Effect of administration of oral contraceptives *in vivo* on collagen synthesis in tendon and muscle connective tissue in young women

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**Running title:** Effect of ethinyl estradiol *in vivo* on collagen turnover

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Abstract

Women are at greater risk than men for certain kinds of diseases and injuries, which may at least partly be caused by sex hormonal differences. We aimed to test the influence of estradiol in vivo on collagen synthesis in tendon, bone and muscle. Two groups of young, healthy women similar in age, body composition and exercise-training status were included. The two groups were either habitual users of oral contraceptives exposed to a high concentration of synthetic estradiol and progestogens (OC, n=11), or non-OC-users tested in the follicular phase of the menstrual cycle characterized by low concentrations of estradiol and progesterone (Control, n=12). Subjects performed one hour of one-legged kicking exercise. The next day collagen fractional synthesis rates (FSR) in tendon and muscle connective tissue were measured after a flooding dose of $[^{13}\text{C}]$ proline followed by biopsies from the patellar tendon and vastus lateralis in both legs. Simultaneously, microdialysis catheters were inserted in vastus lateralis and in front of the patellar tendon for measurement of insulin-like growth factor I (IGF-I) and its binding proteins. S-PINP and urine-CTX-I were measured as markers for bone synthesis and breakdown, respectively. Tendon FSR and PINP were lower in OC compared to Control. An increase in muscle collagen FSR post-exercise was only observed in Control ($P < 0.05$). Furthermore, the results indicate a lower bioavailability of IGF-I in OC. In conclusion, synthetic female sex hormones administered as OC had an inhibiting effect on collagen synthesis in tendon, bone and muscle connective tissue, which may be related to a lower bioavailability of IGF-I.

Key words: Estrogen, exercise, insulin-like growth factor I, ethinyl estradiol, bone.
Introduction

Collagen is the most abundant protein in the human body, and comprises a very high fraction of the tissue organic mass in bone (90%), tendon (60-85%) ligament (70%) and in intramuscular connective tissue (~30% and up to 90%) (31; 57). The frequency of several diseases linked to collagen-rich tissue seems to be biased by sex (30; 34; 36; 65). Furthermore, women are at a greater risk than men for sustaining certain kinds of soft tissue sports injuries (7; 24; 26). Several epidemiological studies have shown that women have up to six times greater risk of anterior cruciate ligament (ACL) ruptures than activity matched men (24). It has been suggested that sex hormones may influence collagen turnover, tissue composition and biomechanical properties of the tissues, which in part may explain sex-specific differences in risk (24). In support of this, tendon collagen synthesis is lower in women compared to men at rest and after exercise (47). In addition, a lower peak stress in female collagen fascicles from patella tendons have been demonstrated compared to fascicles from men (21).

Estrogen receptors have been localized to bone, ligaments and muscle tissue (39; 56; 64), and animal data have shown that tendons express transcripts for estrogen receptors (22). However, the effect of estrogen on collagen synthesis is conflicting, which probably are related to variation between animal species and the applied methods. Several in vitro studies have shown an inhibiting effect of estradiol on the synthesis of collagen and fibroblast proliferation in ACL tissue samples from rabbits and two women (40; 67). In disparity, no effect of estrogen administration on collagen synthesis in sheep ACL samples (56), whereas a stimulating effect of estradiol on gene expression of type I collagen in porcine ACL, has been observed (37). In the latter study an inhibition of gene expression of Type I collagen were observed when estradiol and tensile loading of ACL fibroblasts were combined, in spite of up-regulated the expression of Type I collagen when the stimuli applied separately (37). A negative influence of estradiol administration on the
anabolic response to exercise training in tendons is supported by human data (20; 62). This observation may specifically be of importance when it comes to the tissue adaptation to mechanical loading. This discrepancy in the effect of estradiol on collagen synthesis in tendon and ligament demonstrates the importance of performing in vivo human studies to elucidate the effect of estradiol.

In young animals insulin-like growth factor I (IGF-I) exerts anabolic effects on tendon fibroblasts by increasing collagen synthesis in a dose-dependent manner (25; 49). Oral administration of estrogens is known to reduce serum IGF-I and to enhance the concentrations of IGF binding proteins (IGFBPs) (5; 8; 20; 25). Increased binding by bindings proteins lower bioavailability of IGF-I, and thus potentially result in a lower collagen synthesis in tendon, muscle connective tissue and bones.

The primarily aim of the present study was to measure tendon and muscle collagen synthesis in two groups of women who were either users or OC or non-users of OC (Control), and thereby clarify whether synthetic female hormones either directly or indirectly have an influence on collagen turnover.

**Design and methods**

**Subjects.** Twenty-three young, healthy women were recruited for the study, which was performed at The Institute of Sports Medicine, Copenhagen, Denmark. The subjects were non-smokers, nulliparous, on no medication (except OC where indicated), and absent of orthopaedic and medical conditions, as judged by history and routine medical examination. Women with a cycle length outside the typical range of 21-35 days were excluded (12). The subjects gave informed consent to the protocol adhering to the Declaration of Helsinki and approved by the Ethics Committee of Copenhagen and Frederiksberg Communities (KF-01-032/04).
Eleven women who were long-term users of OC (7.2 ± 2.1 treatment yrs (mean ± SD) range 5-10 yrs), including treatment with Lindynette® (n=7) (30 µg ethinyl estradiol and gestoden 0.0075 mg per day) or Cilest® (n=4) (35 µg ethinyl estradiol and 0.25 mg norgestimate per day). The remaining 12 eumenorrheic women (Control) had never used OC and had a regular cycle length for at least one year. To make the difference in estrogens (endogenous secreted and synthetic ethinyl estradiol) between groups as large as possible, Controls were tested in the early follicular phase of the menstrual cycle where the concentration of estrogen is low, whereas the OC-users were tested in the hours after the last pill ingestion (day 18-21 in pill cycle), where the concentration of ethinyl-estradiol is high (29). The two groups were otherwise similar in the following characteristics: age, height, weight, body mass index (BMI), and body composition as measured by Dual-energy X-ray absorptiometry (DEXA) (Table 1). Finally, the training status of the groups were comparable as determined by a questionnaire asking about frequency and hours spent on regular planned physical training per week, including total time per week spent biking for transportation (usually at low intensity) (Table 1).

**Design.** The influence of OC and exercise on tendon and muscle collagen fractional synthesis rates (FSR) were measured *in vivo* by infusion of stable isotope labeled amino acids and collection of biopsies from the patellar tendon and vastus lateralis of each leg. To determine the effect of exercise a strenuous one-legged exercise bout (1h at 67% of workload maximum (W\textsubscript{max})) was performed 24h prior to the measurements. The time-point for measuring FSR was chosen based on earlier findings in men showing markedly enhanced FSR in muscle and tendon 24 h after a similar exercise protocol (48). The contra-lateral leg represents the resting situation. Patellar tendon CSA was measured by Magnetic resonance imaging (MRI) on a separate day before the experiment (for further details see (20).
**Investigative protocol.** Two weeks prior to the study, subjects visited the laboratory to determine \( W_{\text{max}} \) on a one-legged modified Krogh ergometer. After a 5-min warm-up without resistance, the subjects began one-legged kicking (35 kicks per minute) for 3 min at 0.5 kg load. The load was increased by 0.5 kg every 3 min until the subjects could no longer maintain the cadence. The final workload was defined as \( W_{\text{max}} \). The subjects were instructed to avoid strenuous physical activity the last two days prior to and during the experimental days (47).

At day after the exercise bout the women reported to the lab after an overnight fast. After arrival a urine sample was collected for analyses of a marker for bone degradation (urine C-terminal telopeptides of Type-I collagen (CTX-I). Subsequently, one cannula was inserted into the antecubital veins on each arms; one for tracer infusion and another for blood sampling. Following insertion, blood samples were obtained for measurements of background isotope enrichments, concentrations of hormones and a marker for bone synthesis (serum amino-terminal pro-peptide of Type I collagen (PINP)). Afterwards, the subjects received a standardized commercial clinical nutrient drink (Semper, Frederiksberg, Denmark 15% protein, 64% carbohydrate and 21% fat) in divided doses every 30 min until the end of the experiment. The drink provided energy equivalent to 1.4x basal metabolic rate per 30 min period, with a double dose at initiation of feeding. Basal metabolic rate was estimated from fat free mass of the subject determined by the skin-fold technique (4, 5). Nutrition was standardized to account for the stimulation of protein synthesis by essential amino acids (59) The nutrient drinks were served within regular interval to minimize disturbances in the isotope enrichment. In addition, the subjects completed weighed-food records for three days prior to the experiment to measure habitual intake of energy and macronutrients. There was no difference in energy intake or macronutrient composition between groups (Table 2).
Measurement of collagen synthesis. Measurement of tendon and muscle collagen FSR were performed according to previously applied approaches (4; 48). Briefly, upon baseline blood sampling, a skin biopsy (10 mg) was obtained under local anesthetic (lidocaine 1%) from the posterior hip, just below the waist for determinations of background enrichment of [13C] proline in collagen. A flooding dose of proline (4 g total; 1.0 g labeled L-1-[13C] proline (Cambridge Isotope Laboratories, Andover, MA, USA), 3.0 g unlabeled proline (AppliChem, Darmstadt, Germany)) was intravenously infused over 3 min. The tracer was chemically pure, >99 Atoms % in 13C, and were certified to be sterile and without pyrogens. The tracer was dissolved with 12C-proline on the morning of the infusion in 0.9% NaCl using a sterile technique and then passed through a disposable 0.20 μm filter (Sartorius, Hannover, Germany). After flooding, blood samples were drawn at 10-60 min intervals to determine the area under the 13C-proline enrichment curve in plasma measured as the tracer/tracce ratio. Twenty-four hours after exercise, which correspond to two (Control, n=9, OC, n=8) or three hours (Control, n=3; OC, n=3) after the isotope flood, biopsies were taken from the patella tendon (~10 mg) and the vastus lateralis muscle (50-100 mg) of each leg after previously preparing incision sites with local anesthetic (lidocaine 1%). Tendon biopsies were obtained by using a 16 G Monopty biopsy instrument (Bard Inc, Covington, GA, USA) under ultrasound guidance. Muscle biopsies were obtained by using a 5 mm Bergström needle with suction. Biopsies were cleared of external adipose tissue and blood, frozen in liquid nitrogen, and stored at -80°C for subsequent analysis.

Plasma proline enrichment. Plasma proline was prepared as previously described and analyzed as its t-butyldimethylsilylation (MTBSTFA+1% TBDMS) derivative by gas-chromatography mass-spectrometry (GC, Trace GC 2000 series) (3; 55). We used the plasma 13C-proline enrichment since the flooding technique is assumed to equilibrate all free amino acid pools, including the true precursor pool, prolyl-tRNA (3).
Extraction of collagen from tendon, muscle and skin. Details regarding this procedure have been given elsewhere (4). Briefly, skin (10 mg), tendon (5-10 mg) and muscle (20-30 mg) were homogenized in buffer (0.15 mol L\(^{-1}\) NaCl, 0.1% Triton X-100, and 0.02 mol L\(^{-1}\) Tris-HCl, 5 mmol L\(^{-1}\) EDTA, pH 7.4). Muscles were then centrifuged and the supernatant removed. To the pellet containing myofibrillar and collagen proteins, KCl (0.7 mol L\(^{-1}\)) was added to precipitate non-soluble proteins (collagenous). Thereafter, the pellet containing collagen was washed with acetic acid and acetic acid-pepsin (0.1% wt/vol), dissolving immature collagen and precipitating the insoluble collagen pellet. All the protein fractions were hydrolyzed in 6 mol L\(^{-1}\) HCl at 110°C overnight and the amino acids were extracted through disposable columns using resin (Acidic cation exchanger, Dowex AG-50W, Bio-Rad, Sundbyberg, Sweden).

The amino acids were derivatized as their N-acetyl-n-propyl (NAP) ester (44) and analyzed by gas chromatograph combustion isotope ratio mass spectrometry (GC-C-IRMS) (Delta Plus XL, Thermo Finnigan, Bremen, Germany).

Calculations. The fractional rates of protein synthesis were calculated using the standard equation for the precursor-product principle using the flooding approach (53). Thus, FSR (% h\(^{-1}\)) = \(\Delta E_m/E_p\) \times \(1/t \times 100\%\), where \(\Delta E_m\) is the change in enrichment of proline in the tendon and muscle tissue samples compared to the enrichment in the initial skin biopsy (assuming basal tissue labeling to be identical to that at the natural abundance in the initial skin biopsy), \(E_p\) is the average enrichment of the precursor (plasma \(^{13}\)C-proline) determined as the area under the time-plasma enrichment curve (Figure 1), and \(t\) is the time (h) of tracer incorporation.

Microdialysis. Parallel to the isotope protocol, local concentrations of metabolites were measured by microdialysis technique in the interstitial fluid in front of the patellar tendon and in the skeletal muscle (vastus lateralis) in the leg (35), which had not performed exercise. Under local anesthetic (lidocaine 1%) sterilized (ethylene oxide sterilization) high molecular mass cut-off (3000 kDa,
membrane length 30 mm, i.d. 0.50 mm) catheters were inserted under ultrasound guidance as previously described (35). The microdialysis catheter was perfused at a rate of 2 μL min⁻¹ with a Ringer-acetate solution mixed with radioactive labeled glucose (D-[3-³H]-glucose, 9.25 MBq 0.25 mCi⁻¹; 0.25 mL) in aqueous solution steri-pack (Perkin Elmer Life and analytical science, Boston MA, USA, Net 331A, lot#3559-801). The relative recovery (RR) over the membrane was estimated for each dialysate sample (54) by pipetting 3 μL dialysate and 3 mL scintillation fluid (Ultima Gold, Perkin Elmer, Boston, USA) into a counting vial followed by measuring enrichment of glucose in a β-counter. Samples with RR below 15% were not used for analyses. RR was not significant different between groups either in muscle or tendon dialysate samples. The mean recovery values for the sample vials for the specific parameters ranged from 27±4 to 33±4% for the samples from OC-users, and 25±4 to 32±5% for the samples from Control.

**Dialysate, urine and additional blood analysis.** All blood samples were taken from an antecubital vein into sealed vials. After separation by centrifugation (4°C) the blood samples were either immediately analyzed for serum estradiol (s-estradiol) and s-progesterone or stored at -80°C until analysis for s-testosterone, s-GH, s-IGF-I, s-IGFBP-1, s-IGFBP-3 and s-PINP.

Estradiol was analyzed by chemiluminescent competitive immunoassay (Immune 2500) (NPU 1972, estradiol; Diagnostic Product, Los Angeles, CA, USA). The detection level for the analysis is 10 nmol L⁻¹. Progesterone was analyzed by architect microparticle enzyme immunoassay (Abbott Diagnostics, Wiesbaden, Germany). The analysis for testosterone in serum extracts was performed by liquid chromatography mass spectrometry (LC-MS) using an atmospheric pressure chemical ionization (APCI) interface (CV% <15%).

S-GH was measured using noncompetitive time-resolved monoclonal immunofluorometric assay (TR-IFMA; Wallac Oy, Turku, Finland), while s-IGF-I and dialysate IGF-I were determined by TR-IFMA after acid-ethanol extraction, as previously described (16). All samples were
measured in the same assay run. The intra assay CV for this IGF-I assay is <5%. S-IGFBP-1 was determined by an in-house radioimmunoassay (RIA)(63), with modifications as described previously (33). The within and between assay CVs averaged <5 and <16%, respectively. Serum IGFBP-3 was measured by commercially available IRMA (BioSource Europe, Nivelles, Belgium). The within and between-assay CVs for this assay are <5 and <10%, respectively. Small volumes of tendon dialysates were analyzed for IGFBP-1 to -4 by Western ligand blotting (WLB), as previously described (15).

Serum was analyzed for PINP for indirect quantification of collagen synthesis in bone (s-PINP). The analysis for PINP was performed using a sandwich ELISA utilizing purified alpha 1-chain specific rabbit antibodies (donated by Teisner B, Department of Medical Microbiology, University of Odense, Denmark) (28). The within (double determination) and between-assay CVs averaged 2.2% and 4.9%, respectively.

Urine samples were analyzed for CTX-I and creatinine for quantification of degradation of Type I collagen. Urine-CTX-I was quantified by an enzyme immunosorbent assay (Urine CrossLaps® ELISA, Nordic Bioscience Diagnostics a/s, Denmark). Within- and between-assay CVs were 3.6±2.3% and 2.7±2.0%, respectively. Urine-creatinine was analyzed by a two-point kinetic slide method, with a Vitros 5.1 FS (670 nanometer (nm)).

**Statistics.** Two way repeated measures ANOVA (One Factor Repetition) were used to test for differences between groups in tendon and muscle collagen FSR, and if $P \leq 0.05$ *pos hoc* analyses were performed using pair-wise multiple comparison procedures (Holm-Sidak method). The length of the period from the flooding dose until the biopsies did not influence the calculated mean values for protein FSR, and therefore only pooled data are shown. Student’s unpaired *t*-tests were used to test for differences between groups in the remaining parameters. The baseline characteristics of the subject groups are presented as mean±standard deviation (SD), whereas the
results are presented as mean±standard error of the mean (SE). The statistical analyses were performed using the statistical software packages: Sigma Stat version 3.5 (Systat Software, Chicago, IL, USA) for the two-way repeated measures ANOVA; and Prism version 4.01 (GraphPad, San Diego, CA, USA) (2004) for the remaining tests.

**Results**

The women performed the strenuous exercise bout at an average workload of 67±2% of $W_{\text{max}}$ in Control and 68±2% of $W_{\text{max}}$ in OC without any difference between the two groups (Table 1).

**Sex hormones.** On the day of the experiment, concentrations of s-17-β estradiol were below the analytic detections level in all but two subjects (one from each group: OC, 0.26 nmol L$^{-1}$; Control, 0.16 nmol L$^{-1}$). Serum progesterone was within the lower 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). The difference between groups in s-progesterone was small but significant (Control: 0.94±0.09 nmol L$^{-1}$ vs. OC: 0.66±0.03 nmol L$^{-1}$, $P<0.05$), whereas serum testosterone did not differ between Control (n=10, 1.0±0.1 nmol L$^{-1}$) and OC (n=11, 0.9±0.1 nmol L$^{-1}$).

**Tendon collagen synthesis.** Tendon collagen FSR was lower in OC compared with Control ($P=0.05$). At rest tendon collagen FSR was 57% lower in OC compared to Control ($P<0.05$). Post-exercise tendon collagen FSR not significant lower in OC compared with Control (-41%, $P=0.13$) (Figure 2). No effect of exercise ($P=0.62$) or interaction between sex hormonal levels and exercise ($P=0.45$) was observed 24h post-exercise.

**Tendon cross-sectional area.** Tendon cross-sectional area measured by magnetic resonance imaging was not different between the two groups (Table 1) (20).

**Muscle collagen synthesis.** A general significant effect of exercise was observed ($P<0.05$). Separately, a stimulating effect of exercise was observed in muscle collagen FSR in Control
(\(P<0.05\)) (Figure 3), whereas no significant increase was observed in OC (\(P=0.39\)). The overall difference related to the sex-hormonal differences between groups in muscle collagen FSR was not significant (\(P=0.40\)), and either resting values (\(P=0.75\)) or post-exercise values (\(P=0.23\)) were significantly different between groups.

**Bone collagen turnover.** Bone synthesis (s-PINP) was significantly lower in OC-users (47±7 \(\mu g\) L\(^{-1}\)) than Control (73±10 \(\mu g\) L\(^{-1}\), \(P<0.05\)), indicating a reduced bone formation in OC-users. In contrast, no significant differences between groups were observed in CTX-I, a marker for degradation of bone tissue (228±31 vs. 256±45 \(\mu g\) CTX-I mmol\(^{-1}\) creatinine in Control vs. OC, \(P=0.31\)) or mean bone mass density (BMD) (Control: 1.20±0.02 g cm\(^{-1}\); OC-users: 1.21±0.02 g cm\(^{-1}\), \(P=0.78\)).

**GH, IGF-I and IGFBPs.** After overnight fasting s-GH did not differ between groups, whereas s-IGF-I was lower in OC-user than in Control. S-IGFBP-1 was significant higher in OC than in Control, but no group difference was observed in IGFBP-3 (Table 3).

In the dialysate from the peritendinous tissue a significantly lower IGF-I concentration was observed in OC, whereas the concentrations of IGFBP-1,-3,-4, but not IGFBP-2, were significantly higher compared with Control (Table 3).

In the dialysate from the interstitial tissue in the skeletal muscle IGF-I was lower in OC, as observed in blood and tendons, although the difference did not reach significance (\(P=0.08\)). However, IGFBP-1 to -4 were all significantly higher in OC compared with Control (Table 3).

**Discussion**

The main findings in the present study were that 1) tendon collagen protein synthesis rates both at rest and after exercise were lower in women exposed to a high concentration of synthetic female hormones compared to women exposed to a low concentration of endogenous female hormones
but a higher bioavailability of IGF-I (Controls) in the peritendinous tissue and in the interstitial fluid of the skeletal muscle; 2) muscle collagen FSR was increased 24h post-exercise compared with resting values in Controls, whereas no response to exercise was observed in OC-users; and 3) the concentration of a marker for bone synthesis was lower in OC-users compared to Controls.

**Effects on tendon collagen synthesis**

At rest a lower tendon collagen FSR in OC compared to Control was observed, which indicates a potential diminishing effect of OC administration either directly by OC or indirectly by other hormonal changes introduced by OC. Tendon FSR has been measured once before in vivo in eumenorrheic premenopausal women (47). In that study lower tendon FSR was observed at rest and 72h post-exercise in women compared to men after a similar exercise bout as in the present study. In women, tendon FSR did not differ between the resting and exercise-stimulated leg (47), which is in accordance with the present findings that show no effect of exercise at 24h post-exercise. In contrast, in men a stimulating effect of exercise has been observed 24h and 72h post-exercise (47; 48). Together, these results indicate a sex difference in the regulation of tendon synthesis, which could partly be explained by the discrepancy in the concentrations of sex hormones. In this context, the present results support an inhibiting role on FSR of synthetic female hormones in oral contraceptives in young women.

No response to exercise was observed in tendon collagen FSR in either group. However, when the tendon dialysate was analyzed for PINP, representing local synthesis of collagen, a response to exercise was observed in Control, but not in OC (the results have been published previously) (20). The disparity may be caused by a methodological difference in sensitivity for measuring a change in collagen synthesis in response to exercise. Changes in PINP in response to acute exercise represent an enhanced synthesis of new soluble immature collagen, which is not necessarily built into the structural components (fibrils). In contrast, changes in tendon FSR using
the present procedure represent a change in both soluble, but probably primarily mostly insoluble mature collagen. The isotope method may not be sensitive enough to detect small changes in synthesis of new immature collagen when measuring changes in total tendon collagen FSR in response to acute exercise in contrast to the microdialysis technique.

Tendon FSR was only measured 24h post-exercise. It cannot be excluded that a response to exercise would have been demonstrated if it had been measured at a different time-point. This hypothesis is based on results in men showing an increase in tendon FSR after 6h and a peak in tendon FSR 24h post-exercise (48), whereas the time frame for PINP seems to be delayed (35; 48). Correspondingly, even if the response to exercise is probably smaller in women, an increase in tendon FSR might have been apparent at an earlier time point, whereas at 24h post-exercise only a delayed response to exercise is observable. Nevertheless, based on the present and earlier findings OC administration apparently has an inhibiting effect on tendon synthesis, especially in response to exercise (20). This is supported by in vitro animal findings showing a negative interaction between estradiol and mechanical loading on the expression of type I collagen in ACL fibroblasts (37). Furthermore, no difference in tendon CSA has been observed when untrained women are matched with experienced female runners, whereas experienced male runners were characterized by a bigger tendon CSA compared with untrained men and women (41; 62). The sex difference may be related to the stimulating effect of exercise being counteracted by estradiol in women, whereas in men testosterone and exercise have a combined anabolic effect. In addition, tendon collagen FSR may be lowered by a reduction in the bioavailability of IGF-I in OC-users. This hypothesis is further supported by unpublished results from our lab showing a negative correlation between the concentrations of estradiol and the response to exercise in patellar tendon FSR in eumenorrheic women (n=7, r=-0.84, \( P<0.05 \)).
**Tendon CSA**

Long-term use of OC did not have a significant effect on tendon size (CSA) measured by MRI. Including additional eight subjects to increase the statistical power did not change this conclusion, as discussed in a previous paper (20). An explanation for the comparable tendon CSA could be that the differences between groups in exposure to bioactive female hormones (endogenous and synthetic) is quite small when comparing the accumulated exposure to estradiol through the menstrual cycle with the exposure during a pill cycle including one week without OC administration. Another explanation could be related to the effect of OC on tendon collagen degradation rate not being elucidated in the present study. Along this line, OC may similarly also cause a reduction in tendon collagen breakdown, as has been observed in bone (17), and thereby not change the net collagen content. Finally, we cannot rule out that even though tendon CSA does not differ, tendon composition (collagen content, fibril diameter, fibril density and cross-links) and thereby tendon biomechanical properties may differ between OC-users and Control. In this context, future studies should explore the influence of OC on tendon collagen content and composition Furthermore, the impact of the suggested hypothetic negative interaction between exercise and estradiol on tendon CSA should be clarified by comparing well-trained female athletes who are either long-termed users of OC or have never been using OC (37).

**Effects on muscle collagen synthesis**

An increased collagen turnover in intramuscular connective tissue is important during hypertrophy of the contractile apparatus in response to training to ensure appropriate adaptation of the connective tissue structures. In the present study a significant increase post-exercise in muscle collagen FSR was observed compared to resting values in Controls, whereas the increase in muscle collagen FSR post-exercise was not significant compared to resting values in the women who had ingested an OC. These novel findings indicate that OC has a negative effect on the
response to exercise in muscle collagen synthesis. It cannot be excluded that the present findings are a result of secondary hormonal responses of the OC intake and not a direct effect of estradiol (synthetic or endogenous estradiol). In an earlier study, an approximately 3-fold increase in muscle collagen FSR was observed 24 h post-exercise in eumenorrheic women in either the follicular phase or luteal phase of the menstrual cycle (45). This does not support a direct inhibiting effect of estradiol. However, it cannot be ruled out that the difference in estradiol between menstrual phases was too small to induce a difference in muscle collagen FSR, or that differences in other non-controlled hormonal parameters between phases have counteracted the effect of estradiol (46).

A change in muscle collagen FSR in response to exercise is in discrepancy with the lack of response in tendon FSR. A physiological explanation for the different result between tendon and muscle collagen tissue might be that the tissues differ in distribution and numbers of estrogen receptors (α and β), which thereby may induce tissue differences in collagen synthesis in the response to mechanical loading as observed in bone (38; 43). Another more methodological explanation for the discrepancy between the effect of OC on tendon and muscle collagen FSR might be related to the different preparation procedures of the tissue samples before GC-C-IRMS analysis. Although seemingly sparse (3; 4) some contamination by other proteins cannot be ruled out in the muscle preparations, whereas the risk of this error is minimal in tendon since collagen is the far most abundant protein in tendons.

**Effects on IGF-I and IGFBPs**

Systemic and local concentrations of IGF-I and IGFBPs were markedly influenced by OC in the present study, which indicates a reduced bioavailability of IGF-I by OC. A reduction in free-IGF-I in the blood has been shown by others (17; 60; 66). However, the difference in free-IGF-I concentrations within the tissues is novel. IGF-I is expressed in muscle and tendons (1; 11; 19; 51)
and has been shown to stimulate tendon collagen synthesis in rabbit fibroblasts (1). The specific roles of the individual IGFBPs are still discussed (13; 14). Six distinct IGFBPs (-1 to –6) have been isolated and characterized (14). The most abundant IGFBP is IGFBP-3 was measured in the present study together with IGFBP-1,-2 and –4. The main role of IGFBPs is to inhibit IGF-stimulated events by binding IGF and hinder IGF-binding to the IGF-I receptors. However, IGFBPs proteolysis appears to reverse this inhibition. In addition, the different IGFBPs also seem to have independent functions, which differ depending on the tissue type (13; 14). Still, future studies will be needed to elucidate the physiological function in vivo of the individual IGFBPs. Nevertheless, the significant higher level of IGFBPs and lower IGF-I concentration in OC-users observed in the present study indicates a lower availability of free IGF-I. This observation may at least partly explain the lower tendon collagen synthesis and reduced response to exercise in muscle connective tissue.

Effect of OC on sex hormonal concentrations

The concentration of endogenous 17-β-estradiol was low in both groups due to the chosen time-period of the ovarian cycle in Controls and because endogenous estradiol production was suppressed by ethinyl estradiol in OC-users (2). Thus, the two groups of subjects had contrasting exposure to ethinyl estradiol with very low concentrations of endogenous estrogen. Unfortunately, it was not possible to measure total systemic concentrations of estrogens (endogenous and exogenous estrogens) due to methodological limitations. However, in the present study the flooding dose was started three hours after ingestion of OC, and the biopsies were harvested two hours later. During this period it is known that ethinyl estradiol is enhanced since a 100-fold increase in circulating ethinyl estradiol has been observed in the hours following the daily ingestion of 30 μg ethinyl estradiol in OC-users (29). Therefore, a clear advantage of the present
design is a low concentrations of endogenous estradiol og progesterone, but a distinctive difference in synthetic female hormones.

A difference in sensitivity to the distinct types of estrogens may exist in muscle and tendon tissue. However, *in vitro* studies using estrogen receptor positive cells (breast cancer cells) (52) and rat uterus tissue (27) have shown that sensitivity for ethinyl estradiol is comparable to the response seen during exposure to endogeneous secreted estradiol. Whether this is also true when it comes to the response in skeletal muscle tissue and tendons needs to be elucidated. New results have shown a stimulating effect of estradiol in postmenopausal women (32) (M Hansen et al, *in review*). If it is assumed that the sensitivity in tendons for ethinyl estradiol is comparable to the sensitivity for estradiol, these new results taken together with the present results indicate that the lower tendon synthesis rate in OC-users is not caused by an inhibition of estradiol, but may be introduce by other indirect effects of OC (e.g. lower IGF-I bioavailability) or the synthetic progesterone (progestagens) in OC.

The naturally secreted progesterone has been shown to have both inhibiting (18) and stimulating effects (67) on collagen synthesis. However, the difference in endogenous progesterone between Control and OC was physiologically negligible. The isolated effect of progestogens on tendon and muscle connective tissue turnover is currently not clarified. Furthermore, the different types of progestogens are known to vary in androgenic effects in general, which complicates the picture even more (58). Our results do not support a stimulating androgenic effect of OC on collagen synthesis in the studied tissues. At least, the potentially stimulating effect of the synthetic progesterone on collagen turnover is overruled by the ethinyl estradiol administration. In support, exogenous progestogens have been shown to enhance the IGF-I concentration (50), whereas in the present study IGF-I was reduced after OC administration, which underlines the dominating effect of estradiol compared to the effect of progestogens.
Effects on bone collagen turnover

A marker for bone collagen synthesis was lower in OC compared with Control, whereas no significant group difference was found in CTX-I, a marker of bone breakdown. Hence, a lower bone collagen content could be expected in OC-users, but no difference between groups was detected in BMD as a surrogate measurement for bone collagen. This lack of difference may be due to a type II error since BMD is highly influenced by the genetic endowment of the individual subjects, and the fact that the group sizes in the present study were small. The effect of estrogen administration on bone mass in premenopausal women is not clear (6; 42). However, when studying young eumenorrheic women, several recent reports have shown a detrimental effect of OC on BMD, especially when combined with exercise (23; 61). Findings indicate that OC reduced the ability to achieve a high maximal peak bone mass in young women and may increase the risk of fractures (6; 42). In a prospective study enrolling 46000 women during 482083 person-years of follow-up, a higher risk of bone fracture was observed among women who had used OC compared to those who had never used OC (10). Furthermore, low age at initiation of OC-use in a group of eumenorrheic female endurance athletes was a major determinant of lower BMD (23). Together with the present data this indicates that exposure to OC may inhibit the anabolic effect of exercise on bone formation in young women, which is further supported by animal studies (9).

Conclusions

Manipulation of the concentration of female hormones in vivo by oral contraceptives was associated with a lower tendon collagen synthesis rate in young women. In addition, OC seemed to decrease the stimulating effect of exercise on the synthesis of intramuscular connective tissue, since an increase in muscle collagen FSR was observed in Control, but not in OC-users. Finally, markers for bone collagen synthesis indicated a detrimental effect on the balance between bone
collagen synthesis and breakdown. The observed group differences might be explained by a direct inhibition by synthetic female hormones or indirect effects induced by OC ingestion. Related to the latter, the lower bioavailability of IGF-I in OC may be mechanistically involved in the observed changes in collagen synthesis in tendon, muscle connective tissue and bone.
Acknowledgements

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Figure Legends

**Figure 1**
Plasma enrichment of $^{13}$C-proline after flooding dose of [1-$^{13}$C]-proline (Tracer to tracee ratio).

**Figure 2**
Patellar tendon collagen fractional synthesis rates (FSR) at rest and 24h after exercise in Control and OC-users. Values are mean±SE. Control: Women who had never used oral contraceptives, who were tested in the follicular phase; OC: Oral contraceptive users. ** $P<0.01$, (*) $P=0.13$ unpaired t-test Control vs. OC.

**Figure 3**
Muscle collagen fractional synthesis rates (FSR) at rest and 24h after exercise in Control (n=10) and OC-users (n=11). Values are means±SE. Control: Women who had never used oral contraceptives, who were tested in the follicular phase; OC: Oral contraceptive users. * $P<0.05$ significant difference between rest and exercise leg in Control.
Tables

Table 1. Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th></th>
<th>OC</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>24 4</td>
<td>24 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>65.0 8.4</td>
<td>63.7 8.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.67 0.07</td>
<td>1.70 0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg m⁻²)</td>
<td>23.3 2.4</td>
<td>22.0 2.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>31.1 8.6</td>
<td>27.7 7.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LBM (kg)</td>
<td>42.6 4.1</td>
<td>43.9 4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Work-load (Wattsubmax)</td>
<td>44 9</td>
<td>37 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Work-load (%Wmax)</td>
<td>68 6</td>
<td>67 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PT (times week⁻¹)</td>
<td>2.2 1.3</td>
<td>2.4 1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA (h week⁻¹)</td>
<td>4.9 2.1</td>
<td>5.4 2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bike, transport (% of PA)</td>
<td>61 30</td>
<td>58 23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patellar tendon, mean CSA (mm²)</td>
<td>75 7</td>
<td>82 10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means±SD. No significant differences between groups for any of the parameters were observed. Control: women in the follicular phase who had never used oral contraceptives; OC: high concentration of ethinyl-estradiol in oral contraceptive users. Body composition was measured by DEXA. LBM: Lean Body Mass; BMI: Body Mass Index; PT: Physical training; PA: Physical activity (Bike transportation and PT); CSA: cross-sectional area. The results for tendon CSA are reproduced with permission from Hansen et al (2008).
Table 2. Energy intake and macronutrient intake

<table>
<thead>
<tr>
<th>Habitual diet</th>
<th>Control</th>
<th>OC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy intake (kJ day(^{-1}))</td>
<td>9033 ± 2032</td>
<td>9221 ± 1983</td>
</tr>
<tr>
<td>Energy intake RMR(^{-1})</td>
<td>1.45 ± 0.31</td>
<td>1.49 ± 0.35</td>
</tr>
<tr>
<td>Protein (E%)</td>
<td>15 ± 4</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>Protein (g day(^{-1}))</td>
<td>82 ± 29</td>
<td>90 ± 17</td>
</tr>
<tr>
<td>Protein (g kg(^{-1}))</td>
<td>1.3 ± 0.4</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Protein (g kg(^{-1}) LBM)</td>
<td>2.0 ± 0.6</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>Fat (E%)</td>
<td>23 ± 7</td>
<td>24 ± 6</td>
</tr>
<tr>
<td>CHO (E%)</td>
<td>58 ± 6</td>
<td>57 ± 7</td>
</tr>
<tr>
<td>Alcohol (E%)</td>
<td>4 ± 6</td>
<td>2 ± 5</td>
</tr>
</tbody>
</table>

Values are means±SD. No significant differences between groups for any of the parameters were observed. Control: women in the follicular phase who had never used oral contraceptives; OC: high concentration of ethinyl-estradiol in oral contraceptive users. RMR: resting metabolic rate; LBM: lean body mass measured by Dual-energy X-ray absorptiometry. CHO: carbohydrates.
Table 3. GH, IGF-I and IGFBPs

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>OC</th>
<th>Diff. Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serum</strong></td>
<td>n=12</td>
<td>n=11</td>
<td></td>
</tr>
<tr>
<td>s-GH (µIU ml⁻¹)</td>
<td>11 ± 3</td>
<td>15 ± 2</td>
<td>( P = 0.36 )</td>
</tr>
<tr>
<td>s-IGF-I (µg L⁻¹)</td>
<td>315 ± 24</td>
<td>222 ± 10</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>s-IGFBP-1 (µg L⁻¹)</td>
<td>57 ± 7</td>
<td>121 ± 14</td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td>s-IGFBP-3 (µg L⁻¹)</td>
<td>5166 ± 373</td>
<td>5289 ± 180</td>
<td>( P = 0.78 )</td>
</tr>
<tr>
<td><strong>Tendon dialysate</strong></td>
<td>n=11</td>
<td>n=7</td>
<td></td>
</tr>
<tr>
<td>IGF-I (µg L⁻¹)</td>
<td>22 ± 4</td>
<td>9 ± 2</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td>IGF2BP-1 (AU mm⁻²)</td>
<td>63 ± 13</td>
<td>318 ± 60</td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td>IGFBP-2 (AU mm⁻²)</td>
<td>68 ± 15</td>
<td>54 ± 18</td>
<td>( P = 0.28 )</td>
</tr>
<tr>
<td>IGFBP-3 (AU mm⁻²)</td>
<td>1022 ± 390</td>
<td>2659 ± 399</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>IGFBP-4 (AU mm⁻²)</td>
<td>79 ± 28</td>
<td>161 ± 36</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td><strong>Muscle dialysate</strong></td>
<td>n=7</td>
<td>n=7</td>
<td></td>
</tr>
<tr>
<td>IGF-I (µg L⁻¹)</td>
<td>25 ± 5</td>
<td>16 ± 2</td>
<td>( P = 0.08 )</td>
</tr>
<tr>
<td>IGF2BP-1 (AU mm⁻²)</td>
<td>58 ± 16</td>
<td>477 ± 103</td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td>IGFBP-2 (AU mm⁻²)</td>
<td>53 ± 15</td>
<td>100 ± 21</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td>IGFBP-3 (AU mm⁻²)</td>
<td>2117 ± 1049</td>
<td>4605 ± 1049</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td>IGFBP-4 (AU mm⁻²)</td>
<td>66 ± 23</td>
<td>277 ± 65</td>
<td>( P &lt; 0.01 )</td>
</tr>
</tbody>
</table>

Values are means±SE. Control: women in the follicular phase who had never used oral contraceptives; OC: high concentration of ethinyl-estradiol in oral contraceptive users. AU: arbitrary units (pixel intensity). The results for tendon IGF-I and IGFBP 1-4 are reproduced with permission from Hansen *et al* (2008).
Muscle collagen synthesis (FSR)

FSR (% h⁻¹)

- Rest
- Exercise

Control
OC

*