The effect of estrogen on muscle damage biomarkers following prolonged aerobic exercise in eumenorrheic women

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ABSTRACT: This study assessed the influence of estrogen (E_2) on muscle damage biomarkers [skeletal muscle - creatine kinase (CK); cardiac muscle - CK-MB] responses to prolonged aerobic exercise. Eumenorrheic women (n=10) who were physically active completed two 60-minute treadmill running sessions at ~60-65% maximal intensity during low E_2 (midfollicular menstrual phase) and high E_2 (midluteal menstrual phase) hormonal conditions. Blood samples were collected prior to exercise (following supine rest), immediately post-, 30 min post-, and 24 hours post-exercise to determine changes in muscle biomarkers. Resting blood samples confirmed appropriate E_2 hormonal levels Total CK concentrations increased following exercise and at 24 hours post-exercise were higher in the midfollicular low E_2 phase (p<0.001). However, CK-MB concentrations were unaffected by E_2 level or exercise (p=0.442) resulting in the ratio of CK-MB to total CK being consistently low in subject responses (i.e., indicative of skeletal muscle damage). Elevated E_2 levels reduce the CK responses of skeletal muscle, but had no effect on CK-MB responses following prolonged aerobic exercise. These findings support earlier work showing elevated E_2 is protective of skeletal muscle from exercise-induced damage associated with prolonged aerobic exercise.

INTRODUCTION

It is widely known that unaccustomed exercise results in temporary muscle damage and increased blood creatine kinase (CK) levels. Estrogen has shown to protect skeletal muscle from inflammation in various studies through its attenuation of circulating cytokines and as a result attenuating the inflammatory response [1-6]. However, a clear relationship between estrogen and exercise induced cardiac muscle damage has yet to be examined. As cardiac and skeletal muscles are very similar structurally and functionally, it is logic to assume the protective effect of estrogen observed for skeletal muscle would exist for cardiac too.

Differentiating skeletal muscle damage from myocardial injury is possible by examining the serum CK isoenzyme MB mass measurement to that of the total CK activity and calculating a ratio. Specifically, the ratio is a calculated as a number, where; relative index ratio = [CK-MB (ng·mL⁻¹) ÷ total CK (Units·L⁻¹)] x 100 [7-9]. A ratio less than 3 is consistent with skeletal muscle source of tissue damage, while a ratio greater than 5 is highly indicative of a cardiac source, and a ratio between 3 and 5 represents a somewhat "gray zone" [10].

Estrogen levels fluctuate across a woman's menstrual cycle, being low in the early follicular phase of the cycle and then elevating at ovulation and remaining so until the next menses period. Thus, experimentally examining women at different points in their menstrual cycle allow investigators to have endogenous manipulations of estrogen levels in eumenorrheic women.

Therefore, the purpose of this study was to determine if muscle damage biomarker (CK and CK-MB) responses following moderate intensity prolonged exercise were influenced by the E_2 changes seen in eumenorrheic women.

MATERIALS AND METHODS

Ten healthy, highly trained, pre-menopausal women between the ages of 18-30 were recruited. Samples size was estimated from previous research in the literature to ensure adequate statistical power. Participants eligible for the study needed to be eumenorrheic for the past six months, not taken oral contraceptives or other hormone therapy prior to participation in the study, and not be currently using anti-inflammatory medications, such as ibuprofen, naproxen, or aspirin. Participants were also required to report a current minimum training volume of 3-5 days of aerobic activity lasting between 45-120 minutes per sessions, a VO_2max of 45 ml·kg⁻¹·min⁻¹ and no major in-

TABLE I. Participant physical and menstrual characteristics (n=10)

Measure	Mean ± SD	Range
Age (yrs)	21 ±1	20-23
Height (cm)	164.5 ± 5.7	149.6-172
Body Mass (kg)	61.3 ± 8.3	49.5-80.4
Body Fat (%)	24.0 ± 3.0	17.9-27.4
Menstrual Cycle (days)	28 ± 1	27-30
MF day	7 ± 2	4-10
ML day	23 ± 3	20-27

Note: MF= Midfollicular; ML=Midluteal phases of menstrual cycle

juries that would limit their ability to exercise. See Table 1 for subject characteristics.

Protocol
Orientation Session I

Participants arrived at our laboratory and were informed of the experimental protocol, possible risks and rewards associated with the study, and signed an informed consent form. The Office of Human Research Ethics, Institutional Review Board, of The University of North Carolina, Chapel Hill, approved the informed consent form before beginning the experimental protocol. After giving informed consent, participants filled out the Exercise and Sport Science medical history questionnaire, and underwent a medical screening. Height, weight, and age were recorded [height was measured with a portable stadiometer (Perspectives Enterprises, Portage, MI USA) and body mass was measured using a mechanical scale (Detecto, Webb City, MO USA).

Next, a VO_2max test was performed using a continuous, incremental treadmill protocol on a Quinton Q65 treadmill (Bothell, WA USA). Participants performed a five-minute warm up before beginning the test consisting of cycling at their preferred pace and light stretching. After the warm up, resting VO_2 was measured for three minutes [Respiratory gases were obtained using the Parvo Medics TrueMax 2400 Metabolic System (Parvo Medics, Salt Lake City, UT USA)]. Participants then began the exercise test using the Bruce treadmill protocol [11]. Heart rate and respiratory gases were measured continuously throughout the test as were ratings of perceived exertion (RPE) [Heart rate was taken using the Polar telemetry system (Polar Electro, Inc., Lake Success, NY USA)]. At the conclusion of the test, participants were allowed to recover either actively or passively and were permitted to leave the laboratory when heart rate dropped below 100 beats per minute (bpm). The VO_2 test was determined as a maximal test rather than a peak test through the participants displaying three of the four criteria: a plateau or decrease in VO_2 with increases in workload, respiratory exchange ratio (RER) greater than or equal to 1.1, RPE $\geq$ 18, and a heart rate within 10% of predicted heart rate max (220-age of participant). ACSM guidelines were used to estimate exercise intensity corresponding to ~60-65% of VO_2 [11].

Experimental Sessions

Participants visited the laboratory once during their mid-follicular (MF, low E_2) and mid-luteal (ML, high E_2) phases respectively. Appointment dates were determined using the forward counting method [12]. The two experimental sessions were counterbalanced to prevent order effects. The testing occurred during the MF and ML phases of the menstrual cycle in order to find the largest differences between naturally fluctuating E_2 levels and maximize hormonal effect size.

Participants were asked to refrain from intense physical activity and maintain a similar diet 24 hours prior to the experimental sessions. To ensure they followed all dietary procedures prior to the testing sessions, and a food diary was used to ensure similar nutrient intake between trials, as well as adequate caloric and carbohydrate intake. Once at the laboratory, participants rested supine in a quiet environment for 10 minutes. After this resting period, blood was obtained via venipuncture. The blood sample was split in two and placed in a K^2 –EDTA (purple top) and a sterile SST (yellow top) Vacutainer™ tubes and then immediately put on ice. Participants then completed a five minute warm-up consisting of cycling and light stretching, followed by 60 minutes of treadmill running at their previously determined workload of 65% of VO_2 as determined by the ACSM formula [12]. Blood samples were taken immediately post exercise and 30 minutes post exercise and promptly put on ice. Plasma and sera were separated from blood samples and stored until later analysis.

Participants returned to the laboratory 24 hours post exercise for additional blood draws. Participants were asked to lay supine for 10 minutes in a quiet environment. Blood samples were obtained using the same procedures specified in previous sessions. Participants were asked to refrain from any exercise that is more than what is necessary for activities of daily living for 24 hours before the blood draw.

Blood Procedures
Hematology

Hematocrit (Hct) values were determined in triplicate from all whole blood samples via microcapillary tubes (Fisher Scientific, Pittsburgh, PA) and sealed using Critoseal (Krackeler Scientific, Albany, NY). Whole blood samples were kept in an IEC Centra-8R refrigerated centrifuge (International Equipment Company, Needham Heights, MA USA) and the resultant separated plasma was stored and frozen at -80˚C. Hematocrit was measured using an Adams MHCT II microhematocrit centrifuge (Becton Dickinson, Franklin Lakes, NJ USA) and an International Microcapillary Reader (International Equipment Company, Needham Heights, MA USA). Capillary tubes were spun in a microhematocrit centrifuge for three minutes at 10,000 RPM and then placed on a hematocrit wheel to determine the hematocrit values of each sample.

Hemoglobin (Hb) values were determined in duplicate from the whole blood samples using the Stanbiolab Hemopoint H2 analyzer (Boerne, TX).

Hb and Ht values were used to calculate exercise induced plasma volume shifts according to the Dill and Costill equation [14].

Creatine Kinase (CK and CK-MB), and Estradiol Assays
To separate plasma and serum from whole blood, the blood samples were centrifuged at 3,000 x g for 10 minutes. The separated plasma and serum were transferred to storage tubes and stored until analyses are conducted. Enzyme-linked immunoassay (ELISA) techniques (Abnova Corporation, Taiwan, China) were used to measure plasma estradiol concentrations. The estradiol assay manufacturer reports a minimum detectable concentration of 2.0 pg·mL^{-1}. Serum CK and CK-MB were measured using an Ortho-Clinical Diagnostics Vitros DT60II analyzer (Rochester, NY). The Vitros DT60II blood analyzer employing dry chemistry colorometric procedures was used for assessing CK and CK-MB. The minimum detectable concentration is 1.0 U·L^{-1}L for CK and 1.0 ng·mL^{-1} for CK-MB. All blood assays (unknown samples) were performed in duplicate while standards were done in triplicate.

Data Analysis
SPSS statistical software was used to analyze data (version 18.0, Chicago, IL USA). Significance for all data analysis was set at $p < 0.05$. Descriptive statistics were means ± standard deviations (SD).

A 2x3 (E_2 level x time) totally within, repeated measures ANOVA and where appropriate, Bonferroni post hoc test, were used to assess the effects of estradiol on CK. A 2x2 (estradiol level vs. time) ANOVA was used to evaluate the pre- exercise and recovery during each menstrual cycle phase (estradiol level). These two same procedures were used to assess the effects of E_2 level on CK-MB. A dependent t-test analysis was used to evaluate statistical significance between resting levels of E_2 at MF and ML phase experimental sessions.

RESULTS
VO$_2$Peak Testing. The criteria for a maximal oxygen consumption test was not achieved by all subjects, thus all maximal oxygen consumption tests (VO$_2$max) are referred to as VO$_2$peak tests. The average VO$_2$peak was 53.5 ± 4.7 ml·kg^{-1}·min^{-1} (range 45.1- 60.0 ml·kg^{-1}·min^{-1}). The average peak RPE obtained was 18 ± 1 Borg Units (range 16-20 Borg Units). The average peak heart rate (HR)

was 191 ± 1 bpm (range 180-208 bpm). The time to reach VO$_2$peak ranged between 12:35 to 16:10 mins:sec. Based upon the results of this maximal testing, the average calculated 65% of VO$_2$peak to use in the 60-minute exercise session was 34.8 ± 3.0 ml·kg^{-1}·min^{-1}.

Menstrual Cycle (E$_2$)
Menstrual cycle length was calculated for each subject. The average menstrual cycle length was 28 ± 1 days (range 27- 30 days). Individual menstrual cycle phases were determined using the forward counting method as mentioned in the methodology chapter. The onset of menses was denoted as day 1. Subjects were tested at 7 ± 2 days (range 4-10 days) after the onset of menses during the MF phase. Subjects were tested at 23 ± 3 days (range 20-27) after the onset of menses during the ML phase.

Hormonal analysis of resting blood samples at the MF and ML testing sessions for 17β-estradiol (E$_2$) confirmed the appropriate hormonal status of the subjects. The MF E$_2$ concentration was 39.8 ± 18.3 pg·mL^{-1}, while the ML E$_2$ concentration was 148.1 ± 35.2 pg·mL^{-1}. These concentrations were significantly different ($p < 0.01$) supporting the desired hormonal treatment effect was achieved. The biochemical within assay coefficient of variation for E$_2$ was 5.2%.

Prolonged Runs
The subjects confirmed they followed and complied with protocol guidelines. Specifically, reporting to the laboratory well hydrated (urine specific gravity < 1.030 cc^3) and having replicated their diets, and abstained from strenuous physical activity for the 24 hours prior to the tests. Three subjects participated in light exercise prior to the first testing session, and this exercise was replicated exactly prior to the second prolonged running session. Mean body mass prior to running for MF was 61.4 ± 8.6 kg and for ML was 61.1 ± 8.3 kg.

All subjects completed the 60-minute sessions at the calculated running speed to elicit 65% of the individual's VO$_2$peak. Actual running speed was replicated for each treadmill run at 14.7 ± 1.3 km·hr^{-1} with a corresponding actual VO$_2$ of 61.7 ± 5.0 % during MF and 59.7 ± 2.8 % during ML. Measurements obtained for VO$_2$, HR and RPE were nearly identical for each running session (see Table 2).

TABLE 2. Descriptive data (mean ± SD) for VO$_2$, HR and RPE for each experimental treadmill running protocol in each hormonal condition (MF and MF).

Hormonal Condition	Measure	Time (min)			
		Rest	10	30	60
MF	VO$_2$ (ml·kg^{-1}·min^{-1})	4.9 ± 0.6	30.4 ± 4.2	33.2 ± 3.2	35.2 ± 2.9
	HR (bpm)	63 ± 7	150 ± 15	157 ± 13	165 ± 13
	RPE (Borg units)	-	11 ± 1	12 ± 1	14 ± 1
ML	VO$_2$ (ml·kg^{-1}·min^{-1})	4.9 ± 0.4	30.0 ± 3.2	32.7 ± 3.3	33.1 ± 4.5
	HR (bpm)	61 ± 4	149 ± 7	154 ± 6	164 ± 6
	RPE (Borg units)	-	11 ± 1	12 ± 1	14 ± 2

TABLE 3. Mean (± SD) plasma volume shifts.

Hormonal Condition	Rest to IP (%)	Rest to 30P (%)	Rest to 24P (%)
MF	-8.5 ± 6.9	-3.1 ± 8.0	6.6 ± 16.1
ML	-8.1 ± 5.9	-8.6 ± 7.6	-2.6 ± 6.8

Plasma Volume Shift

Plasma volume decreased over the prolonged treadmill running protocol. Table 3 reports the average percentage plasma volume shifts for fluid moving from the vascular spaces during the exercise and the recovery periods from exercise. The responses for plasma volumes shifts were very comparable in each of the 60-minute running sessions and did not differ ($p > 0.05$). Blood biomarker values (reported Table 4 and 5) are not adjusted for these plasma volume shifts detect based upon the recommendations in the literature [15].

Blood Responses

Total Creatine Kinase. Resting, immediate post-exercise, 30 min post exercise, and 24 hours post-exercise CK activity is reported in Table 4. The main effect for hormonal status was significant ($p < 0.05$; MF>ML). The main effect for time was also significant ($p < 0.01$). Post-hoc revealed levels were significantly increased from rest at 30 min post-exercise and 24 hour post-exercise. Most importantly, the interaction of hormonal status and time was significant ($p < 0.01$). Post hoc tests indicated the increase observed at 24 hour post-exercise was significantly greater in MF than ML.

Creatine Kinase – MB. Creatine kinase - MB (CK-MB) response to the experimental treadmill running protocol is presented in Table 5. The main effect for hormonal status was not significant ($p= 0.72$).

TABLE 4. Mean ± SD for CK at rest, immediately post exercise (IP), 30 minutes post exercise (30P), and 24 hours post exercise (24P).

Hormonal Condition	CK ($U \cdot L^{-1}$)			
	Rest	IP	30P	24P
MF	106.8±34.5	129.9±38.0	161.2±60.9*	378.7±176.5*γ
ML	104.2±3.1	118.6±18.7	153.4 ±28.9*	279.6±100.8*γ

Note: * = significant change ($p<0.05$) from Rest; γ = significant ($p<0.05$) difference between MF and ML responses.

TABLE 5. Mean ± SD for CK-MB at rest, immediately post exercise (IP), 30 minutes post exercise (30P), and 24 hours post exercise (24P).

Hormonal Condition	CK-MB ($ng \cdot mL^{-1}$)			
	Rest	IP	30P	24P
MF	4.7±3.4	4.8±1.4	6.2±1.5	5.6±2.0
ML	5.0±2.3	6.0±2.3	5.4±1.0	5.7±1.4

Note: No significant changes over time or between MF or ML responses were noted.

The main effect for time was also not significant ($p= 0.47$). Furthermore, there was not a significant interaction effect for hormonal condition and time ($p= 0.44$).

DISCUSSION

The purpose of this study was to determine if fluctuations in E_2, corresponding to menstrual cycle phase, influenced blood CK and CK-MB response to prolonged aerobic exercise. It was hypothesized that the higher E_2 levels (ML phase) would attenuate CK and CK-MB concentrations following prolonged aerobic activity.

In the present study, E_2 concentration was approximately four times greater in the ML than MF. These results fell within expected values for the ELISA biochemical procedures used in analysis (MF levels typically range from 30-100pg$\cdot$mL^{-1} and ML levels typically range from 60-400pg$\cdot$mL^{-1}) (Human E_2, Abnova, Walnut, CA & Taiwan, China). Also, the difference in E_2 concentration between hormonal conditions measured in this study agrees with other studies investigating the effect of estrogen on inflammatory markers in eumenorrheic women [16,17].

The running speed of the participants within each of the 60-minute treadmill runs corresponded to an average intensity of 61.7 ± 5.0 % VO$_2$peak during the MF phase and 59.7 ± 2.8 % VO$_2$peak during the ML phase, slightly lower than the desired intensity of 65% VO$_2$peak. However, the mean VO$_2$ responses between hormonal conditions were similar at each time measurement in both of the 60-minute exercise sessions and did not differ. The VO$_2$ (and HR) measurements slightly increased from the start to end of the treadmill run due to the manual adjustments in running speed in an attempt to meet target VO$_2$ levels, and as the result of the cardiac drift phenomenon [18].

Ratings of perceived exertion (RPE) in each of the 60-minute running sessions were nearly identical between hormonal conditions too; although, during both experimental running sessions the RPE did gradually increase which is a typical response. The similar findings of RPE between the two experimental sessions are somewhat at odds with the literature. Previous research has shown an increase in exercise RPE during the ML phase. This is speculated to be due to the increase in body temperature during this phase, which negatively affects a woman's capability of maintaining thermal balance, which can in turn increase the perception of the difficulty of the exercise [19].

Overall, the VO$_2$, HR, and RPE measurements obtained in each of the 60-minute exercise sessions were in line with expected physiological outcomes caused by prolonged aerobic exercise and were very similar between the hormonal conditions [12,13]. This suggests that any differences in blood levels of CK or CK-MB response observed in this study were the result of differences in hormonal condition and not differing exercise conditions.

A key finding was that the CK response observed at 24 hour post-exercise was significantly greater in MF (low E_2) than ML (high E_2). These results are similar and relate to those of Carter et al. who found

naturally elevated estrogen levels during the ML had a protective effect on muscle following eccentric exercise [16]. These and the current results also are in line with previous research supporting the notion that high levels of estrogen attenuate inflammation that causes delayed onset muscle soreness (DOMS) after exercise [20]. To this end, we conducted an exploratory analysis using Spearman correlation procedures (we knew the small sample size prevented the use of correlations as a primary analysis). This analysis was conducted on the change in resting E_2 between the two 60 minute exercise runs and the change in the degree of CK after each exercise run. The Spearman rho value was -0.701 ($p<0.02$), supporting that the change E_2 associated with the change in CK response.

In skeletal muscle, studies on animals have shown that estrogen may potentially prevent the release of CK into the bloodstream [21,22]. The mechanism by which estrogen reduces muscle damage and CK release is not fully understood, but it is thought to be due to estrogens exhibiting antioxidant properties, acting as a membrane stabilizer, and its ability to bind to estrogen receptors and regulate downstream genes inducing decreased inflammation [22].

Exercise did not induce CK-MB changes nor did the response vary with E_2 levels. CK-MB is an indicator of myocardial damage as essentially only myocardium tissue has substantial amounts of this biomarker present. Therefore, this lack of a significant increase in CK-MB response to the exercise suggests that the increase seen in CK levels 24 hours post-exercise was a result of skeletal inflammation and not cardiac muscle damage (i.e., a small component of the CK response is made up of the CK-MB sub-fraction). This notion is supported by the fact that within our calculated relative index ratio values (CK-MB/CK) of our subjects were between 1 and 2 (data not reported), and the literature supports that a ratio less than 3 is consistent with skeletal muscle source of tissue damage [10]. These

CK-MB results, however, are not entirely in line with some previous research. One study on long-distance runners that found elevated CK-MB levels 1, 24, 48, and 72 hours after a marathon race in men [23,24]; while, another study in rats found that after 3.5 hours of swimming with an 8% bodyweight workload attached to their tails, CK-MB concentrations significantly increased [25]. But, these latter studies involved substantially greater amounts of exercise than what was done in the present study, which may explain why we saw no CK-MB response.

A limitation of the present study is blood samples were collected for only 24 hours into recovery, and some researcher's support blood collections should proceed up to 72 hours [23,24]. But, Totsuka et al. [26] have shown that if differences exist at 24 hourr they will persist and typically remain through 72 hour. Thus, our interpretation of our findings would remain unchanged even if sample collection had been extended.

CONCLUSIONS

In conclusion, the muscle damage biomarker CK was significantly increased 24 hourrs post-exercise during periods of low E_2 (MF phase). The lack of a significant increase in CK-MB levels following exercise suggests that this increase in CK is from skeletal inflammation not from cardiac muscle damage. The significant interaction effect for hormonal condition and time for the skeletal inflammatory marker CK suggest that women should consider ingesting an NSAID while training during their MF phase (low E_2) to alleviate exercise induced inflammation.

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