Long Term Estrogen Deficiency Lowers Regional Blood Flow, Resting Systolic Blood Pressure and Heart Rate in Exercising Premenopausal Women

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\textbf{Abbreviated Title:} Cardiovascular Function in Amenorrheic Athletes
ABSTRACT

The cardiovascular consequences of hypoestrogenism in premenopausal women are unclear. Accordingly, the influence of menstrual status and endogenous estrogen (E$_2$) exposure on blood pressure (BP), heart rate (HR), and calf blood flow in young (18-35yrs) regularly exercising premenopausal women with exercise-associated menstrual aberrations was investigated. Across consecutive menstrual cycles, daily urinary ovarian steroid levels were analyzed and the area under the curve calculated to determine menstrual status and E$_2$ exposure. BP, HR, blood flow, vascular conductance and resistance were measured at baseline and following ischemic calf exercise. Exercising subjects consisted of 10 ovulatory (ExOv), 10 short term (anovulatory and ≤100 days amenorrhea; ST-E$_2$ Def), and 8 long-term (>100 days amenorrhea; LT-E$_2$ Def) E$_2$ deficient women. Nine sedentary ovulatory subjects (SedOv) were also studied. All groups were similar in age (24.8±0.7 yrs), height (164.8±1.3 cm), weight (57.9±0.9 kg) and BMI (21.3±0.3 kg/m$^2$). E$_2$ deficient groups had lower (p<0.002) E$_2$ exposure compared with ovulatory groups. Resting systolic BP, HR, blood flow, and vascular conductance were lower (p<0.05), and vascular resistance higher (p<0.05) in LT-E$_2$ Def compared with both ovulatory groups. Peak-ischemic blood flow, vascular conductance and HR were also lower (p<0.05), and vascular resistance higher (p<0.05) in LT-E$_2$ Def compared with all other groups. Our findings show that exercising women with long term E$_2$ deficiency have impaired regional blood flow and lower systolic BP and HR compared with exercising and sedentary ovulatory women. These cardiovascular alterations represent markers of altered vascular function and autonomic regulation of which the long-term effects remain unknown.

Keywords: exercise-associated amenorrhea, menstrual aberrations, vascular resistance.
INTRODUCTION

Exercise-associated amenorrhea (EAA) is the most severe of menstrual disturbances (10, 11). The etiology of EAA is linked to chronic energy deficiency that is communicated as inhibitory signals to the reproductive axis (11). Chronic suppression of ovarian steroids in postmenopausal women is associated with an increased risk of premature chronic diseases, including cardiovascular disease (9). Little is known of the short and long-term cardiovascular consequences of hypoestrogenism in otherwise healthy premenopausal exercising women.

Preliminary data suggest that women with EAA may develop a pro-atherogenic phenotype, including increases in total cholesterol, LDL-cholesterol and apolipoprotein (Apo) B, with concurrent decreases in Apo A-I and Apo AI/Apo B ratio (24, 38), increased susceptibility of LDL-cholesterol to peroxidation (2), and impaired brachial artery endothelium-dependent flow-mediated vasodilation (38, 45, 46). Alarmingly, the severity of endothelial dysfunction in EAA (38, 45, 46) is similar to that previously observed in postmenopausal women (6), and coronary artery diseased patients (7). Reversibility of endothelial dysfunction with oral contraceptive therapy to women with EAA has been reported (38). Since endothelial dysfunction is considered to be an important permissive factor for the development of atherosclerosis, short or long-term impairment of endothelial function in women with EAA may translate into increased long-term cardiovascular risk.

Although exercise is widely believed to be cardioprotective, when associated with chronic hypoestrogenism the benefit may be obviated and potentially contribute to abnormalities in cardiovascular function through actions on the endothelium, myocardium, neurohumoral
activation and the metabolic milieu. The purpose of this study was to examine the association between E2 exposure, quantified via daily urinary assessments, and BP, HR, peripheral blood flow, vascular conductance and resistance measurements in E2 replete and deplete exercising women and E2 replete sedentary women. We hypothesized that the exercising women with the most severe E2 deficiency would exhibit the greatest reduction in regional blood flow.

METHODS

Subjects: Volunteers were recruited by posters targeting both sedentary and physically active women for a study on women’s health. Screening procedures included general questionnaires on exercise, eating, menstrual cycle, and medical health history. Eligibility criteria for the study included, 1) age 18 to 35 yrs; 2) good health determined by a medical exam; 3) no chronic illness, including hyperprolactinemia and thyroid disease; 4) stable menstrual status over preceding 3 months; 5) non-smoker; 6) not currently dieting and weight stable for the preceding 3 months, as determined by self-report; 7) absence of hormonal therapy for at least 12 months; 8) no history or current clinical diagnosis of eating disorders and 9) no other contraindications that would preclude participation in the study. The study was approved by the institutional committee on human research by the Ethics Review Board at the University of Toronto. All volunteers signed an approved informed consent document.

Experimental Design: This study was a sub-study of a large observational project examining relationships between physical activity, metabolism, and reproduction. Volunteers were recruited on a rolling basis over three years. A total of 87 volunteers were recruited, and 52 completed the
entire study, 32 either failed screening or declined study participation and 3 women dropped out due to time constraints. A total of 42 volunteers completed this sub-study that included cardiovascular measurements. Volunteers were retrospectively grouped according to their exercise status (exercising or sedentary) and according to their menstrual status. The details of this classification scheme are outlined below.

**Observational Time Periods:** Menstruating women were monitored for 2 to 3 consecutive menstrual cycles, and amenorrheic women were monitored for 2 to 3 consecutive 30-day monitoring periods. Oligomenorrheic women with irregular cycle lengths of 38-90 days were excluded from analyses. All data presented in this study represent the mean of the 2-3 menstrual cycles or 30 day periods monitored, except for five women who were monitored for only one menstrual cycle or one 30-day monitoring period, including 3 amenorrheic women and 2 menstruating women.

**Subject Grouping Categories:** Subjects were grouped according to exercise status (determined by exercise diaries) and menstrual status (determined by daily ovarian steroid levels). Four combined categories were established: 1) sedentary women with ovulatory menstrual cycles (SedOv; n=10), 2) exercising women with ovulatory menstrual cycles, including women with a luteal phase deficiency (LPD) (ExOv; n=14), 3) exercising women with short-term E₂ deficiency, including those with anovulatory menstrual cycles, and those with short-term amenorrhea, defined as cessation of menses for <100 d (ST-E₂ Def; n=10), and 4) exercising women with long-term E₂ deficiency, including those with long-term amenorrhea, defined as cessation of
menses for >100 d (LT-E2 Def; n = 8). The ST-E2 Def group included 6 anovulatory women, 5 who were consistently anovulatory, and one who was inconsistently anovulatory (one anovulatory cycle, one ovulatory cycle), and 4 short-term amenorrheic women. Mean duration of amenorrhea in the LT-E2 Def group was 270.6 ± 50.8 d at study entry.

Menstrual status, from daily ovarian steroid assessments, was classified as ovulatory, LPD, anovulatory or amenorrheic, as defined in the Determination of Menstrual Status section. Volunteers were categorized into a single menstrual classification according to the predominant presentation of each menstrual cycle. For example, if 2/2 or 2/3 cycles monitored were anovulatory, that individual was classified as anovulatory. For individuals that were inconsistent, i.e., one ovulatory, one anovulatory, menstrual classification was determined by the most severe menstrual disturbance.

Exercise status was defined as “sedentary” when purposeful exercise was less than 2 hours per week and “exercising” when purposeful exercise was more than 2 hours per week. Purposeful exercise, defined as exercise that elicited a heart rate (HR) greater than 55% of maximal HR (220 minus age) for 3 minutes or more, was documented in exercise logs (10).

**Anthropometric Data:** Total body mass was measured to the nearest 0.1 kg on a physician’s balance scale weekly throughout the study, and the mean of these measurements is presented (Detecto, Webb City, MO). Height was measured to the nearest 1.0 cm at the beginning of the study period. Body mass index (BMI) was calculated as average weekly weight throughout the study period divided by height² (kg/m²).
**Body Composition:** Dual-energy x-ray absorptiometry (DXA) was utilized to determine body composition once during the study (Prodigy, General Electric Lunar Corporation, Madison, WI, enCORE 2002 software, version 6.50.069). The DXA scanner has a precision of <1% coefficient of variation for body composition measurements. A 28 volunteer precision study was performed in premenopausal women, and the precision was 0.6% for the total body.

**Peak Aerobic Capacity:** Peak oxygen uptake (VO₂ peak) was measured during a progressive treadmill test to volitional exhaustion using open-circuit spirometry on a single occasion. After a warm-up, subjects ran at a self-selected speed at 0.0% grade for 2 minutes, after which the grade was increased 2.0% every 2 minutes for the first 8 minutes, and then 1.0% for each subsequent minute. Expired gases were collected continuously through a Hans Rudolph valve and corrugated plastic tubing connected to a flow transducer that measured inspired air volumes. Breath-by-breath samples were analyzed using an automated metabolic cart (Moxus Modular VO₂ System, Applied Electrochemistry Inc., Pittsburgh, PA), and reported as 20-second average samples.

**Determination of Menstrual Status:** Daily first morning void urine samples were collected by all volunteers for the duration of the study. Volunteers stored the urine samples in prelabeled cups in the refrigerator for weekly delivery to the laboratory. The urine samples were assayed for LH, pregnanediol 3-glucuronide (PdG), and estrone 3-glucuronide (E1G) to assess ovulatory status, and E₂ exposure.

Ovulatory status was determined by day of the urinary LH surge, identified as a LH peak on the day of or day after the midcycle E1G peak (10), which has been demonstrated to be a good
proxy indicator of ovulation (4). Specific hormonal criteria for positive ovulation included a LH surge concentration above 25 mIU/mL, the E1G peak concentration above 35 ng/mL, and a peak PdG concentration above 5 μg/mL during the luteal phase (19, 41). Menstrual cycle length was defined as the number of days from day 1 of menses to the day before the first day of the next menses. The follicular phase length was defined as the number of days from the first day of menses up to and including the day of the LH surge (10). The luteal phase length was defined as the difference between the cycle length and the follicular phase length (10). LPD was defined as short when the luteal phase length was less than 10 days, or inadequate when the sum of the 3-day midluteal peak PdG (sum of midluteal peak PdG ± 1 day) was less than 10 μg/mL and when the PdG peak concentration was below 5 μg/mL (19, 41). An anovulatory cycle was defined as a cycle in which no increase in E1G was observed in concurrence with a failure of LH to rise at midcycle or when a luteal phase exhibited no increase in PdG concentration from a 5-day baseline or when the peak PdG value was below 2.49 μg/mL (19, 41).

**Estrogen Exposure:** To assess E2 exposure, mean E1G urinary metabolites were compared among the groups using the trapezoidal integrated area under the curve (AUC) for the follicular phase, the luteal phase, and across the entire cycle. Mean E1G levels of daily E1G during the follicular phase, the luteal phase, and across the entire cycle were also assessed.

**Urinary Measurement of E1G and PdG:** The validity of the urinary technique as representative of the 24-h pattern of E1G and PdG excretion has been previously reported (4, 34). Microtiter plate competitive enzyme immunoassays were used to measure the urinary metabolites E1G and
PdG. The secretion of these metabolites in the urine parallels serum concentrations of the parent hormones (34). The E1G (R522-2) and PdG (R13904) assays use a polyclonal capture antibody supplied by Coralie Munroe at the University of California (Davis CA). The competitors for these assays are E1G or PdG conjugated to horseradish peroxidase. E1G and PdG standards (Sigma) are used for the standard curve, and high and low internal controls from in-house samples. The inter-assay coefficients of variation for high and low internal controls are 14.7% and 13.1% for E1G and 15.68% and 17.7% for PdG. Urine samples were corrected for specific gravity using a hand refractometer (NSG Precision Cells, Inc., Farmingdale, NY) to account for hydration status. Specific gravity has been reported to perform as well as creatinine for adjusting hormone concentrations (31). Urinary LH was determined by double antibody radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA). The sensitivity of the LH assay is 0.6 mIU/L. The intra-assay and inter-assay coefficients of variation were 1.6% and 7.1%, respectively.

**Peripheral Blood Flow, Heart Rate and Blood Pressure Testing:** Lower-leg (calf) blood flow, HR and blood pressure (BP) was measured during the early follicular phase (day 2-6) of each menstrual cycle for menstruating subjects and during the first 6-days of each 30-day monitoring period for amenorrheic volunteers, and then averaged for the entire study period. Calf blood flow was assessed using venous occlusion strain-gauge plethysmography methods as previously described (15, 37). Briefly, the subject lay in a supine position with the calf slightly elevated above heart level, and maintained in that position with a foot rest. The calf was isolated using pneumatic cuffs. One cuff was placed at the ankle to isolate calf blood flow from the foot by
inflating to supraarterial systolic pressure (200mm Hg), and a second ‘collecting cuff’ was placed above the knee to occlude venous return. An indium-gallium strain gauge (Vasculab SPG16 Medasonics, Newark, CA) was placed around the widest girth of the calf set to approximately 10 g of tension. Changes detected in limb circumference integrated to the time period by the strain-gauge are assumed to be linearly related to blood flow (16). Baseline resting measures were made by inflating the exclusion pneumatic cuff around the ankle to 200mm Hg and rapidly inflating the collecting venous occlusion cuff to a preset level above venous pressure (~55 mg) for a 7-sec period followed by a 7-sec rest and then reinflated to continue a cycle of 35-sec. Peak-ischemic measures were acquired immediately (starting within 5-sec) following a 5-min resting occlusion period, followed by ischemic plantar flexion exercise to fatigue. Data from the slope of the time-leg volume curve was used to determine blood flow (mL/100mL/min). Blood flow, vascular conductance (blood flow/ mean arterial pressure (MAP); mL/100mL/min/mmHg), and vascular resistance (MAP/blood flow; U) were calculated using custom software. Measurement of HR, BP and mean arterial pressure were recorded continuously throughout each testing session on a beat-to-beat basis using the photoplethysmographic method (Finapres, Ohmeda, Madison, WI).

**Statistical Methods:** All data sets were tested for non-normality, homogeneity of variance, and outliers before statistical hypothesis tests were performed. Data points greater than 3 standard deviations from the mean of the sample were considered to be outliers. Due to the small sample sizes, removal of outliers from analyses minimized the influence on the slope of the regression line and consequently on the value of the correlation coefficient. Only one outlier was detected and removed from analyses. All data sets were normally distributed. Data for demographics,
reproductive hormones, regional blood flow, BP and HR were analyzed using one-way ANOVA. When a significant main (fixed) effect was observed, the least significant squares was used for multiple comparisons to determine where the significant differences existed. Pearson-product moment correlation analysis was used to examine relationships between E₂ exposure and all cardiovascular variables. Data were analyzed using packaged software (SPSS version 12.0; SPSS Inc., Chicago, IL). A significance level of p < 0.05 was used to detect the differences for all statistical procedures. All data are presented as the mean ± SE.

RESULTS

Demographic and Anthropometric Characteristics: Subject characteristics are presented in Table 1. Groups were similar with respect to age (24.8±0.7 yrs), height (164.8±1.3 cm), weight (57.9±0.9 kg) and BMI (21.3±0.3 kg/m²). As expected, the SedOv group had a greater fat mass, (p=0.037) and body fat percentage (p=0.008), compared to the ExOv, ST-E₂ Def, and LT-E₂ Def groups. The SedOv group also had a lower (p=0.003) fat free mass and maximal aerobic capacity (p=0.005) compared to the ExOv, ST-E₂ Def, and LT-E₂ Def groups.

Menstrual Related Characteristics: Menstrual related characteristics are presented in Table 2. Age at menarche was not different (p=0.060) among the groups, but gynecological age, calculated by subtracting the age of the subject at menarche from their chronological age, was lower (p=0.050) in both E₂ deficient groups compared to the SedOv group.
**E1G Cycle Mean and E1G AUC:** Urinary E1G levels are presented in Table 3. Both E2 deficient groups had significantly (p<0.05) lower E1G mean and E2 exposure, assessed by E1G AUC, (Figure 1) for the complete cycle, and during both the follicular and luteal phase compared to the sedentary and exercising ovulatory groups. When expressed as a percentage, the LT-E2 Def group had a 62, 62, and 25% lower E1G cycle mean, and a 66, 68, and 27% lower E2 exposure compared to the SedOv, ExOv, and ST-E2 Def groups, respectively. Although the ST-E2 Def group had lower E1G (p=0.047) concentrations on the day of blood flow testing compared to the other groups, the values for all groups were well within the range expected for the early follicular phase of the menstrual cycle, and within the range expected for E2 deficient premenopausal women, rendering a comparable E2 exposure environment on actual day of testing.

**Resting Blood Flow Responses:** Figure 2 shows resting blood flow and vascular resistance across the groups. Similar (p>0.05) resting blood flow, vascular conductance and resistance values were observed between the ovulatory groups, and between the E2 deficient groups. E2 deficient groups demonstrated lower (p<0.05) resting blood flow, vascular conductance, and higher (p<0.05) vascular resistance compared with ovulatory groups. The LT-E2 Def group had 44 and 48% lower (p=0.006) resting blood flow, and 38 and 47% lower (p=0.010) vascular conductance compared with the SedOv and ExOv groups, respectively. Similarly, resting vascular resistance was 34 and 45% higher (p=0.002) in the LT-E2 Def compared with SedOv and ExOv groups, respectively. The ST-E2 Def group also had lower (p<0.05) resting blood flow, vascular conductance, and higher (p<0.05) vascular resistance compared with the ExOv
group. Resting diastolic BP and mean arterial pressure, as shown in Table 4, were similar (p>0.05) between the groups. Pulse pressure trended toward significantly lower (p=0.057) values in the LT-E2 Def compared with the ovulatory groups. Systolic BP and HR were lower (p<0.05) in the LT-E2 Def compared with the SedOv and ExOv groups, and lower (p<0.05) in the ST-E2 Def group compared with SedOv group.

**Peak Ischemic Blood Flow Responses:** The LT-E2 Def group had between 24-28% lower (p=0.008) peak-ischemic blood flow, 29-47% lower (p=0.016) vascular conductance, and 17-25% higher (p=0.012) vascular resistance compared with the SedOv, ExOv, and ST-E2 Def groups. Figure 2 shows peak blood flow and vascular resistance across the groups. Similar (p>0.05) peak ischemic blood flow, vascular conductance and resistance values were observed between the ovulatory and ST-E2 Def groups.

Peak ischemic BP and HR responses (Table 4) were similar (p>0.05) between groups, except for diastolic BP which was lower (p=0.043) in the LT-E2 Def group compared with both ovulatory groups.

E2 exposure was positively associated with resting measures of blood flow (r=0.407, p=0.008; Figure 3), vascular conductance (r=0.356, p=0.023), and HR (r=0.345, p=0.032), and negatively associated with resting vascular resistance (r= -0.408, p=0.006). Resting BP measures were not correlated (p>0.05) with E2 exposure. Peak ischemic HR was associated with E2 exposure (r=0.385 p=0.017).
**DISCUSSION**

This is the first study to investigate the influence of menstrual status and endogenous E₂ exposure on BP, HR and peripheral vascular function in regularly exercising premenopausal women. Unique to this study, the daily evaluation of urinary ovarian hormone concentrations permitted meticulous characterization of ovarian hormone profile and accurate quantification of E₂ exposure. The novel findings of this study are four-fold. Firstly, this is the first report of reduced regional (calf) blood flow and vascular conductance, and higher vascular resistance at rest in exercising women with E₂ deficiency, independent of duration of E₂ deficiency, whether short-term (up to 100 d) or long-term (>100 d). Secondly, the LT-E₂ deficient women also demonstrated reduced peak ischemic blood flow and vascular conductance, and higher vascular resistance compared to all groups, including the ST-E₂ deficient groups. Thirdly, the LT-E₂ deficient women had lower resting systolic BP and HR, and lower peak ischemic diastolic BP compared with the ovulating women and lower resting pulse pressure and post-ischemic HR than all groups, including the ST-E₂ deficient women. Fourthly, cumulative E₂ exposure was lowest in the exercising women who were anovulatory and amenorrheic, i.e., E₂ deficient, and this finding was associated with reduced resting (but not peak-ischemic) measures of blood flow, vascular conductance, and HR and higher resting vascular resistance. Collectively, these findings are suggestive of altered vascular function and altered autonomic regulation in E₂ deficient exercising women, adding to the literature describing altered lipid profiles, increased lipid peroxidation and endothelial dysfunction in this population (2, 24, 38, 45, 46).

The quantification of E₂ exposure in women across the spectrum of exercise-associated menstrual abnormalities has not previously been performed in association with measures of
cardiovascular function. Consistent with previous studies (10) we have shown that severe menstrual abnormalities like anovulation are often asymptomatic, are inadequately detected by self report, and are markedly E2 deficient. Indeed, ovulatory status determined via characterization of daily ovarian steroid levels is required to distinguish asymptomatic anovulatory cycles from ovulatory cycles and to quantify E2 exposure.

Our finding of reduced resting calf blood flow in association with reduced E2 exposure in healthy exercising premenopausal women is novel. Reduced lower limb blood flow responses have also been observed in hypoestrogenic anorexic patients (13) and in postmenopausal women not taking hormone therapy (33). We also observed that reduced peripheral blood flow was associated with decreased local vascular conductance and increased vascular resistance. Potential mechanisms underlying these are alterations are likely multi-factorial and includes augmented vasoconstriction secondary to elevated autonomic activity (sympathetic tone), or mechanical abnormalities leading to reduced compliance or remodelling of the vessel wall limiting vasodilatory capacity. There is evidence that local vasoregulatory mechanisms can contribute to augment vasoconstriction due to E2 deficiency, including endothelial dysfunction leading to increased synthesis and release of vasoconstrictor substances such as endothelin-1 or thromboxane A2 or conversely reduced bioavailability of endothelial-derived vasodilator including nitric oxide and prostacyclin (5, 36).

Our findings of reduced peak ischemic blood flow and vascular conductance, and increased vascular resistance in LT-E2 deficient women are supportive of an E2-dependent effect on peak vasodilatory responses. However, we show that ST-E2 deficient women demonstrate peak ischemic calf blood flow, vascular conductance and vascular responses that are similar to
that observed in the ovulatory groups, but greater than that observed in LT-E2 deficient women. It is unclear whether the duration of hypoestrogenism, or the existence of a possible E2 threshold conferring benefit to vessel reactivity effected our observations. It is known that numerous vasodilatory substances elicit vasodilation in response to exercise and reactive hyperemia (8). Indeed, we show that peak-ischemic blood flow responses are not associated with E2 exposure. This observation is consistent with redundancy of control among vasodilators, as any one vasoactive factor may be activated when the formation of another is compromised (8). Conversely, inhibition of various vasodilatory factors merely reduces, not eliminates, reactive hyperemia or increased blood flow (21). The lack of evidence regarding the efficacy of any one single vasodilator makes it difficult to ascertain the extent to which hypoestrogenism may impact peak vasodilation, however our observations supports a potential role for adequate circulating E2 in normal vessel function.

During post-occlusive reactive hyperemia the initial vasodilation following blood flow perfusion is deemed to be primarily due to loss of myogenic tone (3). As such, increased peak-ischemic vascular resistance in the face of similar mean arterial pressure in LT-E2 deficient women compared with all other study groups may be attributed, in part, to alterations in structural, rather than functional, changes in lower limb vascular supply. Structural determinants of vascular resistance include vascular diameters, length, and anatomical arrangement of vessels, the most important being radius of the vessel (20). The effects of estrogen deficiency on structural determinants of vascular resistance can be related to studies reporting decreased angiogenesis and arteriogenesis in animal models (23), possibly via estrogens ability to induce the expression of numerous angiogenic factors such as vascular endothelial growth factor and
basic fibroblast growth factor (29). In humans, the normal physiologic cycling of the female reproductive system involves the development and regression of a vascular network which is related to endogenous estrogen levels (25). Estrogen also upregulates (40) and increases the bioavailability of nitric oxide (1), another integral component of the signaling pathway for the remodeling of existing arterial vessels (28). Whether increased vascular resistance in our LT-E2 deficient women is due to lower limb vessel remodeling, or decreased number and/or size of vessels in parallel to the popliteal artery, is not known.

Attenuation of sympathetic activity and increased vagal tone, effecting decreased vascular resistance, is a recognized adaptation to chronic endurance training (35). In contrast to these findings, LT-E2 deficient women demonstrate increased regional vascular resistance despite exposure to chronic aerobic exercise training. Our findings suggest that long-term estrogen deficiency may obviate the beneficial effect of aerobic exercise on vascular resistance, indicating the likelihood of a potent effect of chronic estrogen deficiency on vascular biology. Indeed, estrogen deficiency in postmenopausal women is associated with increased carotid intima-media thickness (43), and endothelial dysfunction (27). In vitro data also show that physiological levels of 17 beta-estradiol inhibits smooth muscle cell proliferation and migration, and decreases collagen synthesis (12). Studies examining the effects of hypoestrogenism on vascular biology on factors other than endothelial function in premenopausal athletes (38, 45, 46) have not yet been reported. In older postmenopausal women, hormone therapy and aerobic exercise training are independently and similarly associated with smaller femoral intima-media thickness compared with sedentary age-matched women not on hormone therapy (32). Discrepancies between
postmenopausal women and younger exercising hypoestrogenic women, however, likely exist due to the known effects of aging per-se on vascular function.

Interestingly, this study also demonstrates a lower resting HR in the both the ST-E₂ and LT-E₂ deficient women. It is well established that increased aerobic fitness due to exercise training induces a physiologic bradycardia (17, 42). Since the aerobic capacity of the exercising groups was similar, it is suggestive of an exercise training-independent effect on resting HR. In anorexic patients, resting bradycardia is particularly evident when coincident with significant weight loss (22). Resting bradycardia is also associated with increased vagal tone (18). Whether resting bradycardia in the LT-E₂ deficient women in this study is also associated with weight loss and/or increased vagal tone remains to be described.

It is also intriguing that we observed lower resting systolic BP in LT-E₂ deficient women compared with both ovulatory groups. Warren et al. (44) similarly reported that amenorrheic athletes demonstrate reduced BP compared to their eumenorrheic counterparts. Anorexic patients also demonstrate lower systolic BP compared to healthy age-matched women (14, 39). The cause of lower resting systolic BP in LT-E₂ deficient women is unclear but in anorexic patients has been attributed, in part, to nutritional status (14, 39). Indeed, caloric restriction has been shown to decrease sympathetic nerve activity in animal models (26). Consistent with the hypothesis that inadequate caloric intake is the cause of EAA (30), caloric restriction, may also have played a mechanistic role in our observed altered systolic BP, and possibly HR, responses in LT-E₂ deficient women. These results appear paradoxical in relation to the vessel function findings, indicative of multiple controls that contribute to the balance of local and systemic cardiovascular control.
In summary, we have shown that women with exercise associated menstrual abnormalities have impaired regional vascular function combined with changes in BP and HR regulation, and that these changes are most profound in women with long-term E2 deficiency. These findings represent altered vascular function and altered autonomic regulation in E2 deficient exercising women. The precise mechanisms responsible for these changes are not known. The long-term implications on cardiovascular health warrant further investigation.
**Figure 1.** Composite graphs for urinary estrone 3-glucuronide (E1G) and pregnanediol 3-glucuronide (PdG), and bar chart for area under the curve (AUC) for E1G for the study groups.

1a, 1b, 1d and 1e Composite graphs for sedentary ovulatory (SedOv), exercising ovulatory (ExOv), short term estrogen deficient (ST-E2) and long term estrogen deficient (LT-E2), respectively. 1c Bar chart for estrogen (E2) exposure, as assessed by AUC. * vs SedOv and ExOv (p=0.001, main effect).

**Figure 2.** 2a and 2b, Resting and peak-ischemic calf blood flow measures (mL/100mL/min), respectively. 2c and 2d, Resting and peak-ischemic calf vascular resistance (U), respectively. * p<0.05 vs ExOv; ** p<0.05 vs SedOv and ExOv; *** p<0.05 vs all other groups.

**Figure 3.** Scatterplot showing the positive correlation between E2 exposure, as assessed by AUC, and resting blood flow measures.
REFERENCES


Figure 1.

A  SedOv Group

B  ExOv Group

C

Day of Menstrual Cycle

Day of Menstrual Cycle

Day of Monitoring Period

Day of Monitoring Period

AUC for E1G (ng/mL)

ST-E2 Def Group

LT-E2 Def Group

Menstrual and Ovulatory Group

E1G (ng/mL)
PdG (µg/mL)

E1G (ng/mL)
PdG (µg/mL)

E1G (ng/mL)
PdG (µg/mL)

E1G (ng/mL)
PdG (µg/mL)
Figure 3

![Graph showing the relationship between Blood Flow (mL/100mL/min) and E1G (ng/mL). The graph includes data points for SedOv, ExOv, ST-E2, and LT-E2. A linear trend line is drawn with the correlation coefficient r=0.407, p=0.008.]
Table 1. Demographics and anthropometric measures for the study groups.

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<th>SedOv (n=9)</th>
<th>ExOv (n=14)</th>
<th>ST-E2 Def (n=10)</th>
<th>LT-E2 Def (n=8)</th>
<th>Probability (Main Effect)</th>
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<td>Age (yrs)</td>
<td>27.3 ± 1.9</td>
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<td>Weight (kg)</td>
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<tr>
<td>Body fat (%)</td>
<td>31.48 ± 2.46</td>
<td>23.91 ± 1.35*</td>
<td>21.01 ± 2.30*</td>
<td>20.58 ± 2.92*</td>
<td>0.008</td>
</tr>
<tr>
<td>Body fat (kg)</td>
<td>17.95 ± 2.17</td>
<td>12.94 ± 0.87*</td>
<td>12.04 ± 1.50*</td>
<td>11.59 ± 1.90*</td>
<td>0.037</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>37.78 ± 1.12</td>
<td>40.91 ± 1.11</td>
<td>44.77 ± 1.55†</td>
<td>43.41 ± 0.92‡</td>
<td>0.003</td>
</tr>
<tr>
<td>VO2 peak (ml/kg/min)</td>
<td>38.60 ± 1.47</td>
<td>46.47 ± 1.40*</td>
<td>45.96 ± 1.69*</td>
<td>45.24 ± 1.83*</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

SedOv, sedentary ovulatory; ExOv, exercising ovulatory; ST-E₂ Def, short term estrogen deficient; LT-E₂ Def, long term estrogen deficient; BMI, body mass index; FFM, fat free mass.

* vs SedOv
† vs SedOv and ExOv
‡ vs SedOv
Table 2. Menstrual cycle characteristics for the study groups.

<table>
<thead>
<tr>
<th></th>
<th>SedOv (n=9)</th>
<th>ExOv (n=14)</th>
<th>ST-E2 Def (n=10)</th>
<th>LT-E2 Def (n=8)</th>
<th>Probability (Main Effect)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menstrual cycle length*</td>
<td>28.3 ± 0.9</td>
<td>28.8 ± 0.7</td>
<td>N/A</td>
<td>N/A</td>
<td>0.631</td>
</tr>
<tr>
<td>Follicular phase length</td>
<td>15.9 ± 0.8</td>
<td>16.3 ± 0.7</td>
<td>N/A</td>
<td>N/A</td>
<td>0.754</td>
</tr>
<tr>
<td>Luteal phase length</td>
<td>12.6 ± 0.5</td>
<td>12.8 ± 0.3</td>
<td>N/A</td>
<td>N/A</td>
<td>0.824</td>
</tr>
<tr>
<td>Age of menarche (yrs)</td>
<td>12.1 ± 0.4</td>
<td>12.0 ± 0.3</td>
<td>13.3 ± 0.4</td>
<td>13.1 ± 0.6</td>
<td>0.060</td>
</tr>
<tr>
<td>Gynecological age (yrs)</td>
<td>15.3 ± 1.9</td>
<td>12.9 ± 0.9</td>
<td>10.6 ± 1.7†</td>
<td>9.1 ± 1.5†</td>
<td>0.050</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

SedOv, sedentary ovulatory; ExOv, exercising ovulatory; ST-E2 Def, short term estrogen deficient; LT-E2 Def, long term estrogen deficient

* Menstrual cycle length and phases are in days.

† vs SedOv
### Table 3. Mean urinary E1G and AUC for E1G for the study groups.

<table>
<thead>
<tr>
<th></th>
<th>SedOv (n=9)</th>
<th>Ex Ov/LPD (n=14)</th>
<th>ST-E2 Def (n=10)</th>
<th>LT-E2 Def (n=8)</th>
<th>Probability (Main effect)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean E1G</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire cycle</td>
<td>70.7 ± 9.4</td>
<td>71.5 ± 10.7</td>
<td>36.1 ± 6.9†</td>
<td>26.9 ± 5.9†</td>
<td>0.002</td>
</tr>
<tr>
<td>FP‡</td>
<td>63.9 ± 11.2</td>
<td>64.1 ± 9.7</td>
<td>29.6 ± 5.1†</td>
<td>23.3 ± 5.3†</td>
<td>0.003</td>
</tr>
<tr>
<td>LP§</td>
<td>85.0 ± 10.4</td>
<td>80.9 ± 11.6</td>
<td>43.1 ± 10.8†</td>
<td>25.0 ± 5.5†</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>AUC for E1G</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire cycle</td>
<td>1944.0 ± 244.6</td>
<td>2037.5 ± 275.1</td>
<td>928.4 ± 163.4†</td>
<td>661.0 ± 158.4†</td>
<td>0.001</td>
</tr>
<tr>
<td>FP</td>
<td>933.4 ± 111.2</td>
<td>1122.8 ± 182.8</td>
<td>469.5 ± 99.2†</td>
<td>331.5 ± 81.9†</td>
<td>0.001</td>
</tr>
<tr>
<td>LP</td>
<td>1004.2 ± 145.4</td>
<td>906.1 ± 123.8</td>
<td>458.9 ± 74.6†</td>
<td>329.5 ± 77.2†</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

SedOv, sedentary ovulatory; ExOv, exercising ovulatory; ST-E2 Def, short term estrogen deficient; LT-E2 Def, long term estrogen deficient; FP, follicular phase; LP, luteal phase.

* All E1G concentrations are expressed as nanograms per millilitre.

† vs SedOv and ExOv

‡ Calculated as first half of cycle or 30day monitoring period for ST-E2 Def and LT-E2 Def.

§ Calculated as second half of cycle or 30day period for ST-E2 Def and LT-E2 Def.
Table 4. Heart rate and blood pressure during resting and peak-ischemic blood flow assessment.

<table>
<thead>
<tr>
<th></th>
<th>SedOv (n=9)</th>
<th>ExOv (n=14)</th>
<th>ST-E$_2$ Def (n=10)</th>
<th>LT-E$_2$ Def (n=8)</th>
<th>Probability (Main Effect)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resting</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR*</td>
<td>61.6 ± 1.7</td>
<td>56.6 ± 2.1</td>
<td>51.9 ± 2.2†</td>
<td>46.3 ± 1.9‡</td>
<td>0.001</td>
</tr>
<tr>
<td>DBP§</td>
<td>56.3 ± 0.8</td>
<td>53.5 ± 1.4</td>
<td>51.8 ± 1.2</td>
<td>52.8 ± 2.7</td>
<td>0.322</td>
</tr>
<tr>
<td>SBP</td>
<td>108.2 ± 3.3</td>
<td>105.3 ± 1.6</td>
<td>101.7 ± 1.4†</td>
<td>96.4 ± 1.5‡</td>
<td>0.003</td>
</tr>
<tr>
<td>MAP</td>
<td>73.1 ± 2.5</td>
<td>70.0 ± 1.4</td>
<td>66.2 ± 1.0</td>
<td>67.1 ± 2.6</td>
<td>0.065</td>
</tr>
<tr>
<td>PP</td>
<td>51.9 ± 2.7</td>
<td>51.8 ± 2.1</td>
<td>49.8 ± 1.9</td>
<td>43.6 ± 2.0</td>
<td>0.057</td>
</tr>
<tr>
<td><strong>Peak Ischemic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>69.4 ± 1.8</td>
<td>69.3 ± 2.0</td>
<td>63.8 ± 2.9</td>
<td>53.9 ± 1$^a$</td>
<td>0.001</td>
</tr>
<tr>
<td>DBP</td>
<td>64.3 ± 2.4</td>
<td>64.0 ± 2.3</td>
<td>58.9 ± 1.9</td>
<td>55.3 ± 2.5‡</td>
<td>0.043</td>
</tr>
<tr>
<td>SBP</td>
<td>121.8 ± 5.0</td>
<td>121.9 ± 2.9</td>
<td>118.4 ± 2.9</td>
<td>109.9 ± 3.0</td>
<td>0.105</td>
</tr>
<tr>
<td>MAP</td>
<td>81.9 ± 2.4</td>
<td>81.1 ± 2.8</td>
<td>75.7 ± 2.2</td>
<td>74.4 ± 1.6</td>
<td>0.138</td>
</tr>
<tr>
<td>PP</td>
<td>57.4 ± 4.0</td>
<td>57.9 ± 2.3</td>
<td>59.6 ± 3.2</td>
<td>54.6 ± 3.1</td>
<td>0.762</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

SedOv, sedentary ovulatory; ExOv, exercising ovulatory; ST-E$_2$ Def, short term estrogen deficient; LT-E$_2$ Def, long term estrogen deficient; HR, heart rate; DBP, diastolic blood pressure; SBP, systolic blood pressure; MAP, mean arterial blood pressure; PP, pulse pressure.

* All heart rate measures are in beats per minute.

† vs SedOv

‡ vs SedOv and ExOv

§ All blood pressure measures are in millimeters of mercury.

$^a$ vs SedOv, ExOv, and ST-E$_2$ Def