁻¹). Serum progesterone was within the low end of normal range in both groups (follicular phase ≤ 4 nmol l⁻¹, luteal phase ≥ 25 nmol l⁻¹, reference values from Hvidovre Hospital, Denmark). Although statistically significant (LE-NOC: 0.9 ± 0.0 nmol l⁻¹ versus HE-OC: 0.7 ± 0.0 nmol l⁻¹, $P < 0.05$), the difference between groups in s-progesterone was so small compared to the normal range over the menstrual cycle (5.5 – 33 nmol l⁻¹) that it was physiologically negligible. Fasting level in testosterone did not differ between LE-NOC ($n = 10$) (0.96 ± 0.09 nmol l⁻¹) and HE-OC ($n = 11$) (0.9 ± 0.09 nmol l⁻¹) ($P = 0.82$).

Tendon collagen synthesis

Because of technical problems with the flow through the catheters, no PINP values were available from any of one subject's legs (LE-NOC). In two HE-OC subjects, dialysate from either the resting leg or the post-exercise leg was missing and a value corresponding to the mean Δ response in PINP ($+1.5 \pm 5.3$ μ g l⁻¹) was inserted to increase the statistical power for detecting a response to exercise.

At rest no difference in interstitial peritendinous PINP between groups was observed ($P = 0.50$). However, the response to exercise in tendon collagen synthesis was lower in HE-OC than in LE-NOC (Δ PINP (exercise – rest): 1.5 ± 5.3 versus 24.2 ± 9.4 μ g l⁻¹ in HE-OC and LE-NOC, $P < 0.05$) (Fig. 1). The increase in tendon synthesis was significant compared with resting values in LE-NOC ($P < 0.05$). Only in one subject in the LE-NOC group was tendon synthesis lower post-exercise compared with resting values. In contrast, only in three subjects in the HE-OC group was a higher tendon synthesis measured 24 h post-exercise compared with resting values, and the average tendon synthesis was not significantly enhanced by exercise ($P = 0.79$).

Tendon CSA

One LE-NOC was excluded from MR scanning due to safety reasons (metal device in the body). The proximal CSA of the patellar tendon was significantly smaller than the distal one ($P < 0.001$). Nevertheless, no difference in CSA between groups was observed either at the proximal or distal level of the patellar tendon (proximal: OC-users ($n = 11$) 65.8 ± 5.2 ; never-OC-users ($n = 11$) 60.4 ± 3.6 mm, $P = 0.40$) (distal: HE-OC 97.3 ± 4.4 ; LE-NOC 93.4 ± 5.6 mm, $P = 0.21$). Mean CSA (mm²) for each group is shown in Table 1.

GH, IGF-I, IGFBPs

Overnight fasting s-GH levels did not differ between groups (LE-NOC ($n = 8$) 11 ± 3 μ IU ml⁻¹; HE-OC ($n = 8$) 15 ± 2 μ IU ml⁻¹, $P = 0.36$), whereas s-IGF-I was lower in HE-OC than in LE-NOC (Fig. 2A). Serum IGFBP1 was significantly higher in HE-OC ($n = 11$) (121 ± 14 μ g l⁻¹) than in LE-NOC ($n = 12$) (57 ± 7 μ g l⁻¹) ($P < 0.001$), whereas no group difference was observed in IGFBP3 (HE-OC ($n = 8$) 5289 ± 180 μ g l⁻¹; LE-NOC ($n = 9$) 5166 ± 373 μ g l⁻¹, $P = 0.78$).

Dialysate samples were missing for IGF-I analysis in 4 out of 11 subjects in the HE-OC group and 1 out of 12 subjects in the LE-NOC group caused by problems with the flow through the catheters or cracking of the catheters caused by movements of the leg. For similar reasons paired dialysate samples from the resting leg and the leg that had

performed exercise the previous day were only available for IGFBP analysis from 9 subjects in each group. In the tendon dialysate a significantly lower concentration of IGF-I was observed in HE-OC compared with LE-NOC (Fig. 2B), whereas a significantly higher level of IGFBP1, -3 and -4 was observed in HE-OC compared with LE-NOC. No effect of exercise on IGFFBPs was observed in a subset of paired samples from each group (Fig. 3).

Discussion

The main finding in the present study was a significantly higher increase in tendon collagen synthesis in LE-NOC compared with HE-OC. The increase in tendon synthesis 24 h post-exercise was significant compared to resting values in LE-NOC, whereas no difference in tendon synthesis was observed in HE-OC in response to exercise (Fig. 1). A secondary finding was that no long-term effect of OC usage on tendon CSA was observed.

Sex steroid exposure

The concentration of endogenous 17- β -oestradiol was low in both groups due to the chosen time period of

the menstruation cycle in the LE-NOC and because endogenous oestradiol production was suppressed by ethinyl oestradiol (Arden *et al.* 1998). Thus, the two groups of subjects had contrasting exposure to ethinyl oestradiol with minimal levels of endogenous oestrogen.

Total circulating oestrogen concentration was not measured, because of the low sensitivity of our method to synthetic ethinyl oestradiol. However, it is known that in the hours following ingestion of OC, ethinyl oestradiol may reach levels comparable to the concentrations of 17- β -oestradiol experienced in the luteal phase of a normal menstruation cycle (Jung-Hoffmann *et al.* 1991). In the present study, we started the 4 h collection period of dialysate one hour after ingestion of OC when others have shown a 100-fold increase in circulating ethinyl oestradiol in users of OCs after ingestion of 30 μ g ethinyl oestradiol (Jung-Hoffmann *et al.* 1991).

In vitro and animal data support a direct effect of oestradiol on collagen tissue turnover (Liu *et al.* 1997; Slauterbeck *et al.* 1999; Yu *et al.* 1999, 2001). Nevertheless, based on present findings and cross-sectional experimental design, we cannot exclude that the observed differences between groups might be due to other hormonal differences between groups such as testosterone,

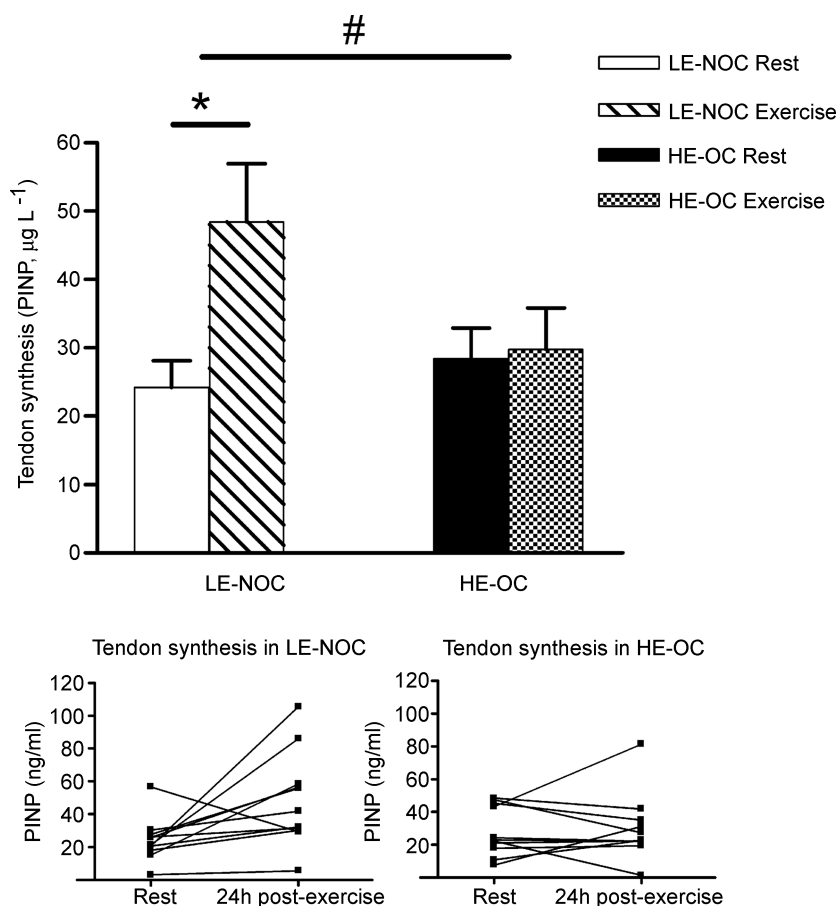


Figure 1

N-terminal propeptide of human collagen type I (PINP, $\mu\text{g L}^{-1}$) measured at rest and after exercise in LE-NOC and HE-OC in the peritendinous tissue in front of the patellar tendon. Values are mean \pm s.e.m. # $P < 0.05$: increase in tendon collagen synthesis (PINP) in response to exercise was significant higher in LE-NOC than HE-OC. * $P < 0.05$: tendon PINP higher 24 h post-exercise than at rest in LE-NOC.

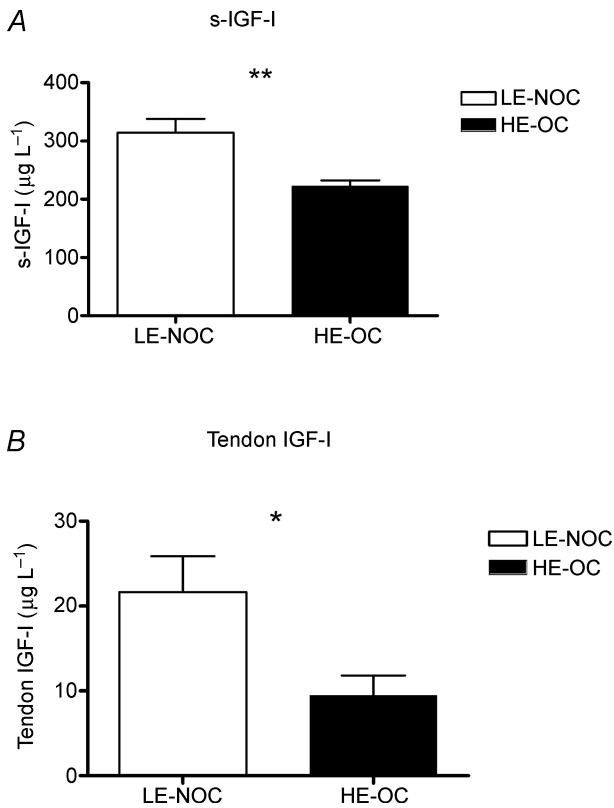


Figure 2
 A, concentration of insulin-like growth factor I (IGF-I) in serum ($\mu\text{g L}^{-1}$) in LE-NOC ($n = 12$) and HE-OC ($n = 11$). B, IGF-I in interstitial tissue fluid surrounding the patellar tendon in LE-NOC ($n = 11$) and HE-OC ($n = 7$) collected as paired samples from the resting leg and the contralateral leg which had performed exercise the previous day. * $P < 0.05$; ** $P < 0.01$: IGF-I higher in LE-NOC than HE-OC.

progesterone, IGF-I or progestogens. Testosterone has been shown to increase collagen content and fibril diameter in the hip joint capsule in rats (Hama *et al.* 1976), but no difference in s-testosterone was observed between groups in the present study. The difference in endogenous progesterone between LE-NOC and HE-OC was physiologically negligible, but we cannot rule out that the observed difference in tendon synthesis between groups was due to exogenous progestogens in OCs. The different types of synthetic progesterone are known to vary in androgenic effects (Sulak, 2004). Our results do not support a stimulating androgenic effect on tendon collagen synthesis of OCs. At least, the potentially stimulating effect of the synthetic progesterone on collagen turnover does not overrule the inhibiting effect of oestradiol, which has been reported in the literature (Fischer, 1973; Liu *et al.* 1997; Yu *et al.* 2001) and is supported by the findings in the present study. The lower IGF-I level in HE-OC further underlines the dominating effect of oestradiol, since exogenous progestogens, in contrast to ethinyl oestradiol, have been shown to enhance the IGF-I level (Nugent *et al.* 2003). However, we cannot rule out that the inhibiting effect of oestradiol is underestimated in the present study caused by a stimulating effect of exogenous progestogens.

Effects on resting and exercise-stimulated collagen synthesis

To our knowledge this is the first time the effect of an enhanced level of oestradiol introduced by OCs on tendon collagen synthesis has been measured *in vivo*.

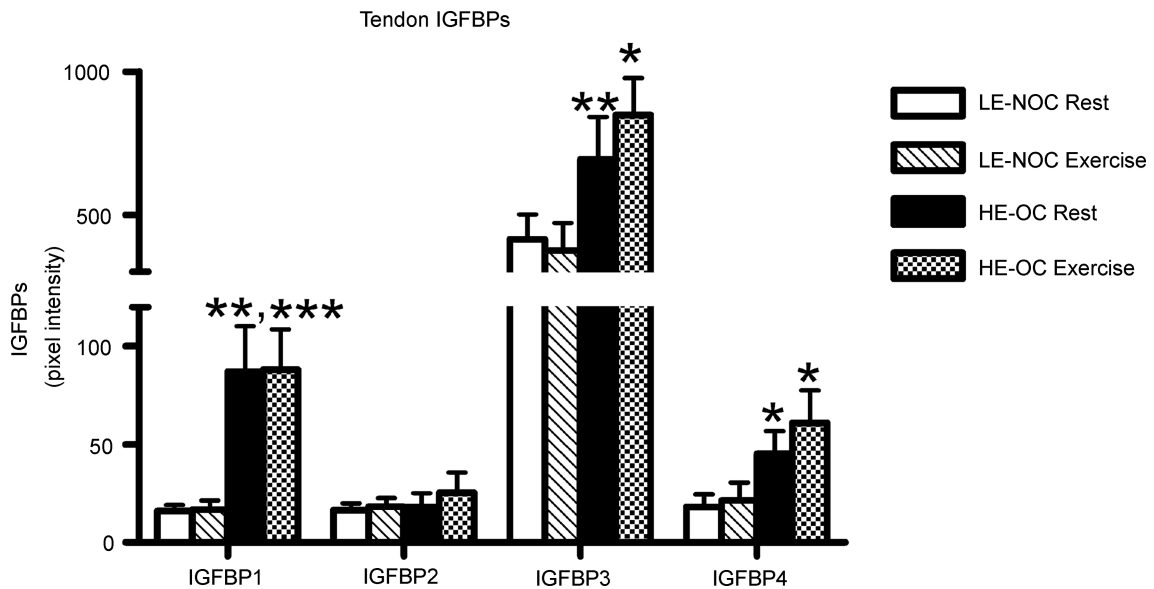


Figure 3
 Insulin-like binding proteins (IGFBPs) 1–4 (pixel intensity) in the interstitial tissue fluid surrounding the patellar tendon in LE-NOC ($n = 9$) and HE-OC ($n = 9$) collected as paired samples from the resting leg and the leg which had performed exercise the previous day. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$: IGFBP higher in LE-NOC than HE-OC at rest and/or post-exercise.

By simultaneously collecting tendon dialysate from the resting and exercise leg 24 h post-exercise it was possible to document that a local exercise-induced change in tendon collagen formation was taking place rather than a general effect on bone collagen formation. This is supported by earlier observations (Langberg *et al.* 2001; Miller *et al.* 2005).

The findings suggest that synthetic oestradiol in OCs inhibits the exercise-induced increases in tendon collagen synthesis. In an earlier study a lower fractional tendon collagen synthesis rate was observed in young eumenorrhoeic women compared to young men, both at rest and in response to an exercise bout similar to the exercise protocol in the present study (Miller *et al.* 2006). The findings by Miller *et al.* (2006) combined with those of the present study support the hypothesis that female sex hormones may inhibit adaptability to an increased training load, which may require repair of microdamage if the tissue is overloaded. In support of this, increases in collagen content and fibril diameter in the hip capsules have been observed in ovariectomized rats (Hama *et al.* 1976). These increases were shown to be inhibited by oestrogen treatment, or oestrogen combined with progesterone (Hama *et al.* 1976). A change in tendon collagen structure, introduced by oestradiol, may affect the strength of the tissue and partly explain why individual patellar tendon fascicles from women have been shown to be less resistant to rupture than fascicles from men (Haraldsson *et al.* 2006) and why greater knee and ankle joint laxities are observed in women compared to men (Beynnon *et al.* 2005; Shultz *et al.* 2005). However, the effect of oestradiol on the biomechanical properties of tendons is not convincingly clarified *in vivo* either in animals (Wentorf *et al.* 2006; Slaughterbeck *et al.* 1999) or in humans (Griffin *et al.* 2006).

Effects on resting and exercise-stimulated growth factors

IGF-I and IGF-BPs were markedly influenced by OCs in the present study. The results indicate that OCs reduce the bioavailability of IGF-I in the blood, as have been shown by others (Garnero *et al.* 1995; Wreje *et al.* 2000; van Rooijen *et al.* 2006), and in the interstitial fluid surrounding the patellar tendon, which to our knowledge is a novel finding. Earlier findings have shown that the exercise-induced increase in IGF-I is only transient and returns to baseline within 1 h (Berg & Bang, 2004). Therefore the dialysate was not analysed for changes in IGF-I in response to exercise.

IGF-I is expressed in tendons in animals and humans (Abrahamsson *et al.* 1991; Olesen *et al.* 2006c) and has been shown to stimulate collagen production by fibroblasts in animals (Abrahamsson *et al.* 1991). Based on our findings we cannot rule out that the difference in local IGF-I was

due to spillover from the blood. Nevertheless, as a growth factor, the consequential local group difference in the bioavailability of IGF-I is of importance *per se* since new findings indicate that IGF-I and exercise-induced changes in IGF-BPs seem to be involved in the observed increase in tendon collagen synthesis in response to exercise. Therefore, we suggest that the higher level of free IGF-I at rest in LE-NOC, potentially combined with a further transient increase introduced by exercise (Berg & Bang, 2004), may at least partly explain the increase in tendon collagen synthesis in response to exercise in LE-NOC, whereas HE-OC may have experienced a direct inhibition of ethinyl oestradiol combined with an indirect effect by a low IGF-I bioavailability.

Differences in patellar tendon CSA

A secondary aim in the present study was to clarify if long-term use of OCs has an effect on tendon size. No group difference in tendon size (CSA) measured by MRI was detected in the present study, even though HE-OC had been exposed to OCs for more than 7 years, but this may have been a Type 2 error ($P = 0.07$). To increase the statistical power, we included data from eight OC users from an earlier study. These women were very similar to our subjects (age: 25 ± 1 years; height: 1.68 ± 0.03 m; weight: 60 ± 2 kg; BMI: 21 ± 1 kg m⁻²; physical activity 5.0 ± 2.8 h week⁻¹). The same scientist, who was blinded for the OC status of the subjects, analysed the data. The increase in group size did not change the conclusion about no difference in tendon CSA between groups (LE-NOC ($n = 11$) 75 ± 2 mm²; all OC users ($n = 19$) 76 ± 3 mm², $P = 0.32$).

A greater tendon size has been observed in post-menopausal women compared with young women, which was suggested to be due to lowered inhibition of tendon collagen synthesis after the menopause, when the level of oestradiol is persistently low (Magnusson *et al.* 2003). Furthermore, results have shown that men both acutely (Miller *et al.* 2006) and in response to strength training (Kongsgaard *et al.* 2007) increase tendon collagen synthesis and tendon CSA. In addition, tendons in young women exposed to a generally higher level of oestrogen do not seem to adapt to training in a similar manner to tendons in men (Miller *et al.* 2006; Westh *et al.* 2008; Magnusson *et al.* 2007). No difference in tendon CSA was observed between experienced female runners and untrained women in a cross-sectional study (Magnusson *et al.* 2007; Westh *et al.* 2008). This observation is in contrast to men, where a larger tendon CSA (Achilles' and patellar tendon) has been observed in male runners compared to untrained men (Magnusson *et al.* 2007).

An obvious explanation for the similar CSA in the present study is that the difference in exposure to

total bioactive oestradiol (endogenous and synthetic) is quite small. Still, it should be kept in mind that the sensitivity for detecting a small difference in tendon CSA is quite low. Therefore, statistical Type 2 error cannot be excluded. Furthermore, there might be a difference in tendon collagen content (fibril diameter, fibril density) and collagen cross-links, which cannot be detected by measuring tendon CSA.

Limitations of the experiment

A potential limitation to the study could be the uncertainty of the difference in sensitivity for the different types of oestrogen existing in tendon tissue. Though, *in vitro* studies have shown that sensitivity for ethinyl oestradiol is comparable to the response seen when oestrogen receptor-positive cells (Prifti *et al.* 2003) and rat uterus (Hyder *et al.* 1999) are exposed to endogenously secreted 17- β -oestradiol. Whether there are differences in sensitivity for ethinyl oestradiol between the different types of oestrogen receptors in tendon tissue needs to be elucidated in order to understand the influences of endogenous and synthetic oestradiol on tendon structure and function.

The collagen content is a result of the balance between collagen synthesis and breakdown. In the present study we only assessed collagen synthesis. Oestradiol may also have an influence on tendon collagen breakdown. This view is supported in a study by Chen *et al.* (2001), which showed that exercise and oestrogen both had an anti-resorption effect in bone tissue in rats. In addition, a consistently lower bone degradation and bone turnover has been observed in postmenopausal women using hormone replacement therapy containing oestrogen (Burkman *et al.* 2004). OC use has also been associated with a lower bone turnover (Garnero *et al.* 1995; Wreje *et al.* 2000). It is tempting to hypothesize that OC use will reduce the tendon collagen turnover and thereby influence the tissue composition and biomechanical properties of tendons. Unfortunately, good markers of tendon collagen degradation in dialysate are not available, which precludes measurements of the net turnover of collagen in tendons.

Another way of examining the effect of oestradiol on tendon tissue *in vivo* would be to measure collagen synthesis in different phases of the menstrual cycle. In an earlier study, we measured fractional tendon collagen synthesis in a group of women and men after an acute exercise bout similar to the exercise protocol in the present study (Miller *et al.* 2006). Half of the women were tested in the early part of the follicular phase and the others in the mid-luteal phase of the menstrual cycle. In the four women tested in the follicular phase we observed a tendency towards an increase in fractional collagen synthesis in response to exercise ($P = 0.09$),

which was not seen in the three women tested in the luteal phase ($P = 0.31$). Because of the limited numbers of samples in each phase of the menstrual cycle, we cannot rule out that the latter observation was due to a Type 2 error. This was not investigated any further with this model for two reasons. Firstly, hormone profiles (e.g. cycle length, hormone phasing, and hormone concentration changes) vary considerably between normal menstruating women (Landgren *et al.* 1980). In the study by Miller *et al.* (2006) phases was established using an ovulation kit and by measuring progesterone, luteinizing hormone and follicle-stimulating hormone besides the oestradiol level. Yet, there was quite a large overlap in the oestradiol level between the two groups tested in the follicular phase ($n = 8$; oestradiol: 0.07–0.42 nmol l⁻¹) and luteal phase ($n = 7$; oestradiol: 0.24–0.79 nmol l⁻¹) separately. At the same time a 15-fold higher level of progesterone was measured in the luteal phase compared to the follicular phase (Miller *et al.* 2006). The cross-sectional design of the present study provided a clearer difference in sex hormones. Secondly, we have noticed that mere repeated insertion of a needle in the microdialysis technique increases collagen synthesis (authors' unpublished observations).

Since fractional tendon collagen synthesis has been shown to peak 24 h post-exercise in men (Miller *et al.* 2005), we also chose to measure collagen synthesis 24 h post-exercise. We cannot exclude that the time frame for the response to exercise is different in women than in men, especially at a high level of oestradiol. An exercise-induced increase in collagen synthesis may have been present in HE-OC before or after the time we measured. If the time pattern for the adaptive response in connective tissue to exercise is dependent upon the level of oestrogen, as suggested in the present study, more sex-specific training protocols are warranted.

Conclusions

In conclusion, we can report that the stimulating effect of strenuous exercise upon tendon collagen synthesis was higher 24 h post-exercise during low exposure to oestradiol compared to a situation where synthetic oestradiol was administered. This indicates that oestradiol may have an inhibiting effect on collagen synthesis *in vivo*. The mechanism behind the inhibiting effect of oestradiol upon collagen synthesis could be either direct or indirect suppression of IGF-I. No differences in patellar tendon CSA between users and non-users of OCs were observed, which may be because exposure to total bioactive oestradiol not differ much over a menstrual cycle. Since no difference in tendon CSA was observed, future training studies will be needed to clarify if chronic use of OCs has an influence on the ability to adapt to an increased training load.

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