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MENSTRUAL CYCLE AND THE VASCULAR PROPERTIES OF REMOTE
PRECONDITIONING

BY

SHELBY RACHEL

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Nutrition and Exercise Sciences

Specialization in Nutritional Sciences

South Dakota State University

2019

MENSTRUAL CYCLE AND THE VASCULAR PROPERTIES OF REMOTE
PRECONDITIONING

SHELBY RACHEL

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Nutrition Exercise Sciences and is acceptable for meeting thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidates are necessarily the conclusions of the major department.

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CONTENTS

ABBREVIATIONS.....	v
LIST OF TABLES AND FIGURES.....	vi
ABSTRACT.....	vii
INTRODUCTION.....	1
METHODS.....	4
RESULTS.....	11
DISCUSSION.....	15
LITERATURE CITED.....	19

ABBREVIATIONS

I/R ischemia/reperfusion

IPC ischemic preconditioning

FBF forearm blood flow

CVD cardiovascular disease

LIST OF TABLES AND FIGURES

Figure 1. Experimental Protocol.....	7
Table I. Subject Characteristics.....	11
Table II. Heart Rate and Blood Pressure.....	12
Figure 2-A. Peak Forearm Blood Flow.....	13
Figure 2-B. Total Forearm Blood Flow Area Under Curve.....	13
Figure 3. Forearm Blood Flow and Reactive Hyperemia.....	14

ABSTRACT

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PRECONDITIONING

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PURPOSE: A promising therapeutic intervention to counteract endothelial ischemia/reperfusion (I/R) injury is ischemic preconditioning (IPC). Limited data from ovariectomized animal models shows that estrogen deficiency abolishes the cardioprotective effects of IPC, whereas estrogen replacement restores protection. This suggests that alterations in female sex hormones, particularly estrogens, that occur naturally during the menstrual cycle is likely to influence the vascular protective properties of IPC. The aim of the present study was to identify how changes in female sex hormones that occur naturally during the menstrual cycle interfere with IPC to provide vascular endothelial protection in women.

METHODS: Nine healthy premenopausal eumenorrheic women (age 21 ± 1 yr) not taking any contraceptive medications were studied in a within-subjects design. Primary outcomes with respect to the assessment of vascular endothelial function were measured during the early follicular (after onset of menses; days 1-6) and at mid-cycle during ovulation (days 10-14 after positive urine ovulation test). Endothelium-dependent vasodilation was assessed by the forearm blood flow (FBF) response to reactive hyperemia using strain-gauge venous occlusion plethysmography in the absence and presence of endothelial I/R injury (20 min

brachial artery ischemia followed by 15 min reperfusion) when preceded by remote IPC (right arm: 3×5 min cycles of ischemia).

RESULTS: In the absence of endothelial I/R injury, peak FBF to reactive hyperemia was similar ($P=0.19$) between the early follicular (19.9 ± 6.1 ml/100ml tissue/min) and ovulation phases (22.1 ± 5.9 ml/100ml tissue/min). In contrast, there was a significant main effect ($P=0.001$) of menstrual cycle phase on the capacity of IPC to protect against endothelial I/R injury. For example, during the early follicular phase, peak FBF was significantly ($P=0.02$) diminished 15% with endothelial I/R injury (from: 19.9 ± 6.1 to 17.1 ± 4.1 ml/100ml tissue/min) despite remote IPC. As a result, total FBF during the initial 30 sec of reactive hyperemia (area under the curve) was decreased ~18% ($P=0.027$) after (41.0 ± 11.4 ml/100ml tissue/min) compared with before (49.6 ± 15.1 ml/100ml tissue/min) endothelial I/R injury. However, during ovulation, remote IPC provided a level of endothelial protection from I/R injury that was not observed in the early follicular phase. Indeed, in the presence of endothelial I/R injury, peak FBF increased 15% (from: 22.1 ± 5.9 to 25.3 ± 6.4 ml/100ml tissue/min; $P=0.012$). As a result, total FBF was well preserved ($P=0.859$) after (58.2 ± 14.6 ml/100ml tissue/min) compared with before (58.0 ± 13.3 ml/100 ml tissue/min) endothelial I/R injury.

CONCLUSIONS: These data suggest that the menstrual cycle differentially influences the capacity of remote IPC to protect against endothelial I/R injury. Greater circulating estrogens during ovulation may be an important mediator contributing to the protective benefits of remote IPC in women.

INTRODUCTION

The most recent data from the American Heart Association states that the prevalence of cardiovascular disease (CVD) in adult women increases with age. For women aged 20-39, 40-59, 60-79 and 80 plus, the prevalence is 11.5%, 39.4%, 68.6% and 86.5% of the population, respectively [1]. In fact, each year nearly 7 million American women develop ischemic heart disease, and of these ~162,000 will die, mostly from acute myocardial infarction. Data in younger women (<55 years of age) indicate that the annual frequency of hospitalizations for myocardial infarction is estimated to be more than 30,000, and this rate increased 2% from 1997 to 2006, mostly in women in their 40s [2]. Moreover, women less than 65 years die more often in the first 30 days after myocardial infarction compared with men, and are more likely to be readmitted for cardiovascular complications with the first month of hospital discharge [3].

Compared with men of similar age, younger women, particularly premenopausal women, more frequently present with plaque erosion versus rupture, less epicardial obstruction, and more extensive coronary microvascular disease [3, 4]. These differences are touted as some of the main reasons why women are less likely to receive timely reperfusion therapy for myocardial infarction and when they do, often have worse outcomes in contrast to men [5-7]. For example, despite successful coronary revascularization with thrombolytic therapy, PCI, or CABG, women develop more severe bleeding complications [8], increased postoperative infarction, greater rates of in-hospital mortality, and non-

cardiac organ injury [2]. In women less than 50 years of age, it is estimated that the risk of death following CABG is 3 times higher than men of similar age [9].

It is logical, therefore, to suggest that despite successful reperfusion therapy, the greater clinical consequences in women stem in part from more severe vascular endothelial ischemia/reperfusion (I/R) injury [4, 8]. In women, we know that I/R injury is worse during the menopause transition when the vascular protective effects of estrogen are lost [10]. What is not clear is how the different phases of the cycle in premenopausal women modulate this injury and interventions to prevent it. Certainly, the menstrual cycle is associated with changes in vascular endothelial function that may intensify injury and interfere with protective therapies. For example, the capacity of the endothelium to increase blood flow (i.e., dilation), a strong predictor of vascular health, is vulnerable to reperfusion damage. In the early follicular phase (when estrogen is lowest), dilation is impaired compared with the high estrogen late follicular phase [11]. Vascular function is also decreased in the early luteal phase (when progesterone is highest) and recovers when progesterone levels decline [12]. As such, the menstrual cycle provides a unique way to gain insight into how changes in female sex hormones impact strategies to reduce I/R injury.

Our tissues have the natural ability to protect against I/R injury through a process known as remote ischemic preconditioning (IPC). Remote IPC triggers tissue protection by exposing an arm to small cycles of arterial occlusion. Mediators released from the preconditioned tissue are released into the circulation and culminate on the remote region exposed to I/R injury to active

cytoprotective signaling. Experimental studies indicate that one of the major players with protection afforded by IPC is estrogens. Animal studies show that low levels of estrogen weaken protection caused by IPC, whereas estrogen replacement restores it [13, 14]. No human studies have been undertaken to determine how phases of the menstrual cycle affect protection induced by remote IPC. Alterations in female sex hormones, particularly estrogens, that occur naturally during the menstrual cycle is likely to influence the vascular protective properties of IPC.

The specific aim of this research was to determine how the early and late follicular phases of the menstrual cycle influences the ability of remote IPC to provide vascular endothelial protection against I/R injury in women. We hypothesize that remote IPC will not work as well in the early phase when estrogen is low but will recover in the late follicular phase when estrogen rises.

METHODS

Subjects

Nine sedentary, healthy, non-medicated, eumenorrheic premenopausal women with no history of pregnancy were studied. Women were included if they reported having regular menstrual cycles for the past year and not taking any contraceptive medications. Women were recruited from the ~6,700 women University students using recruitment flyers posted in the local area and sent via email invitation to the South Dakota State University faculty/staff and student body. Interested participants were e-invited to call/email the investigative team to discuss the nature, risks, and benefits of the project. During the initial phone call/email, a medical history screening form was completed to determine if subjects met eligibility standards. The screening form included contact information and general demographic information (e.g., age, sex, name, and racial/ethnic background) and health information (e.g., history of chronic disease, medication use, and exercise habits, etc.). Before participation, informed consent was obtained from all participants included in the study. This study was submitted to and approved by the Institutional Review Board for the Protection of Human Subjects at South Dakota State University.

Screening Procedures

Blood Pressure measurements

Non-dominant arm auscultatory resting systolic and diastolic blood pressure was measured using an appropriately sized stethoscope and sphygmomanometer

(Diagnostic 700 Series, American Diagnostic Corp, Hauppauge, NY) following 5 min of seated quiet rest using standard procedures. Resting blood pressure measurements were performed twice and separated by 3 minutes and averaged. Resting heart rate was measured using a 60 second radial pulse count.

Anthropometric measurements

Standing height and body weight were measured with a digital scale (Seca 876 digital scale, Seca Corporation, Hamburg, Germany). Abdominal waist circumference was assessed with a Gulick tape measured at the smallest part of the abdomen, above the umbilicus and below the xiphoid process to the nearest 0.1 cm at the end of normal expiration using standard procedures. Waist measurements were completed twice and averaged. Percent body fat was estimated by air displacement plethysmography (BODPOD, COSMED USA Inc., Illinois). Body mass index (BMI) was calculated as weight (kg)/height (m²).

Cardiorespiratory fitness

Estimates of cardiorespiratory fitness were determined using an 8-minute brisk treadmill walk. This walking test is suitable for low risk, apparently healthy adults. The first few minutes of this test consisted of placing a heart rate monitor and wristwatch on the subject to measure the intensity of the walk. Subjects walked at a brisk self-selected walking pace that equates to 50-75% of estimated maximum heart rate. The first 4 minutes of the walking test were at 0% inclination. Thereafter, the ramp of the treadmill was increased to 4% and the

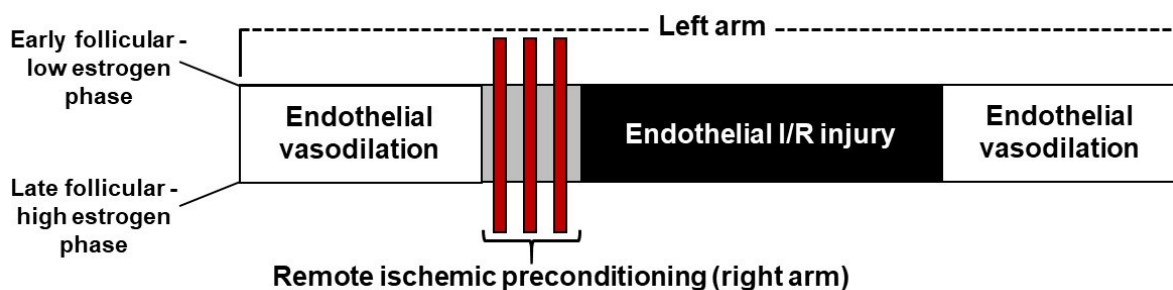
subject maintained the same walking speed for four more minutes. The test was completed in 8-9 minutes following steady-state heart rate. A 3-minute cool-down at a slow walking speed was then completed.

Experimental Design

Determination of menstrual phase

Figure 1 highlights the main experimental protocol. Utilizing a within-subjects crossover design, primary outcomes were assessed during the early follicular (after onset of menses; days 1-6) and at mid-cycle during the late follicular phase (days 10-14; after positive ovulation test; urine ovulation prediction kits, Clearblue Advanced Digital Ovulation, Inverness Medical Innovations, Inc.). The Clearblue system is a common, accurate over-the-counter test kit that uses a urine sample to measure reproductive hormones in 3 minutes (similar to urine pregnancy tests). Current day of the cycle was recorded during the initial screening visit. Subjects were then scheduled for the assessment of endothelial function during the closest cycle phase. As a result, for the assessment of the primary outcome of endothelial function, 6 subjects completed the study during the early follicular phase first, whereas 3 subjects completed it during ovulation first.

Figure 1. Overview of the experimental protocol



Endothelium-Dependent Vasodilation

All procedures will take place in a temperature-controlled (22.5°C) ~500 ft² vascular protection research laboratory in the Department of Health & Nutritional Sciences. Prior to each session, subjects reported to the research laboratory following an overnight fast without caffeine (~10 hours) and were instructed to refrain from purposeful exercise >24 hr prior to the studies.

The capacity of the resistance arteries in the left arm to vasodilate was assessed by the FBF response to reactive hyperemia using strain-gauge venous occlusion plethysmography as previously described [15]. We have elected to use reactive hyperemia as our method to quantify endothelial function because it is most relevant to the physiological circumstances surrounding the abrupt increase in blood flow with I/R injury than other established methods (i.e., vasoactive drug infusions). With the forearm supported above the heart, a high-pressure cuff was inflated for 5 min to interrupt inflow of the brachial artery (DE Hokanson, Inc.). Cuff location and duration of ischemia were chosen to be consistent with established reactive hyperemia protocols by other investigators [16-18]. At 5 min of ischemia, the cuff was deflated. Immediately thereafter, inflation pressure of the same cuff was set to 50 mmHg at a 4:3 sec inflation:deflation cycle [18] to assess the reactive hyperemic response. At this cycle rate, we obtained one FBF measurement every 7 sec during the first min of hyperemia (~8 FBF recordings). Thereafter, a 7:7 sec inflation:deflation cycle was used for the remainder of hyperemia. The total duration for each assessment of endothelium-dependent vasodilation was 15 minutes. This procedure was completed at baseline and

repeated after endothelial I/R injury in the presence of remote IPC.

Remote IPC and Endothelial I/R Injury

Remote IPC was induced after the initial assessment of endothelial function (described above). Briefly, a high-pressure cuff was placed on the right upper arm and inflated to 220 mmHg (EC20 rapid cuff inflator, DE Hokanson, Inc., Bellevue, WA) for 5 min, followed by 5 min of deflation while the subject rested in a supine position. This procedure will be repeated two additional times. A 10 min washout was initiated following the last 5 min reperfusion phase. Immediately thereafter, a high-pressure cuff was inflated to 220 mmHg on the left arm for 20 min, followed by 15 min of reperfusion to induce endothelial I/R injury. The protocol of prolonged brachial arterial occlusion followed by reperfusion is a well-established method to show endothelial I/R injury in human subjects via declines in vascular function that can be alleviated by remote IPC (16). Endothelium-dependent vasodilation to reactive hyperemia in the left arm was repeated immediately following the 15 min reperfusion phase. Heart rate and blood pressure were monitored at baseline, after remote IPC and endothelial I/R injury, and finally at the end of the procedure.

Sample Size and Statistical Analysis

Chan et al. (2001) showed in 15 healthy women, that endothelial function was significantly enhanced during midcycle (flow-mediated dilation of $10.9 \pm 1.4\%$ compared with that during the early menstrual phase ($8.0 \pm 1.1\%$). The equates to

an absolute reduction of in reactive hyperemia of ~30% in the early follicular phase. Prior clinical studies with remote IPC and endothelial function indicates that endothelial I/R-injury blunts forearm vasodilation by 20-50%. Therefore, we applied a 30% decrease in reactive hyperemia-induced vasodilation following endothelial I/R-injury in the presence of remote IPC in women during the early follicular phase. Using this estimate, sample sizes were calculated based on 90% power at an alpha level of 0.05 to detect absolute mean reductions in peak reactive hyperemia vasodilation of 3.3% (i.e., flow-mediated dilation of 11% versus 7.7% after endothelial I/R injury). Corresponding effect sizes were 1.4. Resulting samples size was 12 subjects. Therefore, the selection of 12 women achieves 90% power to detect a mean two-tailed absolute difference of 3.3% in reactive hyperemia with an estimated standard deviation of 1%.

Data were checked for normality and spread and were normally distributed. Measures of central tendency were used to calculate subject characteristic data. The primary qualitative outcome was the difference in FBF before and after endothelial I/R injury between the early and late follicular phases of the menstrual cycle. Differences in peak FBF and total blood flow (area under the curve) responses to reactive hyperemia before and after endothelial I/R injury within menstrual cycle phase was determined by Paired T test. Differences in the capacity of remote IPC to protect against endothelial I/R injury between menstrual cycle phase were calculated as the percent change in peak FBF from baseline and analyzed by repeated measures ANOVA. Statistical significance

was set at $P < 0.05$. Data represent mean \pm SD. Data was analyzed using IBM SPSS Statistics Version 25.0 (Armonk, NY: IBM Corp).

RESULTS

As shown in table 1, participants were normal weight based on BMI, normotensive, and presented with average $\dot{V}O_2\text{max}$ values in 75-80th percentile for age and sex [19].

Table 1. Subject characteristics

Variable	Total Group (N=9)
Age, year	22 ± 1
Body mass, kg	69.1 ± 9.2
BMI, kg/m ²	21.4 ± 1.4
Body fat, %	26.3 ± 6.4
Waist circumference, cm	76.5 ± 7.9
$\dot{V}O_2\text{max}$, ml/kg/min	43.2 ± 4.9
Systolic BP, mmHg	119 ± 8
Diastolic BP, mmHg	67 ± 4
RHR, bpm	70 ± 9

Values are mean ± SD. BMI: body mass index; $\dot{V}O_2\text{max}$: maximal oxygen uptake; BP: blood pressure; RHR, resting heart rate.

As shown in Table 2, resting heart rate remained stable during the assessment of endothelial function in the early (P=0.89) and ovulation phases (P=0.78). There was no change in blood pressure during the early follicular (P=0.24) or ovulation phases (P=0.39). There were no significant heart rate (P=0.79) or blood pressure interactions between menstrual cycle phase (P=0.80).

Table 2. Heart rate and blood pressure at baseline, with remote IPC, and before and after endothelial I/R injury during the assessment of endothelial function in the early follicular and ovulation phases.

Heart rate, bpm				
	Baseline	Remote IPC	Endothelial I/R injury	Study end
Early follicular	67±10	60±6	60±9	59±9
Ovulation	69±11	61±8	59±8	60±8
Systolic blood pressure, mmHg				
	Baseline	Remote IPC	Endothelial I/R injury	Study end
Early follicular	124±10	115±8	117±11	120±10
Ovulation	121±12	115±6	110±8	122±12
Diastolic blood pressure, mmHg				
	Baseline	Remote IPC	Endothelial I/R injury	Study end
Early follicular	67±7	65±6	62±9	68±10
Ovulation	65±7	64±6	59±8	68±12

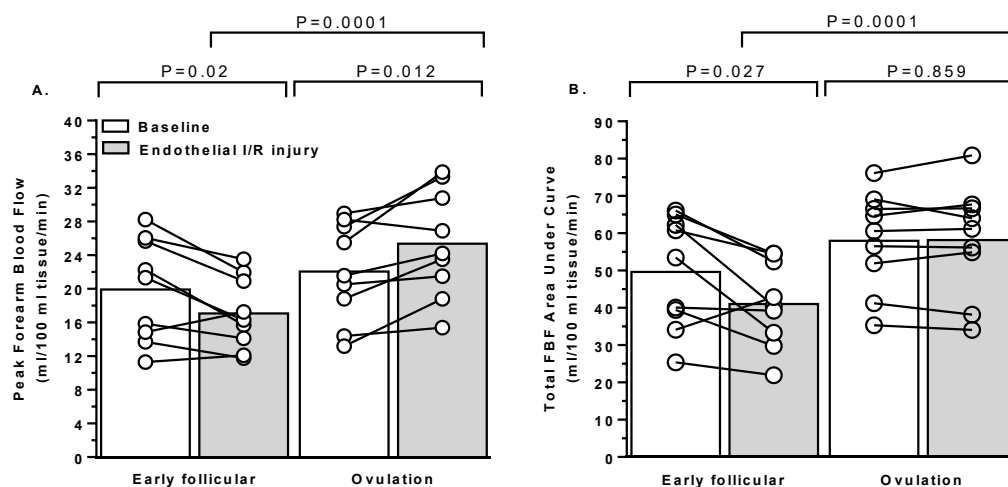
IPC: ischemic preconditioning; I/R: ischemia-reperfusion. Values are means±SD.

Menstrual Cycle and Endothelium-Dependent Vasodilation

Baseline resting FBF were similar between the early follicular phase (3.31±0.98 ml/100 ml tissue/min) and ovulation (3.53± 1.01 ml/100 ml tissue/min). As shown in Figure 2-A below, in the absence of endothelial I/R injury, peak FBF to reactive hyperemia was similar (P=0.19) between the early follicular (19.9±6.1 ml/100ml tissue/min) and ovulation phases (22.1±5.9 ml/100ml tissue/min). However, during the early follicular phase, peak FBF was significantly (P=0.02) diminished 15% with endothelial I/R injury (from: 19.9±6.1 to 17.1±4.1 ml/100ml tissue/min) despite remote IPC (Figure 2-A), a significant

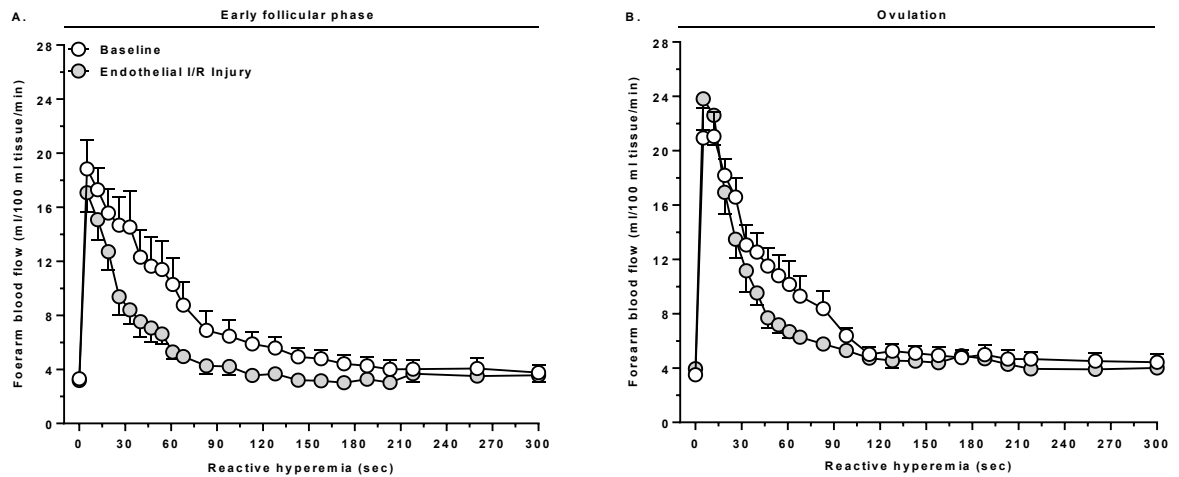
difference ($P=0.0001$) compared with ovulation. As a result, total FBF during the initial 30 sec of reactive hyperemia (area under the curve) was decreased $\sim 18\%$ ($P=0.027$) after (41.0 ± 11.4 ml/100ml tissue/min) compared with before (49.6 ± 15.1 ml/100ml tissue/min) endothelial I/R injury (Figure 2-B). During ovulation, remote IPC provided a level of endothelial protection from I/R injury that was not observed in the early follicular phase. In the presence of endothelial I/R injury, peak FBF increased 15% (from: 22.1 ± 5.9 to 25.3 ± 6.4 ml/100ml tissue/min; $P=0.012$) (Figure 2-A). As a result, total FBF was well preserved ($P=0.859$) after (58.2 ± 14.6 ml/100ml tissue/min) compared with before (58.0 ± 13.3 ml/100 ml tissue/min) endothelial I/R injury (Figure 2-B). Total FBF with endothelial I/R injury was markedly higher ($P=0.0001$) during ovulation compared with the early follicular phase (Figure 2-B). There was no influence of menstrual cycle sequence on the FBF responses to endothelial I/R injury.

Figure 2. Peak forearm blood flow response to 5 min reactive hyperemia (A) and total forearm blood flow (B) at baseline and in the presence of endothelial I/R injury with remote IPC during the early follicular (A) and ovulation phase (B). Bars represent means, circles represent each subject.



The FBF response to 5 min reactive hyperemia is shown in Figure 3. During the early follicular phase (Figure 3-A) FBF responses at 30-second intervals up to 3 mins were approximately 15-50% lower (Figure 3-A) with endothelial I/R injury despite attempts to induce remote IPC. The FBF responses during ovulation were well preserved despite endothelial I/R injury (Figure 3-B).

Figure 3. FBF response to 5 min reactive hyperemia at baseline and in the presence of endothelial I/R injury with remote IPC during the early follicular (A) and ovulation phase (B).



DISCUSSION

The main findings of the present are that the menstrual cycle differentially influences the capacity of remote IPC to protect against endothelial I/R injury in premenopausal women. The early follicular phase caused a significantly diminished peak FBF and total FBF when compared to the ovulation phase, despite IPC. This suggests that the greater circulating estrogens during ovulation were an important factor contributing to the protective benefits of remote IPC. When endothelial I/R injury was caused, the early follicular phase had decreased FBF during the reactive hyperemia phase suggesting that blood flow is diminished at that time in comparison to before endothelial I/R injury. During the ovulation phase, remote IPC delivered protection from I/R injury and increased peak FBF significantly. Baseline resting FBF were similar between the early follicular phase and ovulation, as well as in the absence of endothelial I/R injury creating an ideal situation to detect changes in FBF in the presence of endothelial I/R injury.

In women, we know that I/R injury is worse during the menopause transition when the vascular protective effects of estrogen are lost. What is not clear is how the different phases of the cycle in premenopausal women modulate this injury and interventions to prevent it. Certainly, the menstrual cycle is associated with changes in arterial function that may intensify injury and interfere with protective therapies. For example, the capacity of the endothelium to increase blood flow (i.e., dilation), a strong predictor of vascular health, is vulnerable to reperfusion damage. In the early follicular phase (when estrogen is lowest), dilation is impaired compared with the high estrogen late follicular phase

[3]. Vascular function is also decreased in the early luteal phase (when progesterone is highest) and recovers when progesterone levels decline [4]. Indeed, while it is clear that estrogen exerts many vascular protective effects (e.g., stimulates dilation by enhancing nitric oxide production) [20], its deficiency contributes to alterations in cardioprotection and declines in vascular endothelial function across the menopause transition [21]. Limited data from animal models suggests that estrogen deficiency interferes with ischemic conditioning to defend the blood vessels from injury [5], yet these findings have not been documented in women. As such, the menstrual cycle provides a unique way to isolate the independent effects of female sex hormones on reperfusion injury.

Understanding how estrogen and the menstrual cycle play a role in protecting the endothelium can clinically impact how women are treated when presenting with cardiovascular implications. In the present study, we showed that protection in the endothelium from I/R injury was diminished in the early follicular phase.

Ovulation on the other hand was linked with significantly better protection.

Impaired endothelial function plays a critical role in the development of I/R injury and is a significant predictor of atherosclerotic events [22]. These findings are important because they may shed light on why, despite successful reperfusion therapy, younger women are at risk of greater clinical consequences associated with I/R injury. Many of these consequences are due to more severe injury to the microvasculature endothelium [4, 8]. Future research should establish guidance in scheduling planned medical interventions that induce I/R injury during the phase of the cycle with the greatest potential for cell protection.

Currently, most research investigating IPC is completed with healthy animal models that do not translate well or mimic humans in clinical settings. This study is novel in that it is the first human study to investigate the influence of the menstrual cycle on IPC-mediated protection. The findings of the study are in line with prior animal models. Previous data from ovariectomized animal models shows that estrogen deficiency abolishes the cardioprotective effects of IPC, whereas estrogen replacement restores protection. These models show that Oestrogen replacement restored the IPC effect in comparison to a placebo group where the cardioprotective effect of IPC was lost [13]. Using selective activation of protein kinase C-mediated signaling could reestablish the IPC effect without using Oestrogen replacement, which would facilitate the anti-inflammatory protection provided by the IPC in females [13]. Demerouti et al. found that IPC in ovariectomized rabbits resulted in weaker or lost protection and post conditioning significantly reduced infarct sizes [14].

This study has limitations. First, the sample size was small and limited to Caucasians. In addition, we did not determine changes in the mid-luteal phase when progesterone levels are high. Another limitation is that we focused exclusively on premenopause. Future work should investigate how perimenopause and postmenopause affect IPC-mediated endothelial protection. Lastly, it is also likely that various contraceptive medications could impact IPC-mediated protection. Future work should address this.

In conclusion, the menstrual cycle differentially influences the capacity of remote IPC to protect against endothelial I/R injury. Greater circulating estrogens

during ovulation may be an important mediator contributing to the protective benefits of remote IPC in women. As emphasized in the Scientific Statement from the American Heart Association on Acute Myocardial Infarction, “CHD remains understudied, underdiagnosed, and undertreated” in American women [23]. Having a greater understanding of the effects of remote IPC during the early follicular and ovulation phases of the menstrual cycle can help lead further research to reduce the mortality rates for CVD in premenopausal women.

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