Effects of the Menstrual Cycle and Acute Aerobic Exercise on Cytokine Levels

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Abstract

Objective: The purposes of this study were: 1) to investigate the effects of menstrual cycle on the basal levels of salivary cytokines (interleukin (IL)-6 and IL-8) in young healthy women; 2) to examine the influences of prolonged aerobic exercise at ventilatory threshold (VT) on salivary and circulating IL-6 and IL-8 concentrations during menstrual phase.

Methods: Eight healthy women participated in three visits (saliva sampling during menstrual, follicular and luteal phases) and two trials (exercise and resting trials during a separate menstrual phase). The exercise trial involved a 60-min cycling at VT and the resting trial involved resting for 90 min. Saliva and blood samples were taken at before, immediately after and 30 min post-exercise and equivalent times during the resting trial.

Results: Salivary IL-6 level was higher during the follicular phase than the luteal phase at rest. Salivary IL-8 immediately post-exercise was higher than pre-exercise, and circulating IL-6 was significantly increased from pre-exercise to immediately post-exercise and 30 min post exercise.

Conclusion: The results showed that salivary cytokines are influenced by menstrual cycle and prolonged exercise during menstrual phase. However the levels of IL-6 and IL-8 in saliva do not closely reflect those in plasma, and therefore might reflect a local level of inflammation.

Keywords: Menstrual cycle; Inflammation; Ventilatory threshold; Endurance exercise; Interleukin-6; Interleukin-8

Introduction

It has been shown that physical and psychological stresses are associated with the phases of the menstrual cycle [1,2]. Only few studies have examined the relationship between stress level and inflammation status during different phases of menstrual cycle using biological stress markers and cytokines such as cortisol and interleukin (IL)-6, respectively [1-3]. Cortisol is a well-known biological stress marker whereas IL-6 is a multifunctional cytokine that is secreted by endometrial cells, immune cells and muscle cells [4,5] and is a major factor in the regulation of the inflammatory process, immune response, metabolism and the acute phase response [5,6]. In the previous studies, healthy women with a normal menstrual cycle demonstrated higher serum cortisol levels during luteal phase compared with the follicular phase [1] yet plasma IL-6 levels were higher during the follicular phase compared with the luteal phase [2].

On the other hand, other studies report similar serum IL-6 levels between follicular and luteal phases [3]. To date, no studies have examined markers of inflammation specifically during the menstrual phase of the cycle. This is because the menstrual phase has been considered as a part of the follicular phase [2,3]. Female sex hormones, especially progesterone, influence immune responses by decreasing basal IL-6 levels [2]. Moreover, menstruation, initiated by progesterone withdrawal, is highly inflammatory in nature and involves feedback loops that enhance inflammatory pathways [7]. Therefore, it is important to specifically focus on menstrual phase as a distinct phase.

Recently, saliva sampling has received attention as an alternative to blood sampling to measure stress markers [8-11]. Saliva collection has the advantage over blood sampling in that it is a non-invasive method of sample collection which does not require any specialized training. However, the influence of menstrual cycle on stress markers using saliva has received comparatively little attention. In healthy women with a normal menstrual cycle, saliva samples were taken every evening to compare cortisol levels among menstrual, follicular, ovulatory, luteal and premenstrual phases and the analysis revealed that salivary cortisol level did not differ across the phases [12]. In another study, female participants provided a saliva sample directly after awakening and 30, 45 and 60 min thereafter to measure cortisol levels during the menstrual phase, follicular phase, ovulation and luteal phase. The results showed that there was a significant interaction between time and menstrual cycle and the net increase in cortisol level was larger during the ovulation than during the menstrual, follicular and luteal phases [13,14]. It is well known that saliva cortisol exhibits diurnal variations with concentrations highest in the morning and this may account for the disparities in the apparent influence of menstrual phase on cortisol levels between these studies.

Several studies have examined salivary IL-6 and/or IL-8 concentrations in relation to systemic or local disease conditions including oral cancers [15,16] and Sjögren’s syndrome [15], an autoimmune disease that affects mucosal exocrine functions. Other studies have assessed the relationships between salivary IL-6 levels and...
physiologic factors in middle-aged people [17] and in postmenopausal
women [18]. Therefore, there is a lack of information regarding salivary
IL-6 and IL-8 concentrations on resting healthy women during different
phases of menstrual cycle. In addition, previous studies reported that
salivary and circulating IL-6 levels do not relate to each other at rest
[15,17,18] and at post-exercise [10]. Such findings need to be confirmed
in healthy women at different timings of menstrual cycle to demonstrate
whether salivary sampling can be employed as an alternative to blood
sampling for an examination of inflammation status.

Moderate intensity aerobic exercise is important for maintaining or
promoting physical fitness. However, exercise has been shown to induce
an inflammatory response and estrogen is associated with an attenuation
of the response [19]. Moreover, the secretion of estrogen changes during
the menstrual cycle yet reported circulating inflammatory responses
to exercise during the different phases of the menstrual cycle are equivocal [20], which may reflect differences in participant fitness, oral
contraceptive use and differing exercise protocols used. Considering
women’s health and safety and the effectiveness of exercise, it is
important to assess the influence of exercise or changes in the stress and
inflammatory markers during the menstrual cycle [21]. Only one study
has examined an influence of prolonged moderate intensity exercise on
salivary IL-6 and reported that salivary IL-6 level was higher at 30 min
post treadmill running (1 h at 65% VO2peak) compared to the baseline
level during follicular and luteal phases in females [22]. However, the
magnitude of increases did not differ between the follicular and the
luteal phases. Hence, an influence of exercise on inflammation status
during menstrual phase is still unclear.

Therefore, the aims of this study were: 1) to investigate the effects
of menstrual cycle on the basal levels of salivary cytokines (IL-6
and IL-8) in young healthy women; 2) to examine the influences of
prolonged aerobic exercise at ventilatory threshold (VT) on salivary
and circulating IL-6 and IL-8 levels during menstrual phase. It was
hypothesised that: 1) basal salivary cytokine levels would be affected
by menstrual cycle; and 2) salivary cytokine levels would be affected by
acute aerobic exercise at VT.

Materials and Methods

Subjects

Eight healthy young women enrolled in this study (age: 20.5 ± 0.8
years (means ± standard deviation (SD)), body mass: 48.3 ± 5.9 kg,
height: 155.4 ± 4.9 cm, BMI: 20.0 ± 2.2 kg/m², estimated body fat: 23.7
± 4.0 % and VO2peak: 1790 ± 281 ml/min). None of them were taking
any medications including oral contraceptives or dietary supplements.
The intra-assay coefficients of variation
for all participants and they were free to withdraw from the study at
any time. The experimental procedure was approved by the Research
Ethics Committee at Waseda University.

Experimental design

The subjects attended the laboratory on seven separate occasions
for two preliminary trials, three short visits and two main trials. Two
preliminary trials were arranged for orientation, to familiarize the
subjects with the experimental procedures and to determine their
VT, height, body mass, and estimated body fat (OMRON BODY FAT
MONITOR HBF-903, Omron Healthcare, Kyoto, Japan). The visits
took place during the menstrual phase (day 1-4 from the start of
menstrual flow), follicular phase (day 7-11 after the start of menstrual
flow) and mid-luteal phase (5-9 days before estimated date of the next
menstrual phase) and main trials were conducted during menstrual
phase. The subjects refrained from performing any strenuous physical
activity and drinking alcohol for at least 24 h prior to the testing trials,
and consumed a small meal at 12:00 pm before the trials. To control
for diurnal variations in the hormones, all trials were performed at the
same time of day from 2:00 to 3:00 pm.

Determination of VT

In a preliminary session, peak oxygen uptake (VO2peak) was
estimated using a continuous incremental exercise test performed on
an electromagnetically braked cycle ergometer (AEROBIKE 75XL, Combi,
Shinagawa, Tokyo, Japan). Oxygen consumption, ventilation and heart
rate were measured during an incremental cycle ergometry test (2
Watts increase every 10 seconds, starting at 20 Watts) with a bicycle
ergometer. Ventilation was measured and gas analysis was performed
during the test on a breath-by-breath basis (AEROMONITOR AE-
300S, Minato, Yodogawa, Osaka, Japan). VT was determined by the
modified V-slope method, which is from a plot of VCO2 as a function
of VO2 [23].

Visits and main trials

For the three visits, the subjects reported to the laboratory in a
rested state during the menstrual phase, follicular phase and mid-luteal
phase to provide saliva and blood samples. For the main trials, subjects
reported to the laboratory at 2:00 pm for the exercise and control trials
in a separate menstrual phase. In the exercise trial, subjects cycled for
60 min at the predetermined VT from 2:30 pm and they seated quietly
for 30 min after the cycling. Saliva and blood samples were taken at
pre-, immediately post- and 30 min post-exercise. Subjects drank water
ad libitum until the day of assays. All salivary samples were centrifuged again on the day of
assay to remove particulate matter.

Sample collection and analyses

Each participant was instructed to empty her mouth of pre-existing
saliva before sampling. One and a half ml of unstimulated saliva was
collected before blood sampling by passive drool for determination of
hormone and cytokine levels. Saliva samples were centrifuged at 3,000
rpm for 15 min at room temperature and stored at -30°C until the day
of analyses. All salivary samples were centrifuged again on the day of
the assay to remove particulate matter.

Capillary blood samples were obtained from the fingertip into at
least three heparinized capillary tubes for hematocrit measurement.
The capillary tubes were immediately centrifuged at 12,000 rpm for
5 min at room temperature. Hematocrit was measured to confirm
changes in plasma volume and hydration status. After the hematocrit
measurement, the plasma was put into microtubes and stored at -30°C
until the day of assays.

To confirm the menstrual cycle, the hormonal status was
determined by measuring salivary 17β-estradiol and progesterone
concentrations [24]. The salivary 17β-estradiol, progesterone, cortisol
and Dehydroepiandrosterone (DHEA) concentrations were measured
using high sensitivity enzyme immunoassay (EIA) kits (Salimetrics
LLC, Sate College, PA, USA). The intra-assay coefficients of variation
for 17 β-estradiol, progesterone, cortisol and DHEA were 8.1%, 6.2%,
3.6 and 5.5%, respectively. The salivary amylase activity was measured using an enzymatic method (AMYLASE MONITOR, Nipro Co., Osaka, Japan) [25]. The saliva concentrations of IL-1β and TNF-α were measured using Quantikine high sensitivity (HS) enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). The intra-assay coefficients of variation for IL-1β and TNF-α were 4.4 and 8.5%, respectively. The saliva concentrations of protein were measured by the Bradford method using a protein assay kit purchased from Bio-Rad (Hercules, CA, USA) [26]. Resting and exercise-induced salivary and plasma concentrations of IL-6 were measured using Quantikine HS ELISA kits (R&D Systems, Minneapolis, MN, USA) and IL-8 was measured using OptEIA kits (Becton Dickinson Bioscience, San Diego, CA, USA). The intra-assay coefficients of variation for IL-6 and IL-8 were 7.8 and 5.5%, respectively, and inter-assay coefficients of variation were 14.2 and 10.0%, respectively. These measurements were performed with a microplate reader (VERSAMAX, Molecular Devices Inc. Sunnyvale, CA, USA). Plasma and saliva samples from each individual subject were tested in the same assay run.

Statistical analyses

A one-way analysis of variance with repeated measures was used to assess the differences in basal salivary hormone and cytokine levels among the menstrual, follicular and luteal phases. A two-way (trial x time) analysis of variance with repeated measures was used to examine the differences in cytokine levels. When a main effect was observed, post hoc tests using Bonferroni adjustment was employed for multiple comparisons. Normality plots with tests (Shapiro-Wilks) were performed and log transformed before statistical analysis when data were not normally distributed. After log transformation, data were investigated again and considered to be normally distributed. Effect sizes (η2) were calculated using guidelines of Cohen [27], and were considered as follows: 0.01 = small effect; 0.06 = moderate effect; and 0.14 = large effect. Data are presented as mean ± SD. Spearman’s correlation coefficients were employed to assess the relationships between the salivary and plasma values. Statistical significance was set at P<0.05. Statistical analyses were performed using PASW Statistics 18 software (SPSS, Chicago, IL, USA).

Results

Menstrual cycle effects

The descriptive data for resting salivary hormones and plasma cytokines during each menstrual phase are presented in Table 1. Salivary 17β-estradiol concentration was not different between menstrual and follicular phases, however, the concentration increased by 41% from follicular phase to luteal phase (Table 1). Salivary progesterone concentration was increased by 156% from menstrual phase to luteal phase and 177% from follicular phase to luteal phase (Table 1).

Salivary IL-6 concentration significantly differed between different phases of menstrual cycle (P = 0.023, ηp² = 0.42). When Bonferroni adjustment was conducted, salivary IL-6 concentration was significantly higher during the follicular phase compared with the luteal phase (P = 0.036) (Figure 1). Salivary TNF-α, IL-1β and IL-8 concentrations tended to decrease from the menstrual phase to the luteal phase but the concentrations were not significantly affected by menstrual cycle (TNF-α: P = 0.059, ηp² = 0.33, IL-1β: P = 0.474, ηp² = 0.10, IL-8: P = 0.256, ηp² = 0.18) (Figure 1).

Similarly, plasma IL-6 and IL-8 concentrations tended to decrease from the menstrual phase to the other phases but these were not significantly affected by menstrual cycle (IL-6: P = 0.153, ηp² = 0.18, IL-8: P = 0.180, ηp² = 0.16) (Table 1). There were no significant relationships between salivary and plasma IL-6 concentrations and between salivary and plasma IL-8 concentrations.

Salivary cortisol, DHEA concentrations and salivary amylase activity at rest were unaffected by the menstrual cycle (Table 1).

Exercise effects

During the 60-min cycling at VT, subjects cycled at 63.3 ± 9.3 Watts, corresponding to 78.5 ± 15.4% HRmax (using Karvonen formula). The changes in salivary and plasma IL-6 and IL-8 concentrations during the exercise and control trials are presented in Figure 2. A significant interaction (trial by time) was found in plasma IL-6 (P = 0.001). Immediately and 30-min post-exercise plasma IL-6 concentrations were significantly higher than that of pre-exercise (pre: 0.92 ± 1.03 pg/mL, immediately post-exercise: 1.85 ± 1.23 pg/mL, 30 min post-exercise: 2.38 ± 2.43 pg/mL, P = 0.001) (Figure 2). No significant time and trial effects were found in plasma IL-8, salivary IL-6 and protein. However, a significant interaction (trial by time) was found in salivary IL-8 (P = 0.004). Immediately post-exercise salivary IL-8 concentration was significantly higher than that of pre-exercise (P =

| Salivary hormone   | Menstrual | Follicular | Luteal |
|--------------------|-----------|------------|--------|
| 17β-estradiol (pg/mL) | 2.43 ± 0.70 | 2.51 ± 0.60 | 3.53 ± 1.18 |
| Progesterone (pg/mL)   | 105.21 ± 49.21 | 97.23 ± 37.30 | 269.56 ± 145.35 |

| Stress marker     | Cortisol (pg/mL) | DHEA (pg/mL) | Amylase activity (KIU/L) | Plasma cytokine |
|-------------------|------------------|--------------|-------------------------|----------------|
|                   | 0.19 ± 0.13      | 174.60 ± 115.99 | 67.50 ± 52.37          | IL-6 (pg/mL) |
|                   | 0.20 ± 0.11      | 258.68 ± 282.79 | 60.50 ± 33.95          | 1.23 ± 0.90 |
|                   | 0.23 ± 0.14      | 149.19 ± 55.29 | 60.13 ± 61.33          | 5.38 ± 2.65 |
|                   |                  |              |                         | 2.38 ± 2.43 |

| Figure 1: Salivary inflammatory cytokine data at baseline with regard to menstrual cycle phases. Data are presented as means ± SE (n = 8). IL-6: Interleukin-6, IL-8: Interleukin-8. * P < 0.05 follicular versus luteal phase; * P < 0.05 menstrual versus luteal phase.

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Table 1: Salivary hormone, stress marker and plasma cytokine data at baseline with regard to menstrual cycle phases. Data are presented as means ± SD (n = 8). DHEA: Dehydroepiandrosterone, IL-6: Interleukin-6, IL-8: Interleukin-8. * P < 0.05 follicular versus luteal phase; * P < 0.05 menstrual versus luteal phase.
No significant relationships were found between the concentrations of saliva and plasma IL-6 and IL-8 (IL-6: \( r = 0.366, P = 0.019 \)). In the current study, salivary IL-6 level showed small (1.9 fold) insignificant decreases from menstrual phase to luteal phase, but no differences were observed between the follicular phase and luteal phase. However, Angstwurm et al. [2] reported that plasma IL-6 decreased from follicular phase to luteal phase. This may be related to the timing of sample collection during the menstrual cycle. This conflicting result could be attributable to the current study having used three phases of the menstrual cycle (menstrual, follicular and luteal) as opposed to two phases (follicular and luteal) used in the study by Angstwurm et al. [2].

However, no significant relationships were found between saliva and circulating IL-6 (and IL-8) concentrations in recreationally active healthy women at rest in the current study. Previous studies agree with the current study as they reported that salivary IL-6 level was not related to circulating IL-6 at rest and post-exercise conditions in patients and mixed gender athletes [10,15,17,18]. Moreover, it has been discovered that IL-6 mRNA are up regulated in salivary glands of mouse models [28] and in humans [29]. Furthermore, salivary IL-6 (and IL-8) has been used to assess immunological patterns relevant to systemic or local disease conditions and its levels were altered in patients with oral health problems [15,16,30]. Therefore, salivary IL-6 is possibly reflecting a local level of inflammation and as such it is understandable that saliva IL-6 concentrations are not a good reflection of circulating values.

In the present study, salivary IL-6 level was the lowest when concentrations of female sex hormones (17\(\beta\)-estradiol and progesterone) were the highest (luteal phase). This is possibly because female sex hormones reduce inflammations as previous studies reported that estradiol showed an anti-inflammatory action and down regulated IL-6 in vitro [31,32].

All other salivary and plasma cytokines were not significantly affected by menstrual cycle. However, salivary levels of TNF-\(\alpha\), IL-1\(\beta\) and IL-8 tended to decrease between the menstrual and luteal phases. Although a significant difference was not found, moderate or large effect sizes were demonstrated for these relationships, perhaps suggesting that these markers are also worthy of future attention.

In the present study, cortisol and DHEA in saliva and salivary amylase activity did not significantly differ among the menstrual cycle phases. Generally, these hormones have been used as stress markers [11,25,33]. Bernstein et al. [34] investigated an association between sympathetic responsiveness and the serum concentration of IL-6 in young women. However, changes in salivary amylase activity, an index of sympathetic tone according to menstrual cycle was not observed in the current study. Therefore, salivary IL-6 concentration at rest seems to reflect the inflammatory conditions which is influenced by menstrual cycle, but not influenced by sympathetic tone.

Exercise effects

In the present study, there were no differences in salivary IL-6 concentration among before, immediately after and 30-min post cycling at VT during menstrual phase in recreationally active women with a normal menstrual cycle. However, a previous study reported that salivary IL-6 concentration increased after a prolonged moderate intensity exercise in sedentary women [22]. In the previous study, salivary IL-6 concentration increased significantly from baseline to 30 min post 1 h treadmill running at 65% VO\textsubscript{2peak} during follicular and luteal phases. Potential rationales for the different outcomes are an employment of different exercise intensity and conducting the studies during different phases of menstrual cycle. In general, 65% VO\textsubscript{2peak} is higher than VT and ventilation rates increase at higher exercise intensities which may lead to increased airway inflammation. Hence, higher exercise intensities may be linked with a greater local inflammation which is reflected in an increased concentration of salivary IL-6. Moreover, resting salivary IL-6 concentration is the highest during menstrual phase compared to the other phases as observed in the current study; this may limit further elevations in saliva IL-6 concentration in response to exercise during menstrual phase.

In the current study, salivary IL-8 concentration significantly increased from pre- to post-exercise. In contrast to the salivary IL-8 concentration, circulating IL-8 concentration did not change throughout the trial. IL-8 is a neutrophil chemotactic factor, released by immune cells and endothelial cells to recruit neutrophils to sites of tissue damage (i.e. inflammation) [35-37]. Hence, the increase in salivary IL-8 observed in the present study after exercise may also simply reflect local inflammation due to increased ventilatory rates with exercise. Currently, there are no other studies which examined an influence of exercise on cytokine levels during menstrual phase using both salivary and circulating IL-8 concentrations. Therefore, further research is required to confirm the findings from the present study.
In conclusion, salivary cytokines (IL-6) is influenced by menstrual cycle at rest in recreationally active healthy women. Moreover, salivary IL-6 and IL-8 responded differently to both the menstrual cycle and to exercise compared with their counterparts in plasma. Therefore saliva sampling should not be considered as a non-invasive alternative to blood sampling for examining the inflammation status in women as it may simply reflect local inflammatory events related to increased ventilatory rates.

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