Estrogen and progesterone exposure is reduced in response to energy deficiency in women aged 25–40 years

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BACKGROUND: Alterations in circulating steroids are believed to be important mediators of the impact that diet and exercise have on breast cancer risk and changes in bone density. This study aimed to test the hypothesis that moderate exercise training combined with caloric restriction would produce significant menstrual disturbances and alterations in ovarian steroids in premenopausal women.

METHODS: Sedentary premenopausal women (25–40 years; body mass index: $23.6 \pm 0.6 \text{ kg/m}^2$) assigned to either a light conditioning (LC, $n = 9$) or an exercise combined with caloric restriction group (EX + CR, $n = 24$) were studied for one screening, one baseline and four intervention periods equivalent to the length of subjects' menstrual cycles. Exercise consisted of supervised training sessions, i.e. two LC or four EX + CR times per week, 30–60 min at a moderate intensity. The EX + CR group was prescribed a diet representing a caloric restriction of 20–35% below baseline energy requirements, whereas the LC group remained eucaloric. Ovarian steroid exposure was determined with daily urinary estrone-1- and pregnanediol glucuronides (E1G and PdG, respectively) and mid-cycle urinary LH measures. Fitness, body composition, and serum sex hormone binding globulin (SHBG) and serum estradiol (E2) were assessed repeatedly.

RESULTS: The intervention produced significant increases in VO_2 max and decreases in both body weight ($-3.7 \pm 0.5 \text{ kg}$; ranged from -8.8 to $+1.8 \text{ kg}$) and percent body fat ($-4.5 \pm 0.7\%$; ranged from -12 to $+0.3\%$), which were attributable primarily to changes in the EX + CR subjects (time $\times$ group; $P < 0.05$). Serum E2 and urinary E1G and PdG concentrations declined significantly across the intervention period (time; $P < 0.05$), whereas SHBG increased transiently (time; $P < 0.05$) in the EX + CR subjects, with no significant changes observed in the LC group. The decrease in E1G area under the curve was significantly related to the daily energy deficit ($R = 0.61$; $P = 0.003$), not the amount of weight lost. There was no significant impact of the intervention on menstrual cyclicity or the incidence of menstrual disturbances in either group.

CONCLUSIONS: A moderate aerobic exercise training program combined with modest weight loss in accordance with recommended guidelines produces significant reductions in ovarian steroid exposure without disrupting menstrual cyclicity in premenopausal women aged 25–40 years. Exposure to a daily energy deficit is a stronger predictor of the decline in estrogen exposure than decreases in body weight.

Key words: Estrogen / progesterone / menstrual cycle / exercise / energy balance

Introduction

Ovarian steroids play important and diverse roles in physiological processes independent of their primary role in the reproductive function. For example, the importance of estradiol in the maintenance of optimal bone health (Balasch, 2003), and the promotion of breast cancer tumorigenesis (Miki *et al.*, 2009) has been established. Alterations in circulating steroids are believed to be important mediators of

the impact that diet and exercise have on breast cancer risk (Colditz *et al.*, 2003; Bernstein *et al.*, 2005) and bone density (De Souza *et al.*, 2008; Misra, 2008).

Menstrual disturbances and concomitant changes in circulating estradiol and progesterone occur with a greater frequency in exercising women than in non-exercising women (Otis *et al.*, 1997; De Souza *et al.*, 2010). However, the effects of physical stress of exercise *per se* are unlikely to explain these changes, as prospective studies that

employed exercise training in the absence of weight loss have produced only mild disruptions of menstrual cyclicity (Williams, 2003). In contrast, several laboratory-based studies which demonstrate that reductions in circulating ovarian steroids and gonadotrophins occur predominantly when exercise energy expenditure is higher than energy from food intake, illustrate the importance of negative energy balance in the suppression of reproductive hormones with exercise (Loucks *et al.*, 1998; Williams *et al.*, 2001; Loucks and Thuma, 2003; Williams, 2003). However, age-related changes have been suggested to alter the responsiveness of the reproductive axis to exercise perturbations (Rogol *et al.*, 1992; Loucks, 2006). To date, no prospective studies have been performed in premenopausal women aged 25–40 years to test the impact of exercise combined with caloric restriction on menstrual cyclicity. Prospective laboratory-based studies are needed to test whether, and to what degree, older (>25 years) premenopausal women who are also gynecologically older than subjects in previous studies, are susceptible to the impact of lifestyle interventions on menstrual cyclicity. The purpose of this study was to test the hypothesis that moderate exercise training combined with caloric restriction would produce significant changes in menstrual cyclicity and ovarian steroids in premenopausal women aged 25–40 years. A secondary goal was to describe the time course of changes in these factors and the magnitude of energy deficit associated with these changes.

Materials and Methods

Subjects

Inclusion criteria were: (1) age 25–40 years; (2) weight 50–90 kg; (3) body mass index (BMI) 18–35 kg/m²; (4) <1 h of self-reported exercise per week; (5) years since menarche (gynecological age) ≥10; and (6) eumenorrheic with ovulatory menstrual cycles. Exclusion criteria were: (1) history of or current serious medical conditions; (2) history of or current diagnosis of depression, disordered eating or other affective disorders; (3) smoking; (4) medication use that would alter metabolic or

reproductive hormone levels; (5) significant weight loss/gain (± 2.3 kg) in the past year; and (6) taking hormonal contraceptives within the last 6 months. Fliers, newspaper ads and electronic notices targeted the local community and the Pennsylvania State University campus. Informed consent was obtained from subjects as approved by the Penn State University Biomedical Institutional Review Board. Recruitment took place on a rolling basis throughout the year.

Experimental design overview

The study design is depicted in Fig. 1. This study was conducted at the Penn State University General Clinical Research Center (GCRC) over 3 years. Subjects were studied for a screening period consisting of one menstrual cycle (screening cycle), followed by a baseline period equivalent to one menstrual cycle (baseline cycle) and then an intervention period equivalent to four menstrual cycles (interventions 1–4) that consisted of supervised exercise combined with caloric restriction. Repeated assessments of menstrual status, urinary measures of reproductive hormones, aerobic capacity and body composition were obtained across the intervention and then during a post-study (post-study) period of up to 1 week following the end of intervention 4.

Treatment

The intervention consisted of a combination of caloric restriction and moderate aerobic exercise; dietary intake was prescribed by GCRC dietitians and closely monitored, and all exercise sessions took place in the laboratory and were supervised. To achieve comparable levels of energy deficiency among subjects, prescriptions for reductions in caloric intake and calories expended during workouts were individualized based on subjects' baseline energy needs. Results for subjects who participated in the intervention (EX + CR) were compared with a smaller reference group of subjects who performed light conditioning (LC). Both groups of subjects participated in all study procedures with the main difference being that the LC subjects' diet prescription remained eucaloric and their exercise duration and workouts per week were significantly lower than those of EX + CR subjects.

Screening	Baseline	Intervention 1	Intervention 2	Intervention 3	Intervention 4	Post-Study (Cycle Days 1-7)
Medical History Menstrual History Physical Activity Eating Attitudes CBC/Chemistry Panel Endocrine Screen Physical Exam Body Weight Psychological Interview 3 Day Diet Log Daily Urine Collection Menstrual Calendar Ovulation Detection Kit Mid-luteal Progesterone	3-Day Diet Record Body Weight Body Composition Menstrual Calendar Ovulation Detection Kit Mid-luteal Progesterone Daily Urine Collection Dietary Counseling VO2max RMR RT3 Serum Estradiol SHBG	3-Day Diet Record Body Weight Body Composition Menstrual Calendar Daily Urine Collection Supervised Exercise Dietary Counseling RT3 Exercise Kcals SHBG	3-Day Diet Record Body Weight Body Composition Menstrual Calendar Daily Urine Collection Supervised Exercise Dietary Counseling RMR RT3 Exercise Kcals SHBG	3-Day Diet Record Body Weight Body Composition Menstrual Calendar Daily Urine Collection Supervised Exercise Dietary Counseling VO2max RMR RT3 Exercise Kcals SHBG	3-Day Diet Record Body Weight Body Composition Menstrual Calendar Daily Urine Collection Supervised Exercise Dietary Counseling RT3 Exercise Kcals Serum Estradiol SHBG	3-Day Diet Record Body Weight Body Composition Menstrual Calendar Daily Urine Collection Supervised Exercise Dietary Counseling VO2max RMR RT3 Exercise Kcals SHBG
Menses	Menses	Menses	Menses	Menses	Menses	Menses
Menstrual Cycle 1	Menstrual Cycle 2	Menstrual Cycle 3	Menstrual Cycle 4	Menstrual Cycle 5	Menstrual Cycle 6	Menstrual Cycle 7

Figure 1 Study phases showing Screening through Post-Study each represent a subject's menstrual cycle marked by the onset of menses, such that all subjects were studied for 6+ cycles. Post-study period represents approximately one week. CBC, Complete Blood Count; SHBG, sex hormone binding globulin; RMR, Resting Metabolic Rate; RT3, research accelerometer for non-purposeful exercise kcals.

Screening

Screening procedures assessed body weight, medical history, menstrual history, current and past physical activity, and eating attitudes and behaviors using previously published methods (Westerlind and Williams, 2007). The screening and baseline periods were initiated by the onset of menses. Blood samples during screening via venipuncture were obtained once during menstrual cycle Days 1–7 after an overnight fast to be analyzed for a complete blood count (CBC), basic chemistry panel and endocrine screen. Each subject had a physical examination by a GCRC clinician, underwent a clinical interview under the supervision of a clinical psychologist to determine mental health status and was instructed how to complete a 3-day diet log by a GCRC dietician. Subjects were weighed at each visit. Daily urine collections to assess menstrual status were begun on Day 1 of the menstrual cycle, and continued for the rest of the study. Subjects also tracked menstrual symptoms using a menstrual calendar; this continued for the rest of the study.

Baseline

During baseline subjects completed a 3-day diet log during Days 1–7 and underwent testing to determine body weight, body composition, baseline energy needs (see Assessment of baseline energy needs and energy balance during the intervention) and aerobic capacity (see Assessment of baseline energy needs and energy balance during the intervention). Daily urine collections and menstrual calendars were collected, and subjects underwent serial blood sampling for the determination of serum estradiol (see below), and testing for sex hormone binding globulin (SHBG).

Menstrual status and ovarian steroids

Menstrual status was determined by self-report for the prior 6 months and prospectively with menstrual calendars documenting menstrual characteristics throughout the study. Daily urine samples were collected during the screening, baseline and intervention cycles according to previously published methods (Westerlind and Williams, 2007). Each day subjects collected an aliquot of their first morning void of urine using a sponge-like collection device. These samples were then stored in their freezer and transported to the lab approximately every 2 weeks. Estrogen and progesterone exposure, confirmation of ovulatory status, the presence or absence of luteal phase defects, and the lengths of the follicular and luteal phases were determined by analysis of daily urinary metabolites of estrone-1-glucuronide (E1G), pregnanediol glucuronide (PdG) and mid-cycle luteinizing hormone (LH) after completion of the study using previously published methods (De Souza et al., 1998). Subjects who did not ovulate during screening and baseline were excluded. Ovulation during screening and baseline was determined using ovulation detection kits (First Response, Church and Dwight, 2004) and confirmation of a luteal phase rise in serum progesterone. Estrogen exposure was also determined using measures of serum 17- β estradiol which were collected on 10 different days across each subjects' menstrual cycles during the baseline and intervention 4 cycles.

Assessment of baseline energy needs and energy balance during the intervention

Twenty-four hour energy expenditure was determined and assumed to represent baseline energy needs, as all subjects were deemed to be weight stable during screening. To determine 24 h energy expenditure, the resting metabolic rate (RMR) (kcal/24 h) and calories attributable to physical activity throughout the day were utilized. RMR was measured on one day during Days 1–7 of the baseline cycle using indirect calorimetry with previously reported methods (Leidy et al., 2004). To determine

physical activity calories, subjects wore a research accelerometer (RT3, Stayhealthy, Monrovia, CA) for 24 h per day, 7 days per week during Days 1–7 of the baseline cycle. The calories from the RMR and the calories representing the average of 7 days of physical activity calories recorded from the RT3 accelerometer were added together; this sum was operationally defined as the baseline energy needs calorie level (Leidy et al., 2004). The target dietary intake for the intervention was calculated as a percentage reduction from the estimate of baseline energy needs (see below). To estimate the energy balance during the intervention, measures of energy intake (3-day diet logs) and energy expenditure were repeated during the study and these data were used to calculate a daily value for energy balance. The calories from the RMR and the RT3, and the calories expended during the training sessions were summed to represent the 24 h energy expenditure. RMR was assessed on one day during the follicular phase of baseline, intervention 2, intervention 3 and the post-study period. Subjects completed 3-day diet logs and simultaneously wore the RT3 during the early follicular phase of each intervention cycle. Calculations for the parameters of energy balance were updated continually throughout the intervention as new measurements were obtained.

To calculate an estimate of the actual daily energy deficit across the intervention for each subject, it must be taken into account that energy balance measures are obtained throughout the intervention in a non-energy balance state. Thus, since energy is neither created nor destroyed, measures of energy expenditure obtained during a non-energy balance state must take into account the loss/gain of body energy stores (de Jonge et al., 2007). Therefore, the caloric equivalent of the changes in fat mass and fat-free mass from baseline to post-study in each subject was calculated using appropriate energy coefficients for fat mass lost/gained (9.3 kcal/day) and fat-free mass lost (1.1 kcal/g) or gained (1.8 kcal/g) (Del Corral et al., 2009). This number of calories was then divided by the number of days each subject was in the intervention to estimate the kcal/day lost/gained with changes in fat mass and or fat-free mass. Each subject's average total 24 h energy expenditure (kcal/day) for the intervention months 1–4 was calculated i.e. overall 24 h expenditure. This value was then adjusted for the kcal/day lost/gained, as described in (Del Corral et al., 2009) i.e. adjusted overall 24 h expenditure = overall 24 h expenditure + kcal/day (lost/gained). Each subject's average total 24 h dietary intake (kcal/day) for the intervention months 1–4 was calculated i.e. overall 24 h dietary intake. Overall daily energy deficits (kcal/day) were then calculated as: overall daily energy deficit (kcal/day) = overall 24 h dietary intake – adjusted overall 24 h energy expenditure.

Target dietary intake for intervention

Dietary intake was prescribed and closely monitored by GCRC dietitians. To promote weight loss, a reduction in dietary intake equal to 20% for the first 1–2 weeks of intervention 1 and progressing to 35% by the last 2 weeks of intervention 1 of the estimated baseline energy needs was prescribed as the target dietary intake during the diet intervention for the subjects. The macronutrient composition of the target dietary intake during the intervention prescription was 55% carbohydrate, 30% fat and 15% protein. During the baseline cycle the subjects were taught by a GCRC dietician how to use the food exchange system (American Diabetes Association, 2003 Edition, Chicago, IL) to achieve their target caloric intake and prescribed macronutrient composition. Subjects monitored their daily food exchanges for 7 days at a time, every other week and met with the GCRC dietitian every other week throughout the intervention to review these records and discuss their progress. They also completed a 3-day diet log (two week days and one weekend day) during

the first week of each menstrual cycle they were in the study. Total calories and macronutrient content were determined using Nutritionist Pro (First Data Bank, Indianapolis, IN). Dietary counseling sessions also included discussion of food education modules, including shopping tips, low-fat/low-calorie food, food preparation, dining out, iron, calcium, fiber and vitamins in food.

Exercise training

All exercise training took place in the laboratory and was supervised by trainers who had experience in fitness assessment and personal training. Workouts were performed four times per week and consisted of a 5-min warm-up, 40–90 min of aerobic activity at $79 \pm 0.7\%$ of the maximal heart rate and then a 10-min cool-down. The duration of exercise (min) was equal to that required to expend a prescribed number of calories, determined to be $\sim 20\%$ of the subjects' baseline energy needs. For example, if the baseline energy needs represented 2000 calories, the subject's prescribed workout calorie target would be 400 calories. Subjects increased their exercise minutes gradually over the first two weeks with the goal of attaining their target prescription by the third or fourth week of intervention 1. Exercise was not prescribed on 'off' days, and subjects were asked to maintain their non-purposeful physical activity at levels equivalent to baseline throughout the study. The total amount of calories expended during each exercise session was measured using the OwnCal feature on the Polar S610 heart rate monitor (Polar Electro Oy, Kempele, Finland). Modes of aerobic activity for both groups included treadmill walking and running, stationary cycling, stair stepping and the use of an elliptical ergometer. The duration of exercise was progressively increased throughout the intervention. Average exercise duration was 56 ± 2 min. The EX + CR subjects expended 13%, 19%, 25% and 28% of their baseline energy needs expressed in calories during intervention 1–4, respectively.

Body composition and body weight measurements

Body weight and body composition were determined in the follicular phase (Days 1–7) of the baseline cycle, each intervention cycle and during the post-study period. Hydrostatic weighing was used to measure percent body fat, fat mass, and fat-free mass using previously described methods (Leidy *et al.*, 2004). Body weight was measured using a digital scale to the nearest 0.01 kg (Seca scale, Hamburg, Germany) on the same day that body composition was determined and twice per week throughout the intervention.

Aerobic capacity

Measurement of the maximal aerobic capacity (VO_2 max) was performed on the treadmill during the follicular phase of the baseline cycle, intervention cycle 3 and during the post-study period using indirect calorimetry and the modified Astrnd protocol (Westerlind and Williams, 2007).

Blood sampling

Blood samples for serum estradiol were collected every 2–3 days across the entire baseline and intervention 4 cycles only so that each subject had ~ 10 samples equally spaced across each of these two cycles. Blood samples for SHBG were obtained (follicular phase Days 1–7) during the baseline cycle, each intervention cycle and during the post-study period. Subjects were fasted overnight and did not exercise in the morning prior to blood collection, which occurred between 0700 and 1000 h at the GCRC after lying supine for at least 15 min. Blood samples were processed as previously described (Westerlind and Williams, 2007).

Light conditioning subjects

To serve as a reference group, nine subjects performed exercise training under the same conditions as the EX + CR subjects, although only 1–2 times per week. Sessions were comprised of 36 ± 2.9 min of aerobic activity at $77 \pm 0.8\%$ of maximum heart rate, and 10 min of light stretching and calisthenics. These sessions were designed to provide only a modest training stimulus and to expend a low percentage of baseline energy needs, i.e. average of $10 \pm 0.75\%$ of baseline energy needs expressed as kcals per session. The dietary prescription for these subjects was similar in macronutrient content but caloric intake was unrestricted with the goal that body weight would be maintained. All study measures undertaken by LC subjects were identical to the procedures for the EX + CR subjects.

Biochemical analyses

CBC/Chem 24 and the endocrine screen determinations were completed by Quest Diagnostics (Lyndhurst, NJ). The endocrine screen included LH, follicle-stimulating hormone, prolactin, progesterone, estradiol, thyroxine and thyroid-stimulating hormone. Serum 17- β estradiol was measured using a radioimmunoassay (Coat-a-Count TKE21) (DPC, Los Angeles, CA). Assay sensitivity was 8 pg/ml. The intra-assay and inter-assay coefficients of variation were 4.3% and 6.8%, respectively. SHBG was measured using an immunoradiometric (DSL-7400) assay from Diagnostic Systems Laboratories (DSL, Webster, TX). The intra- and inter-assay coefficients of variation were 3.4% and 10.3%, respectively. Assay sensitivity was 3 nmol/L. Competitive enzyme immunoassays were used to measure the major urinary estrogen metabolite estrone-1-glucuronide (E1G) according to previously published methods (McConnell *et al.*, 2002). All biochemical determinations were assayed in duplicate; all samples from a given subject were tested in the same assay.

Strategies to maintain compliance

Study staff maintained contact with subjects several times a week, especially during exercise sessions. This provided opportunities to query subjects for questions they may have, and provide reminders. E-mail reminders were sent prior to all visits with detailed instructions regarding the nature of the appointment, and special instructions, i.e. fast overnight and do not exercise prior to blood draws. Attention was paid to maintaining a positive attitude in the training room, in the GCRC, in the lab and by the nutritionists who counseled the subjects. Compliance to urine collection was achieved by checking urine samples and records for collection, including menstrual symptom calendars each time the subjects dropped off samples (approximately once every 2 weeks).

Statistics and data analysis

We calculated sample size based on expected differences and standard deviations from other published studies examining ovarian hormonal changes in response to diet and exercise. A power coefficient of 0.80 was expected with an initial sample size of 15 subjects in each of two groups ($\alpha = 0.05$, $\Delta/\sigma = 1.11$ –2.22) with $P < 0.05$ considered to be significant. All data sets were tested for non-normality and outliers before statistical hypothesis tests were performed. Outliers detected were rejected. To describe estrogen and progesterone exposure, area under the curve (AUC) was calculated for each menstrual cycle using daily urinary measures of E1G and PdG using the trapezoidal rule (Whittaker, 1967). AUC was also calculated for each subject from serum concentrations of 17- β estradiol obtained during the 10 blood samples spread across the baseline and intervention 4 cycles. Analyses were conducted using general linear model procedures for repeated measures on each or both groups while controlling for baseline variables as necessary. Post

hoc testing to reveal when significant time effects occurred was performed using *t*-tests with a Bonferroni correction, comparing the baseline cycle to each intervention cycle for a total of four tests; thus $P < 0.0125$ was considered significant. In the case of post hoc comparisons being made that included the post-study time point, $P < 0.010$ was considered significant. Pearson bivariate correlational analyses were used to determine associations between variables of interest. To determine differences between groups in the incidence of menstrual disturbances, χ^2 analyses were conducted between LC and EX + CR groups for baseline and all intervention cycles. To determine the impact of the intervention on incidence of menstrual disturbances in each group, Friedman's test was performed comparing baseline to all intervention cycles in LC and EX + CR groups separately. Data were analyzed using SPSS for Windows (version 15.0; Chicago, IL). All data are expressed as mean $\pm$ SEM.

Results

Dropouts

Fig. 2 depicts the flow of subjects through the study. Eighty-five women were assessed for eligibility. Thirty-eight women were eliminated during the screening cycle or baseline cycle, i.e. 12 for menstrual irregularities, 8 for medical reasons, 5 for BMI outside inclusion criteria and 13 for other reasons. Thirty-six women were allocated to the EX + CR group, and 11 were allocated to the LC group. All subjects

received the intervention and none were lost to follow-up. Fourteen subjects dropped out during the intervention (30%), 12 from EX + CR and 2 from LC. Reasons for dropout included pregnancy ($n = 2$), injuries ($n = 2$), non-compliance ($n = 1$), moving ($n = 1$), medical reasons ($n = 1$) and time constraints ($n = 7$). Thirty-three women completed the study, i.e. 24 in the EX + CR group, and 9 in the LC group. Of the 33 women that completed the study, 27 were Caucasian (79.4%), 3 were Asian (8.8%), 1 was Hispanic (2.9%) and 2 were 'other' (5.8%). Finishers and dropouts did not differ in initial body weight, age, height, VO_2 max or ethnicity. Dropouts differed significantly from finishers in percent body fat (dropouts = $35.7 \pm 1.5\%$ versus finishers = $32.3 \pm 5.0\%$; $t = 2.082$; $P = 0.043$) and in fat mass (dropouts = 24.5 ± 1.8 kg versus finishers = 20.7 ± 0.9 kg; $t = 2.056$; $P = 0.046$).

Compliance to diet and exercise prescriptions

The EX + CR group attended $99 \pm 1\%$ of the nutrition counseling sessions and $96 \pm 1\%$ of the exercise sessions. They achieved their target exercise energy expenditure during $95 \pm 2\%$ of their workouts. The LC groups attended $95 \pm 3\%$ of the nutrition counseling sessions and $92 \pm 2\%$ of the exercise sessions. They achieved their target exercise energy expenditure during $95 \pm 2\%$ of their workouts.

Figure 2 CONSORT diagram showing flow of participants through the study.

Table 1 Subject characteristics at baseline.

Variable	EX + CR <i>n</i> = 24			LC <i>n</i> = 9			P-value
	Mean ± SEM	Median	Range	Mean ± SEM	Median	Range	
Age (years)	31.5 ± 0.9	31.0	(25–40)	35.3 ± 1.0	36.0	(29–39)	0.024
Weight (kg)	63.3 ± 1.8	61.4	(53–84)	65.2 ± 1.9	65.4	(58–73)	0.556
Height (cm)	164 ± 1.3	164	(152–175)	165 ± 2.2	166	(154–177)	0.598
BMI (kg/m ²)	23.6 ± 0.6	23.2	(19–29)	24.1 ± 1.1	23.9	(20–30)	0.682
Body fat (%)	31.6 ± 1.0	32.0	(22–43)	34.0 ± 1.6	33.6	(27–41)	0.217
Fat mass (kg)	20.2 ± 1.1	19.5	(12–35)	22.2 ± 1.8	21.1	(16–30)	0.360
Fat-free mass (kg)	43.1 ± 1.0	42.2	(35–56)	42.1 ± 0.7	42.1	(39–45)	0.619
Age of menarche (years)	12.8 ± 0.3	13.0	(9–15)	12.9 ± 0.5	13.0	(10–15)	0.928
Gynecological age (years)	18.7 ± 1.0	23.0	(11–29)	22.4 ± 1.1	23.0	(11–17)	0.047
VO ₂ max (ml/kg/min)	32.7 ± 1.2	33.8	(21–41)	29.9 ± 0.5	29.8	(27–31)	0.037
Average dietary intake (kcal/day)	1930 ± 61	1882	(1323–2493)	1855 ± 133	1759	(1287–2448)	0.566

BMI, body mass index.

Subject characteristics

Baseline subject characteristics for those who completed the study are shown in Table 1. The LC subjects were somewhat older than the EX + CR subjects ($P = 0.024$). The groups did not differ in anthropometric measures, aerobic fitness or reproductive maturity.

Effects of the intervention on body weight, body composition and aerobic fitness

Fig. 3 depicts data for body weight and body composition. Body weight declined significantly (time effect $F = 19.5$; $P = 0 < 0.0001$) attributable primarily to changes in the EX + CR subjects (time $\times$ group $F = 7.9$; $P < 0.0001$). Fat loss accounted for most of all of the change in weight (time effect $F = 31.5$; $P < 0.0001$) observed in the EX + CR group (time $\times$ group $F = 8.9$; $P < 0.0001$); since no significant changes in fat-free mass were observed. Subjects' weight changes in the EX + CR group ranged from -8.8 to $+1.8$ kg, with an average weight loss of -3.7 ± 0.5 kg. In the LC group subjects' weight changes ranged from -2.4 to $+1.1$ kg; the average change was -0.6 ± 0.4 kg. Percent fat declined significantly (time effect $F = 28.8$; $P < 0.001$), mostly attributable to EX + CR subjects (time $\times$ group $F = 7.8$; $P < 0.001$). Percent fat losses in the EX + CR group ranged from -12.3 to $+0.3\%$, with an average loss of -4.5% . In the EX + CR subjects' average fat mass declined by -3.8 ± 0.5 kg, whereas in the LC subjects it declined by -0.9 ± 0.4 kg. The time course of changes in body weight and body fat was rapid as significant reductions were noted in the first and second intervention cycles, respectively ($P < 0.0125$). Training resulted in a significant increase in maximal aerobic capacity (time $F = 20.9$; $P < 0.0001$). VO₂ max increased from baseline to post-study in the EX + CR group from 33.2 ± 1.2 to 41.9 ± 2.0 ml/kg/min ($P < 0.001$) and in the LC group from 30.0 ± 0.4 to 37.1 ± 1.3 ml/kg/min. Significant improvements in VO₂ max were noted by the mid-point of the study, i.e. the follicular phase of intervention cycle 3 but only in EX + CR subjects (data not shown).

Energy balance parameters during the intervention

Energy balance data are presented in Table 2. The EX + CR group consumed $\sim 30\%$ fewer calories when compared with baseline kcals as determined with 3-day diet logs (time $\times$ group effect $F = 6.8$; $P < 0.0001$). Overall they consumed significantly fewer calories than the LC group (group effect $F = 8.5$; $P = 0.007$). RMR did not change significantly in either group. The calories expended during non-purposeful activity as measured using the RT3 did not change in either group. Exercise caloric expenditure is expressed as the average kcal/day on a weekly basis, and as the kcals/workout session for workout days. EX + CR subjects expended significantly more calories than LC during the week and during workouts (time $\times$ group effect $F = 123.9$; $P < 0.0001$). Total energy expenditure did not change in either group. The average daily deficit expressed as kcals (intake – expenditure) differed significantly between EX + CR and LC (time $\times$ group effect $F = 6.2$, $P < 0.0001$). The actual kcals lost/gained based on changes in fat mass and fat-free mass was -310 ± 24 kcal/day for EX + CR subjects, and -70 ± 26 kcal/day for LC subjects. The overall average daily energy deficit across the intervention, when calculated and expressed as a percentage of baseline energy needs, was $-38 \pm 2\%$ for EX + CR and $-22 \pm 6\%$ for LC subjects ($t = -266$; $P = 0.003$). In all subjects the daily energy deficit correlated with the change in fat mass ($R = 0.41$; $P = 0.025$) but not with the change in body weight.

Effects of the intervention on menstrual cyclicity

The effects of the intervention on menstrual cyclicity are presented in Table 3. No significant changes in the average menstrual cycle length, follicular phase length or luteal phase length were observed in either the EX + CR or the LC groups. There was also no significant impact of the intervention in either group upon the incidence of menstrual disturbances of any type. The most frequent menstrual disturbance was the occurrence of a short (< 10 days) luteal phase. There

Figure 3 Effects of exercise training and caloric restriction (EX + CR: open circle) and light conditioning (LC: filled circle) on (A) fat-free mass, (B) fat mass, (C) body fat and (D) body weight in all subjects over time. Data are expressed as the mean $\pm$ SEM. Asterisk, $P < 0.010$ versus baseline, post hoc t -test using Bonferroni correction; B, baseline; Int, intervention; and Post, post-study.

were two subjects during the baseline cycle that had short (< 10 days) luteal phases (8%), and short luteal phases were observed in four subjects during intervention 1 (17%), two subjects during intervention 2 (8%), four subjects during intervention 3 (17%) and two subjects during intervention 4 (8%) in the EX + CR group. Only one subject out of 24 in the EX + CR group experienced an anovulatory cycle in intervention 4 (4%) which was preceded by an inadequate luteal phase in intervention 3 (4%). No other anovulatory cycles were observed and no other inadequate luteal phases were observed in the EX + CR group. Oligomenorrheic cycles, defined as longer than 36 days, occurred very infrequently, with two subjects in the EX + CR group experiencing this disturbance in the intervention 4 cycle (8%).

In LC subjects, the most commonly observed menstrual disturbance was, again, the presence of short luteal phases. Short luteal phases were observed in one LC subject during baseline (11%), four subjects during intervention 1 (44%), three during intervention 2 (33%), three during intervention 3 (33%) and two during intervention 4 (22%). Inadequate luteal phases were observed in one LC subject during baseline (11%), one during intervention 1 (11%) and one subject during intervention 3 (11%). There were no anovulatory cycles observed in the LC group (0%) although there were three oligomenorrheic cycles, i.e., one during Intervention 1 (11%), one during Intervention 2 (11%), and one during Intervention 4 (11%).

Overall, there are no differences in the incidence of any type of menstrual disturbance during any intervention cycle when the EX + CR and LC groups are compared with each other ($P > 0.05$ χ^2 -test). In addition, there was no significant incidence of any type of menstrual disturbance across the intervention cycles when EX + CR and LC subjects were compared with the baseline ($P > 0.05$ Friedman's test).

Effects of the intervention and ovarian steroid exposure

In EX + CR subjects, urinary EIG AUC exhibited a progressive decline (time effect $F = 4.110$; $P = 0.004$) with a significant decline noted by intervention cycle 4 (Fig. 4). Fig. 5 illustrates a composite representation of EIG data for all EX + CR subjects during the baseline cycle and intervention cycles. An additional analysis of the change from baseline in EIG AUC during the luteal phase (defined as the day after the day of ovulation until the day before the next menses) revealed that the AUC during the luteal phase was largely responsible for the decline observed in the total cycle AUC (time effect $F = 3.445$; $P = 0.012$). A similar result was observed for estrogen AUC as calculated using serum 17- β estradiol. The total AUC (representing the entire menstrual cycle) calculated using the 10 serum samples for serum estradiol obtained during the baseline cycle and then during the intervention 4 cycle declined significantly from the baseline cycle to intervention 4 cycle (from 2596 ± 144 to 2060 ± 163 pg/ml $\times$ day) ($t = 3.194$; $P = 0.004$) in the EX + CR group. In the EX + CR group, the change in urinary EIG AUC was significantly correlated with the overall study energy deficit ($R = 0.61$; $P = 0.003$) (Fig. 6), but not correlated with the change in body weight. The latter correlation was also significant ($R = 0.39$; $P = 0.037$) when the LC group is

Table II Energy balance data.

	Baseline			Intervention 1			Intervention 2			Intervention 3			Intervention 4			Post-study			Intervention mean		
	LC	EX+CR	LC	EX+CR	LC	EX+CR	LC	EX+CR	LC	EX+CR	LC	EX+CR	LC	EX+CR	LC	EX+CR	LC	EX+CR	LC	EX+CR	LC
Self-reported energy intake (3-day logs) ^{a,b,c} (kcal/day)	1818 ± 118	1907 ± 59	1553 ± 108	1334 ± 57	1698 ± 108	1319 ± 54	1754 ± 136	1309 ± 51	1711 ± 143	1308 ± 50	1648 ± 151	1338 ± 53	1676 ± 112	1319 ± 48							
RMR (kcal/day)	1303 ± 30	1314 ± 28	1303 ± 30	1314 ± 28	1305 ± 31	1296 ± 28	1306 ± 34	1287 ± 30	1306 ± 34	1287 ± 30	1352 ± 39	1278 ± 37	1303 ± 29	1295 ± 28							
Physical activity (RT3) (kcal/day)	913 ± 114	689 ± 63	965 ± 172	669 ± 53	710 ± 107	654 ± 48	726 ± 79	658 ± 60	843 ± 151	627 ± 64	836 ± 145	612 ± 64	807 ± 79	648 ± 47							
Exercise ^{a,b,c} (kcal/day; workout days)	0	0	228 ± 20	250 ± 7	230 ± 17	375 ± 10	239 ± 17	503 ± 16	245 ± 17	503 ± 16	-	-	235 ± 17	419 ± 13							
Exercise ^{a,b,c} (kcal/day; weekly average)	0	0	62 ± 5	131 ± 4	64 ± 5	210 ± 6	61 ± 5	270 ± 12	61 ± 6	264 ± 19	-	-	62 ± 4	217 ± 8							
Total energy expenditure (kcal/day)	2215 ± 101	2003 ± 68	2308 ± 153	2087 ± 65	2059 ± 107	2127 ± 61	2074 ± 70	2181 ± 72	2188 ± 123	2147 ± 72	2135 ± 110	2106 ± 71	2149 ± 63	2132 ± 60							

RMR, resting metabolic rate. Mean ± SEM.
^a*p* < 0.05 ANOVA Main effect of time.
^b*p* < 0.05 ANOVA time × group interaction.
^c*p* < 0.05 ANOVA main effect of group.

included. Urinary PdG AUC across the menstrual cycle declined significantly over time in the EX + CR group (time effect $F = 3.46$; $P = 0.011$) from 160 ± 15 ng/ml × day during the baseline cycle to 139 ± 10 ng/ml × day during intervention cycle 4 (Fig. 4C). In LC subjects no changes were observed in urinary E1G, serum 17-β estradiol or urinary PdG over time (Fig. 4).

At baseline, BMI was negatively correlated with SHBG ($R = -0.42$; $P = 0.020$) when all the subjects were considered. Similarly, percent body fat was inversely correlated with SHBG ($R = -0.40$; $P = 0.03$). The intervention produced a biphasic response in SHBG such that concentrations in the EX + CR group significantly increased by intervention 2 and intervention 3 and then decreased somewhat by intervention 4 (time effect $F = 3.921$; $P = 0.003$). Fig. 4 shows the changes in SHBG in comparison with those in E1G and PdG AUC. In the EX + CR group, a significant increase in SHBG during intervention 2 preceded the significant decline in E1G observed at intervention 4. No changes in SHBG were noted in the LC group.

Discussion

We conducted a controlled exercise and diet intervention to assess the magnitude and time course of changes in menstrual cyclicity and ovarian steroids in premenopausal women aged 25–40 years. Our results, the first in this particular age group of premenopausal women, show that a moderate aerobic exercise program combined with caloric restriction can produce significant reductions in estrogen and progesterone exposure and transient increase in SHBG. We observed a 15% and 20% reduction in both serum estradiol and urinary E1G, respectively, which occurred progressively across the four intervention months. Urinary PdG was also significantly reduced. Although body weight, fat mass and percent body fat also progressively declined, the change in estrogen exposure was not correlated with the loss of weight or fat mass. Rather, it was correlated with the magnitude of the average calculated daily energy deficit throughout the intervention, such that the greater the daily energy deficit, the greater was the decline in estrogen exposure from the baseline period to the last intervention cycle. This suggests that the signals conveying nutritional status to areas involved with the central modulation of the reproductive axis (and estrogen exposure) may be more sensitive to the day-to-day fluctuations in energy balance than to more slowly changing measures of body weight or body fat. This is a novel finding for a study examining chronic changes in reproductive hormone outcomes over a long duration but one that is supported by similar observations aimed at detecting changes in LH pulsatility in response to short-term (days) declines in energy availability (Loucks and Thuma, 2003). Our methods of measuring energy intake and energy expenditure have limitations in terms of accuracy. It is likely that our calculations reflect overestimations of negative energy balance due to under-reporting that occurs with 3-day diet logs (Del Corral et al., 2009), and the fact that our post-study measures were not obtained during a period of weight maintenance (de Jonge et al., 2007; Del Corral et al., 2009).

Whether or not a 20% reduction in estrogen exposure in response to a lifestyle intervention consisting of diet and exercise changes to induce weight loss would represent a chronic and sustained adaptation or be associated with significant physiological outcomes such as effects on bone or breast cancer risk is unknown.

Table III Effects of the intervention on menstrual cycle length.

	Baseline		Intervention 1		Intervention 2		Intervention 3		Intervention 4		
LC											
Cycle length (days)	27.7 ± 0.3	(25–31)	28.2 ± 0.9	(21–37)	27.8 ± 1.4	(21–45)	28.7 ± 0.8	(23–35)	28.5 ± 1.0	(24–39)	
Follicular length (days)	16.0 ± 0.9	(13–21)	17.0 ± 1.4	(12–24)	17.8 ± 2.1	(12–33)	18.2 ± 1.7	(12–25)	17.2 ± 1.8	(11–29)	
Luteal phase (days)	11.4 ± 0.5	(9–14)	11.2 ± 1.0	(6–16)	11.1 ± 0.8	(8–15)	10.5 ± 0.8	(7–15)	12.3 ± 1.3	(7–21)	
EX+CR											
Cycle length (days)	29.7 ± 0.5	(24–36)	28.6 ± 0.5	(23–33)	28.6 ± 0.70	(25–36)	28.7 ± 0.7	(23–38)	28.8 ± 1.0	(23–42)	
Follicular phase (days)	12.5 ± 0.4	(11–21)	16.2 ± 0.6	(11–22)	16.2 ± 0.6	(12–25)	17.3 ± 0.8	(11–28)	17.0 ± 1.0	(10–28)	
Luteal phase (days)	12.6 ± 0.3	(8–15)	12.4 ± 0.4	(8–15)	11.5 ± 0.3	(8–14)	11.4 ± 0.4	(6–14)	11.8 ± 0.3	(9–15)	

Values are the mean ± SEM (range).

Although more work is required to directly assess the physiological and clinical significance of these exercise-related changes in estrogen and progesterone, it is important to note that the dose of exercise employed in our intervention was well within recommended guidelines for physical activity (Haskell et al., 2007). In addition, the weight loss achieved by our subjects over 4 months was well within recommended guidelines for dieting (US Department of Agriculture; Haskell et al., 2007). Moreover, our subjects exhibited high compliance and few injuries.

The results of our study challenge the notion that reductions in ovarian steroid exposure in response to an exercise and diet intervention occur in association with significant disruptions in menstrual cyclicity (Bullen et al., 1985). Our results suggest that modest disruptions in ovarian synthesis and/or secretion of estradiol can occur in the absence of significant disruptions in ovulation, significant suppression of folliculogenesis such that follicular length is increased, and without a significant impact on corpus luteum function as is usually indicated by a shortening of luteal phase length or inadequate luteal phases (Beitins et al., 1991; De Souza et al., 1998; Williams et al., 1999). The reductions in estrogen and progesterone exposure occurred in the absence of significant alterations in menstrual cycle, follicular or luteal phase length, and without severe disturbances in the menstrual cycle such as anovulation or oligomenorrhea. A significant decline in estradiol has been noted previously in the absence of changes in menstrual cyclicity in response to a year of marathon training, but this study did not impose weight loss (Boyden et al., 1984). The absence of a significant incidence of menstrual disturbances observed in our study stands in contrast to other prospective studies in younger women, where menstrual cyclicity has been shown to be disrupted by exercise combined with weight loss, or by diet-induced weight loss (Bullen et al., 1985; Kurzer and Calloway, 1986; Schweiger et al., 1987). In particular, our results differ from those of Bullen et al. (1985) who observed that up to 65% of subjects experienced luteal phase disturbances and up to 75% experienced a loss of the LH surge, despite similar weight loss (average 4.0 kg versus average 3.7 kg in the current study). Although gynecological maturity has been cited (Rogol et al., 1992) and demonstrated (Loucks, 2006) to be a factor associated with both reduced responsiveness of the hypothalamic–pituitary–gonadal axis to exercise and with exercise combined with caloric restriction interventions, other examples of a suppressive effect of caloric restriction upon LH pulsatility and on

follicular development in subjects in the age range of those in this study exist (Kurzer and Calloway, 1986; Schweiger et al., 1987; Alvero et al., 1998). Whether increased age, a higher initial body weight (one-third of them were overweight) (Alvero et al., 1998) or other unknown factors played a role in the low susceptibility to menstrual disturbances induced by energy deficiency in our subjects is unclear. It is possible that individuals in the same age range, who exhibit prior menstrual irregularity and in whom more weight is lost, could experience a suppression of menstrual cyclicity. To examine whether age impacted the results within our group of subjects, we performed a sub-analysis comparing those under and over 35 years found no differences in effects on ovarian steroids. It has been theorized that advancing age might offer resistance to energetic stresses so as to maximize reproductive success as opportunities to reproduce become limited (Wingfield and Sapolsky, 2003). Our results, combined with other reports of associations between estradiol and body composition (Emaus et al., 2008; Ziolkiewicz et al., 2008) in premenopausal women with normal cycle lengths, may suggest that menstrual disturbances represented by changes in cycle length are not a prerequisite for varying estradiol concentrations. As the mechanism for the observed reduction in estrogen exposure in the present investigation is unclear, our results underscore the need for more studies in peak reproductive aged women, including women older than our subjects, i.e. those entering the perimenopausal years, where comprehensive measures of estrogen exposure across the menstrual cycle in response to exercise and diet interventions are employed.

The observed decline in estrogen exposure was associated with a transient increase in SHBG, suggesting that the bioavailability of estrogen was further reduced during that time. Others have reported higher SHBG in association with exercise alone or exercise combined with caloric restriction (Franks et al., 1991; Tymchuk et al., 2000; McTiernan et al., 2006; Akin et al., 2007). Hepatic production of SHBG is regulated by many factors including but not limited to insulin, thyroid hormone, sex steroids and dietary factors. As insulin has been shown to inhibit SHBG synthesis (Tymchuk et al., 2000), it is possible that the observed increase in SHBG in our study was secondary to a decline in circulating insulin. Notably, the increase we observed was transient, and thus our study does not support a purported impact of physical activity to chronically alter sex hormone exposure through chronic alterations in sex steroid binding (Friedenreich and Orenstein, 2002).

Figure 4 Effects of exercise training and caloric restriction (EX + CR: open circle) and light conditioning (LC: filled circle) on (A) SHBG, (B) mean EIG AUC and (C) mean PdG AUC for each cycle in all subjects across the intervention. Data are expressed as the mean $\pm$ SEM. Asterisk, $P < 0.0125$ versus baseline, post hoc t -test using Bonferroni correction; B, baseline; Int, intervention; and Post, post-study.

Our LC group showed no changes in ovarian steroid exposure, perhaps indicating that larger changes in body weight, body fat or an increased exercise volume are necessary to produce significant changes. Resources prohibited continued recruitment into the LC group, and thus our results must be interpreted in light of the relatively smaller sample size of this group. In addition, our LC subjects were a few years older than our EX + CR subjects, which may have

Figure 5 Composite graphs of the effects of exercise training and caloric restriction on the EIG AUC in all subjects in EX + CR during baseline and all intervention cycles. Data are expressed as the mean $\pm$ SEM. AUC for Intervention Cycle 4 was significantly lower than that vs. Baseline ($P < 0.0125$; post hoc t -test using Bonferroni correction).

Figure 6 Correlation between average energy deficit calculated during all intervention cycles and changes in E1G AUC from baseline to intervention 4 in response to exercise training and caloric restriction in EX + CR subjects ($R = 0.43$; $P = 0.046$). Int, intervention.

influenced their susceptibility to our intervention. Our results do not provide information on whether caloric restriction, exercise, weight loss, some other factor or even synergistic actions of these parameters caused the observed changes. A larger sample size is likely necessary to perform regression analyses that would allow us to test whether the changes observed could be attributed to independent contributions of these factors. We chose to combine caloric restriction and exercise to mimic what is more likely to result in weight loss, as exercise alone is often not a successful weight loss strategy (Sodlerlund et al., 2009). Additionally, it is important to note that our rather large dropout rate (30%) warrants caution with respect to the generalizability of our results. Lastly, we excluded women who displayed menstrual disturbances during screening or baseline. In light of this, the 'robustness' exhibited by our subjects in terms of the susceptibility to menstrual disturbances during the intervention should be interpreted in consideration of these baseline cycle characteristics.

In conclusion, the results of this study suggest that a moderate aerobic exercise training program combined with modest weight loss in accordance with recommended guidelines produces significant reductions in ovarian steroid exposure without disrupting menstrual cyclicity in premenopausal women of advanced gynecological age. Although larger studies using more precise measures of energy balance are required to confirm this finding, the change in estrogen exposure may be more related to daily fluctuations in energy balance than longer-term changes in body weight or body fat.

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