DOES MENSTRUAL CYCLE PHASE IMPACT THE BRACHIAL ARTERY FLOW MEDIATED DILATION RESPONSE TO ACUTE HYPERGLYCEMIA?

by

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Abstract

Acute hyperglycemia transiently impairs endothelial function in healthy males when assessed via flow-mediated dilation (FMD). However, research in female participants is lacking and the impact of menstrual phase (early follicular (EF) and late follicular (LF)) on vulnerability to acute hyperglycemia-induced endothelial dysfunction is unknown. It is hypothesized that hyperglycemia-induced endothelial dysfunction will be attenuated in the LF phase. Seventeen healthy, naturally menstruating women (21 ± 1 years [mean ± SD]) participated in three experimental visits. During two visits (one EF phase, one LF phase), endothelial function was assessed via brachial artery FMD before and 60-, 90-, and 120-min after an oral glucose challenge (75g glucose/300mL solution). Blood samples were taken to assess blood glucose, insulin, estradiol, progesterone and viscosity. During a third visit in the EF phase, participants ingested 300mL of water as a time-control for FMD. Blood samples were only taken for blood glucose and viscosity during this visit. Additionally, ovulation testing was used to confirm the LF phase. Data are [mean ± SD]. Glucose and insulin levels increased 30min post-glucose ingestion, with no difference between visits ($p < 0.001$). FMD was reduced 90min post-glucose ingestion (Post90) during the EF glucose visit (Pre: 8.7 ± 2.9%, Post90: 6.2 ± 2.8%, $p = 0.001$; Cohen's $d = 0.864$). FMD-impairment during $LF_{Glucose}$ was not significant (Pre: 8.4 ± 2.4%, Post120: 6.8 ± 2.7, $p = 0.089$; $d = 0.649$). A direct comparison of EF and $LF_{Glucose}$ delta-FMD did not reach significance ($p=0.117$) revealing a small effect of phase ($d = 0.431$) on vulnerability to hyperglycemia-induced FMD impairment. These results suggest that the cyclic elevation in estrogen that occurs during the menstrual cycle confers a small degree of protection from acute hyperglycemia-induced impairment in endothelial function. In order to fully characterize the

impact of acute stimuli on vascular function in premenopausal women, it is essential to examine responses across multiple phases of the menstrual cycle.

Co-Authorship

All chapters of this thesis were written by Jennifer Williams, with supervision from Dr. Kyra Pyke. Jennifer Williams was responsible for reviewing the literature, developing the research question and study design, coordinating and participating in data collection analyzing the data, interpreting the analysis, and writing the manuscript. Taylor Stimpson assisted with data collection, in line with her Undergraduate Thesis project. Josh Tremblay assisted with data collection and blood viscosity analysis. Alyssa Fenuta assisted with acquiring blood draws. Dr. Pyke assisted with study design, interpretation of data and edited the thesis document. Dr. Pyke was the principal investigator on the research grant funding this study.

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List of Abbreviations

AbsFMD – absolute FMD

AUC – area under the curve

BMI – body mass index

BP – blood pressure

CVD – cardiovascular disease

E - estrogen

E1 - estrone

E2 - 17β estradiol

E3 – estriol

ECG – electrocardiogram

EE - ethynylestradiol

EF – early follicular

EL – early luteal

eNOS – endothelial nitric oxide synthase

ER – estrogen receptor

F – female

FMD – flow mediated dilation

HOMA-IR – homeostatic model assessment for insulin resistance

HR – heart rate

IR - ischemia-reperfusion

LF – late follicular

LH – luteinizing hormone

LL – late luteal

M - male

MAP – mean arterial pressure

MDA - malondialdehyde

NO – nitric oxide

NT - nitrotyrosine

O – ovulation

OCP – oral contraceptive pill

ROS – reactive oxygen species

RH – reactive hyperemia

SR – shear rate

TBARS – thiobarbituric acid reactive substances

Chapter 1: Introduction

The vascular endothelium is a single layer of cells that lines the arteries, and is important in the regulation of vascular tone. In response to increases in blood flow associated shear stress, endothelial cells release molecules involved in vasodilation. These molecules include nitric oxide (NO; Joannides *et al*, 1995; Mullen *et al*, 2001; Mitchell *et al*, 2008; Green *et al*, 2014), prostacyclin (PGI2; Mitchell *et al*, 2008) and endothelium derived hyperpolarizing factors (EDHF; Rubanyi *et al*, 1986; Komori & Vanhoutte, 1990). The resulting vasodilation is termed flow-mediated dilation (FMD). The most common non-invasive technique used to assess endothelial function using high frequency ultrasound is called reactive hyperemia FMD (Celermajer *et al*, 1992; Coretti *et al*, 2002). This test assesses FMD in response to a transient shear stress stimulus created with the release of limb occlusion. A larger FMD response is indicative of healthy endothelial function, whereas a small FMD response is indicative of dysfunction. Although there is evidence of variability (Pyke *et al*, 2010; Parker *et al*, 2011; Wray *et al*, 2013), NO has been shown to have an important role in this response (Green *et al*, 2014).

Acute hyperglycemia, referring to the transient increase in blood glucose levels, has been shown to result in impairments in brachial artery FMD by many studies performed primarily in men, (Akabari *et al*, 1998; Kawano *et al*, 1999; Title *et al*, 2000; Ceriello *et al*, 2002; Zhu *et al*, 2007; Baynard *et al*, 2009; Ceriello *et al*, 2011; Mah *et al*; 2011; Watanabe *et al*, 2011; Grassi *et al*; 2012; Suzuki *et al*, 2012; Nakayama *et al*, 2013; Mah *et al*, 2013; Wang *et al*, 2013; Greyling *et al*, 2015; Walsh *et al*, 2016). While not all studies have reported this impairment in FMD (Bagg *et al*, 2000; Siafarikas *et al*, 2004; Dengel *et al*, 2007; Weiss *et al*, 2008; Xiang *et al*, 2008), a recent meta-analysis by Loader *et al* (2015) has concluded that acute hyperglycemia results in a transient decrease in FMD in healthy participants and in those living with metabolic disease(s). Mechanistically, there is evidence that acute hyperglycemia may increase reactive oxygen species (ROS), which can react with NO and decrease its bioavailability (Tesfamariam & Cohen, 1992; Cosentino *et al*, 1997; Konukoglu *et al*, 1997; Koska *et al*, 1997; Du *et al*, 1999; Kawano *et al*, 1999). For example, estimates of ROS have been reported to increase with hyperglycemia *in vivo* and *in vitro* (Tesfamariam & Cohen; Konukoglu *et al*, 1997; Kawano *et al,* 1999; Mah *et al*, 2011). Additionally, several studies have reported an attenuation of hyperglycemia-induced impairments in FMD following antioxidant supplementation, including dark chocolate (Grassi *et al*, 2012), alpha-tocopherol and ascorbic acid (Sheikh-Ali *et al*, 2009), onion extract/quercetin (Nakayama *et al*, 2013), and y-tocopherol (Mah *et al*, 2013).

While acute hyperglycemia may have a negative impact on endothelial function, there is evidence that estrogen – specifically estradiol (E2) – may provide some vasoprotection to the endothelium. In postmenopausal women, hormone replacement therapy has been associated with improvements in FMD (Liberman *et al*, 1994; Kawano *et al*, 1997; Hashimoto *et al*, 2002; Harvey *et al*, 2005; Sherwood *et al*, 2007; Vitale *et al*, 2008; Moreau *et al,* 2013). Likewise, premenopausal women using oral contraceptive pills also appear to experience vascular benefits during the active phase of the cycle, when estrogen is elevated (Torgrimson *et al,* 2007; Meendering *et al,* 2009; Meendering *et al,* 2010; Thompson *et al,* 2011).

Similarly, in premenopausal women with a natural menstrual cycle, some studies have observed improvements in FMD during the late follicular (LF) phase of the cycle (Hashimoto *et al*, 1995; Kawano *et al*, 1996; Adkisson *et al*, 2010; Harris *et al*, 2012; Jarrard *et al*, 2015). However, this has not been observed by all studies exploring natural menstrual cycle phase and FMD (Williams *et al*, 2001; Saxena *et al*, 2012; Rakobowchuk *et al*, 2013; Brandao *et al*, 2014;

Luca *et al*, 2016; D'Urzo *et al*, 2017). While it appears that high levels of estradiol may have a beneficial impact on FMD, it is less clear how cyclic fluctuation in estrogen interacts with transient insults to endothelial function. In particular, it is unknown how cyclic estrogen fluctuation with menstrual phase impacts vulnerability to hyperglycemia induced endothelial dysfunction.

There is some evidence that cyclic variation in estrogen attenuates vulnerability to transient insults to endothelial function (Luca *et al*, 2016). Research by Luca *et al* (2016) found that vulnerability to ischemia-reperfusion (IR) induced FMD impairment varied depending on menstrual cycle phase. Specifically, Luca *et al* (2016) found that IR injury blunted FMD during the early follicular (EF) phase of the menstrual cycle, but this was prevented during the LF phase.

IR induced endothelial dysfunction is thought to involve ROS (Gao *et al*, 2008; Loukogeorgakis *et al*, 2010) and the decreased vulnerability to IR impairment in the LF phase observed by Luca *et al* (2016) may be explained by estrogen's antioxidant effects (Stirone *et al*, 2005; Lam *et al*, 2006; Haas *et al*, 2012). Importantly, a recent in vivo study in human umbilical vein endothelial cells found that estradiol suppressed hyperglycemia induced increases in superoxide anion production (Haas *et al*, 2012). This has been supported by animal research in diabetic male rats, which observed amelioration of elevated glucose, insulin, and ROS levels during hyperglycemia, with estradiol supplementation (Hamden *et al*, 2008; Ahmed & Hassanein, 2012). Given the findings described above, indicating that hyperglycemia induced FMD impairment may be a result of ROS, this evidence provides support for the hypothesis that high levels of estradiol may protect against hyperglycemia-induced impairment in FMD.

In conclusion, the primary objective of the study presented in Chapter 3 is to determine whether menstrual phase (EF vs LF phase), has an impact on the FMD response to acute hyperglycemia. The primary hypothesis of this study is that FMD will be impaired by acute hyperglycemia during the EF phase, but that this impairment will be attenuated during the LF phase. Overall, the proposed study will provide significant contributions to understanding the vascular impact of acute hyperglycemia in young, premenopausal women, during two phases of the menstrual cycle.

Chapter 2: Literature Review

This review will explore 1) the role of the endothelium and its assessment, 2) estrogen and its effects on endothelial function, 3) acute hyperglycemia and its effects on endothelial function, and 4) the putative interaction between menstrual cycle phase and hyperglycemia.

2.1 Role of the Endothelium and its Assessment

Arteries are lined by a single layer of cells called the endothelium, which are vital in the regulation of vascular tone. In response to changes in blood flow associated shear stress, the frictional force exerted on the endothelium as blood flows past the artery's walls, endothelial cells will release key molecules involved in the vasodilator response. These vasodilator molecules include: nitric oxide (NO; Joannides *et al.*, 1995; Mullen *et al.*, 2001; Mitchell *et al.*, 2008; Green *et al.*, 2014), prostacyclin (PGI2; Mitchell *et al.*, 2008) and endothelium derived hyperpolarizing factors (EDHF; Rubanyi *et al.*, 1986; Komori & Vanhoutte, 1990). The release of these molecules elicits a vasodilation response, termed 'flow mediated dilation (FMD)'. The focus of this review will explore NO, as it is discussed later that NO may be mechanistically involved in the impact of both estrogen and acute hyperglycemia on FMD.

Researchers have developed non-invasive techniques to assess endothelial function. One of the most widely used techniques to assess endothelium-dependent vasodilation is called reactive hyperemia FMD (FMD; Celermajer *et al.*, 1992). A pneumatic cuff is placed around the forearm or leg and inflated to approximately 250 mmHg for five minutes. This period of occlusion will result in dilation of the arterioles that lie distal to the cuff's placement (Celermajer *et al.*, 1992). Following the cuff release, there is a transient increase in blood flow associated shear stress, known as reactive hyperemia, resulting in a delayed vasodilation of the conduit artery. This dilation of the artery can be captured using ultrasound technology, to assess the diameter of the artery before and at peak dilation (Celermajer *et al.*, 1992). Endothelium-dependent vasodilation, represented by this difference in artery diameter from baseline to peak dilation (FMD), is a non-invasive index of endothelial function. FMD has been shown to predict risk of future cardiovascular events in adults with and without diagnosed cardiovascular disease (CVD; Inaba *et al*, 2010).

There is evidence that endothelium-dependent vasodilation, stimulated via a reactive hyperemia induced transient shear stress stimulus, is predominantly NO-dependent. Research by Joannides *et al* (1995) assessed radial artery vasodilation using a FMD protocol, with and without the infusion of a NO synthase inhibitor, NG-monomethyl-L-arginine (L-NMMA). The introduction of L-NMMA resulted in a marked decline in endothelium-dependent vasodilation. Subsequent research by Mullen *et al.* (2001) confirmed this finding. Although not all studies have found that FMD is NO dependent (Pyke *et al*, 2010; Wray *et al*, 2013), a recent meta-analysis by Green *et al* (2014) of twenty studies using FMD techniques and L-NMMA infusion concluded that FMD is impaired by the infusion of L-NMMA (%FMD: 8.2% [CI: 6.8% - 9.6%] saline infusion vs. 3.7% [CI: 3.1% - 4.3%] L-NMMA infusion). These studies provided evidence that FMD is mediated, in part, by NO bioavailability.

In response to increases in shear stress, endothelial nitric oxide synthase (eNOS), a calcium-calmodulin-dependent enzyme expressed in endothelial cells, will convert an NO precursor, L-arginine, to NO and L-citrulline (Palmer *et al*, 1988). NO will then diffuse into smooth muscle cells that surround the artery endothelium layer. This will activate an enzyme called cytosolic guanylyl cyclase (GC) to convert guanosine triphosphate (GTP) to the secondary messenger cyclic guanosine monophosphate (cGMP; Moncada *et al*, 1991). Cyclic GMP will interact with intracellular receptor proteins in smooth muscle cells, such as GMP-dependent kinases, which will decrease intracellular calcium (Moncada *et al*, 1991). This cascade will result in smooth muscle cell relaxation and subsequent vasodilation.

2.2 Impact of Estrogen on Endothelial Function

2.2.1 What is Estrogen?

Estrogen is the primary female sex hormone responsible for proper primary and secondary sexual characteristic development and sexual reproductive function (Arnal *et al*, 2010). Estrogen is produced mainly in the ovaries, adipose tissue, and placenta. There are three forms of physiological estrogen: estrone (E1), estradiol (17β- estradiol; E2), and estriol (E3), all produced from cholesterol biochemical synthesis, as reviewed in Armstrong and Dorrington, 1977, and more recently in Cui *et al*, 2013. E2 is the major product from cholesterol synthesis into estrogen, and is the most potent endogenous estrogen formed during premenopause mainly from the follicles of the ovaries (Cui *et al*, 2013). In postmenopausal women, E1 will play a greater role, as it is synthesized from adrenal dehydroepiandrosterone in adipose tissue (Cui *et al*, 2013). Finally, E3 is the least potent estrogen, and is mainly produced by the placenta during pregnancy (Cui *et al*, 2013).

Estrogen exerts its physiological effects on various systems in the human body by binding to estrogen receptors (ER) – mainly ERα and ERβ. Relevant to the cardiovascular system, while both ERs are present in endothelial cells, ERα is more prevalent (Pare, 2002). Estrogen binding to ERs on endothelial cells will trigger nongenomic and genomic effects. Specifically, binding will increase NO bioavailability and trigger increased gene expression of factors related to NO production (Caulin-Glaser *et al*, 1997; Chambliss & Shaul, 2002).

2.2.2 Fluctuations in Estrogen Levels with the Natural Menstrual Cycle

Natural menstrual cycles, not regulated by a form of hormonal contraception, occur in regularly menstruating women from shortly after the onset of puberty until menopause. Following menopause, women will experience chronically low levels of estrogen. The menstrual cycle involves cyclic variations in estrogen, specifically estradiol, and progesterone. These variations occur during two phases of the cycle: the follicular phase and the luteal phase, over approximately 28 days on average (Owen, 1975; See Figure 1). During the early follicular phase (days 1-7), menstruation occurs. Estradiol levels during this time will be on average 19-140 pg/mL (Stricker *et al*, 2006; Cui *et al*, 2013). Following menstruation, estradiol will gradually increase over time during the follicular phase. This phase ends when ovulation occurs during the late follicular phase, typically within days 14 or 15. Just prior to ovulation, there is a progressive increase in estrogen of approximately 110-410 pg/mL, resulting in a surge in luteinizing hormone (LH), which triggers ovulation approximately 24-36 hours following (Stricker *et al*, 2006; Cui *et al*, 2013).

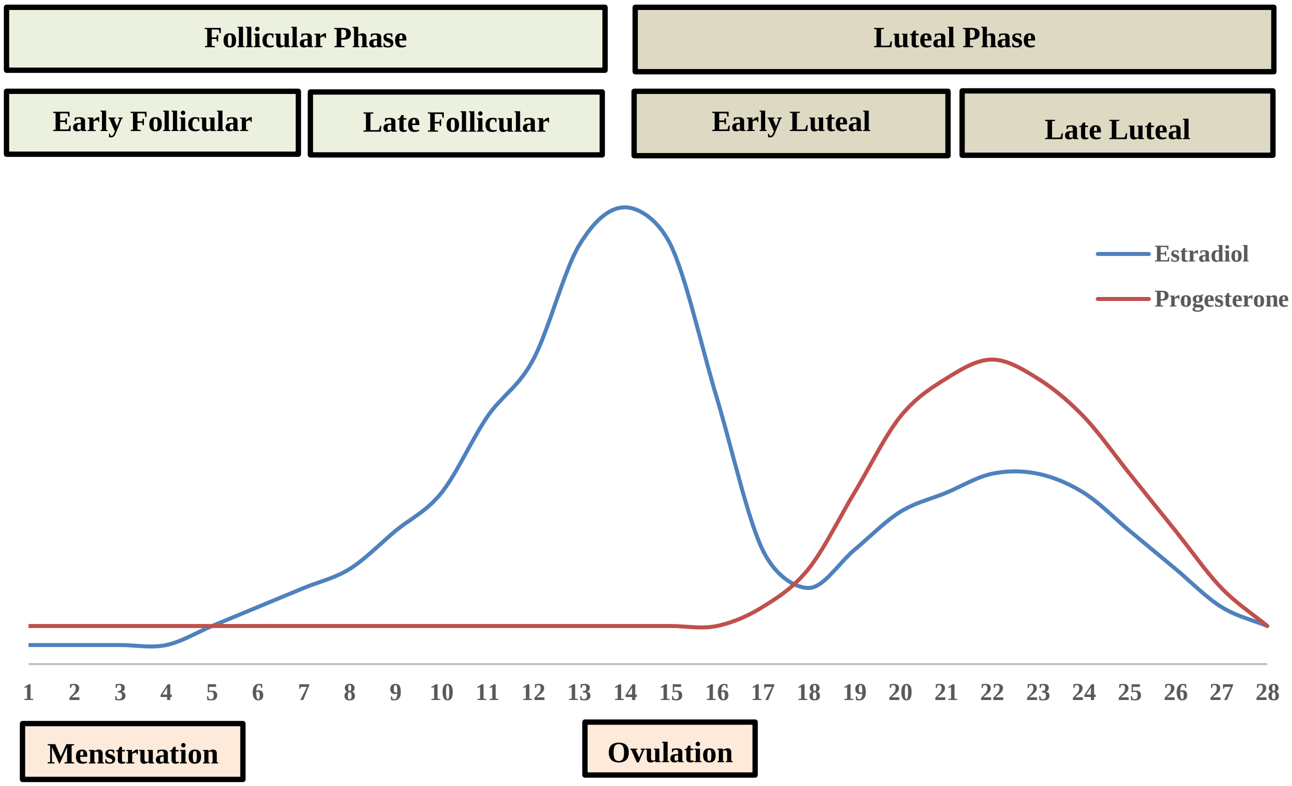

Figure 1. The menstrual cycle, demonstrating estradiol and progesterone fluctuations throughout a 28-day cycle. Adapted from "Menstruation" (Clayton, 2008).

2.2.3 Models to Investigate the Impact of Estradiol on FMD

Researchers have utilized three primary techniques in assessing the impact of estrogen on FMD: synthetic estrogen replacement therapy in postmenopausal women, oral contraception "active" synthetic estrogen vs. placebo pill phases of oral contraceptive pills (OCPs) in premenopausal women, and natural menstrual cycle phase fluctuations in estrogen in premenopausal women. The following sections will focus on the impact of synthetic and natural estrogens on FMD, using evidence derived from these models.

2.2.3.1 Impact of Hormone Replacement Therapy in Postmenopausal Women

The overwhelming majority of studies in postmenopausal women have observed improvements in FMD associated with synthetic estrogen hormonal replacement therapy (HRT; See Table 1: Liberman *et al*, 1994; Kawano *et al*, 1997; Hashimoto *et al*, 2002; Harvey *et al*, 2005; Sherwood *et al*, 2007; Vitale *et al*, 2008; Moreau *et al*, 2013). However, there appears to be an effect of age, such that improvements in FMD appear in women in their fifties, but not into their sixties and seventies (Sherwood *et al*, 2007; Vitale *et al*, 2008). For example, Sherwood *et al* (2007), assessed brachial artery FMD in 100 postmenopausal women (51 healthy, 49 with CAD), prior to and 18h following the use of an acute transdermal estrogen patch. Compared with age-matched controls given a placebo, women in their 50s experienced improvements in FMD (control: 4.81 ± 5.97%, transdermal patch: 7.69 ± 4.79%). However, women in their sixties and seventies did not experience this improvement with HRT. Other research with acute and chronic estrogen administration has found similar results (Vitale *et al*, 2008). For example, in a population of 134 postmenopausal women, FMD was elevated following both acute (1h) and chronic (3 months) administration of oral estradiol (baseline: 5.6 ± 1.5%; acute: 9.3 ± 0.6%; chronic: 9.6 ± 1.3%). Overall, researchers have concluded that FMD increases following acute and chronic estrogen treatment to levels similar to premenopausal women during their follicular phase (Harvey *et al*, 2005), and remains elevated over the period of use (e.g. 36h, Hashimoto *et al*, 2002).

Table 1. A summary of studies that have examined the effects of estrogen replacement therapy on FMD in postmenopausal women.

Citation	Population & Age	Study Design	FMD	Estradiol	Mechanisms
Liberman *et al*, 1994	n = 13 postmenopausal women Age: 55 ± 7 years	FMD assessed before and 9 weeks following treatment – one of: Placebo Daily E2 1mg/day Daily E2 2 mg/day	↑FMD with E2 supplementation (no diff. b/w dosage groups) ↔ FMD with placebo	Not reported.	None.
Kawano *et al*, 1997	n = 15 postmenopausal females (Age: 63.6 ± 2.6 years) and 15 males (Age: 63.1 ± 0.7 years)	FMD assessed before and 36h following transdermal E2 supplementation (both male and female)	↔ FMD in men ↑FMD in women with E2 supplementation	↑ E2 in men and women post E2	↑ Nitrite/Nitrate (estimate of NO bioavailability) in women post E2 *Conclusion: Estimate of NO bioavailability increases with E2 treatment.*
Hashimoto *et al*, 2002 (Abstract)	n = 18 postmenopausal women Age: 53.7 ± 1.1 years	FMD assessed post-estrogen (0.625 mg and medroxyprogesterone acetate 2.5 mg/day) or placebo (calcium) at 3, 6, 12, 18, 24, 26 months	↑FMD in women with E2 supplementation, elevated whole 36 months	Not reported.	None.

Citation	Population & Age	Study Design	FMD	Estradiol	Mechanisms
Harvey *et al*, 2005	n = 13 postmenopausal women (Age: 54 ± 2 years n = 14 premenopausal women (Age: 28 ± 1 years)	FMD assessed before and 4 weeks post supplementation (2mg/day) for postmenopausal group; premenopausal assessed during follicular phase (days 5-13) of menstrual cycle	↑FMD in postmenopausal, post-treatment; reaching premenopausal levels	↑Estradiol (pmol/L) with treatment 22 ± 3.3 (Pre) 998 ± 220 (Post) 187 ± 28 (premenopausal)	None.
Sherwood *et al*, 2007	n = 100 postmenopausal women (51 healthy, 49 with CAD) Age: 50-80 years	FMD assessed 18h following one of: 1) Placebo 2) 0.05 mg/day E2 3) 0.05 mg/day E2 + norethindrone acetate (NETA; 0.14 mg/day)	↑FMD in women in 50s (not different between E2 groups) ↔ FMD in women in 60s and 70s	Not reported.	None.
Vitale *et al*, 2008	n = 134 postmenopausal women Age: 62 ± 6 years	FMD assessed acute (1h post 1mg/s E2) and chronic (3 months 1mg/day E2) supplementation	↑FMD acute and chronic (same magnitude)	↑E2; greater with acute > chronic	None.
Moreau *et al*, 2013	n = 36 postmenopausal women Age: 45-65 years n = 10 placebo (56 ± 7 years) n=12 oral, =11 transdermal	FMD assessed before and after 12 weeks of one of: 1) Placebo 2) Oral E2 (1mg/day) 3) Transdermal E2 (0.05mg/day)	↑FMD with both E2 groups (same magnitude)	↑E2 for both groups	None.

Table 1. Literature review of studies exploring the impact of estrogen on endothelium-dependent vasodilation assessed by RH-FMD, in postmenopausal participants on synthetic estrogen replacement therapy, in chronological order. EE = ethinyl estradiol; E2 = 17β estradiol; FMD = flow mediated dilation.

2.2.3.2 FMD Fluctuation with Oral Contraceptive Pill (OCP) Use

Oral contraceptive pills (OCPs) come in the form of an estrogen-progestin combination pill or progestin-only pill, also called a mini-pill. Given the focus of this review, only studies involving the combination pill, with a synthetic estrogen compound, typically ethynylestradiol (EE) and a synthetic progestin compound, will be examined. Over the years, there have been four generation upgrades to the progestins found in OCPs to negate side effects and risk profiles of previous generations of OCPs (Petitti, 2003).

Generally, first generation OCPs contained EE and either mestranol or norethindrone; second generation OCPs have lower dosages of EE with levonogestrel; third generation OCPs contain EE and either gestodene or desogestrel; and, finally, fourth generation OCPs contain EE and chlormadinone acetate or drospirenone (Petitti, 2003; Shufelt *et al*, 2009). It has also been found that some progestins may counteract the benefits associated with estrogen on FMD (Table 2, Miller *et al,* 1991; Torgrimson *et al,* 2007; Meendering *et al,* 2009; Meendering *et al*, 2010; Thompson *et al*, 2011; Friedman *et al*, 2011; Franceschini *et al*, 2013). OCPs can also come in the form of a monophasic (active: 3 weeks of the same doses daily, placebo: 1 week placebo pills) or biphasic/triphasic (active: 3 weeks of varying dosages weekly, taken daily, placebo: 1 week placebo pills) combinations, for a 28 day cycle (Petitti, 2003; Shufelt *et al*, 2009). For the purposes of simplicity in this review, literature associated with a monophasic OCP will be examined.

In studies of participants taking monophasic oral contraceptive pills, FMD was assessed during the active phase (days 5-7 of the third week of the 28-day cycle) and the placebo phase (days 5-7 of the placebo pills) (See Table 2). Torgrimson *et al* (2007) assessed FMD during the active and placebo phases of participants taking two dosages of a second generation OCP.

Participants in the low dose group used 150 μg levonorgestrel/30 ug EE OCP compared to participants taking 100 ug levonorgestrel/20 ug EE in the very low dose group. FMD decreased in the active phase for participants in the very low condition, with no changes in the low condition. Additionally, two participants took only EE and there was a consistent increase in FMD with EE ingestion only. Altogether, this 2nd generation progestin may counteract the beneficial effects of EE alone on FMD, according to dosage.

Likewise, researchers Meendering *et al* (2009) replicated the study by Torgrimson *et al* (2007), looking at a 3rd generation progestin: desogestrel. Participants in the very low dose group took 20 mcg ethynyl estradiol (EE)/150 mcg desogestrel compared to 30 mcg EE/150 mcg desogestrel in the low dose group. Participants in the very low dose group also repeated their placebo week, but took 10 mcg EE instead of placebo pills. FMD was enhanced during the low dose group's active phase, and did not change with the very low dose group. However, FMD was enhanced in the very low dose group with EE alone. Taken altogether, the 3rd generation OCP progestin seems to only antagonize the effects of estrogen on FMD when in the very low dose group.

Finally, follow-up research by Meendering *et al* (2010) found that participants taking a low dose 4th generation OCP: 30 mcg EE/3.0 mg drospirenone had an elevated FMD during their active pill stage (placebo: 6.86 ± 0.48% vs. active: 10.97 ± 0.68%). The notion that 3rd and 4th generation low dose OCPs do not prevent the FMD enhancement associated with EE, but 2nd generation OCPs do, has been further confirmed by a comparison study by Thompson *et al* (2011). In conclusion, it appears that in newer generations of low dose OCPs EE enhances FMD during the active pill phase, compared to the placebo phase, further demonstrating the positive impact of synthetic estrogen on vascular function.

Table 2. A summary of studies that have examined the effects of oral contraceptive pill phase on FMD in premenopausal women.

Citation	Population & Age	Dose & Generation	Study Design	FMD
Torgrimson *et al*, 2007	n = 15F Age: 21.0 ± 2.0 years	*2nd Generation* Very low dose: n=7, 100 ug levonorgestrel/20 ug EE Low dose: n =7, 150 ug levonorgestrel/30 ug EE	FMD assessed during placebo and active cycles. FMD also assessed in n=2F who received 10ug EE only	↓ FMD in active phase of very low dose ↔ FMD in low dose ↑ FMD in EE only
Meendering *et al*, 2009	n = 22F Age: 18-30 years	*3rd Generation* Very low dose: n = 11, 150 mcg desogestrel/ 20 mcg EE (Weeks 1-4); 10 mcg EE only week 4 Low dose: n = 11, 150 mcg desogestrel/30 mcg EE	FMD assessed during placebo and active pill phases for both groups. FMD also assessed in VLD group with only EE	↔ FMD in very low dose (active vs. placebo) ↑ FMD in low dose (active vs. placebo) ↑ FMD in EE only (vs. very low dose active and placebo phases)
Meendering *et al*, 2010	n = 12F Age: 22 ± 1 year	*4th Generation* 30 mcg EE/3.0 mg drospirenone	FMD assessed during active and placebo phase of OCP	↑ FMD in active phase
Thompson *et al*, 2011	n = 29F Age: Unknown	30 ug EE w/: n = 10: 150 ug desogestrel (*3rd Generation*) n = 10: 150 ug levonorgestrel (*2nd Generation*) n = 9: 3 mg drospirenone (*4th Generation*)	FMD assessed during active and placebo phase of OCP	↑ FMD in active phase for desogestrel and drospirenone ↔ FMD in active phase for levonorgestrel

Citation	Population & Age	Dose & Generation	Study Design	FMD
Friedman *et al*, 2011	n = 23F Age: 21-35 years	*2^{nd}, 3^{rd}, 4^{th} Generation* Using OCP with varying estrane/gonane and drosperinone progestins	FMD assessed in the inactive phase only	↑ FMD in drosperinone OCP vs. estrange/gonane OCP
Franceschini *et al*, 2013	n = 43F Age: 28.3 ± 3.7 years	Three groups: 1) Control 2) Oral contraception #1: 30 mcg EE/150 mcg chlormadinone acetate (*3^{rd} Generation)* 3) Oral contraception #2: 30 mcg EE/150 mcg levonogestrel (*2^{nd} Generation*)	FMD assessed before (baseline) and after 6 months of control/pill use. FMD assessed in early follicular phase of control group and 3^{rd} week of active pills in contraception groups	↑ FMD in levonogestrel at baseline, but fell to levels of control & chlormadinone acetate at 6 months post ↔ FMD for control and chlormadinone acetate baseline to 6 months post.

Table 2. Literature review of studies exploring the impact of estrogen on endothelium-dependent vasodilation assessed by RH-FMD, in premenopausal participants on synthetic oral contraceptives, in chronological order. EE = ethinyl estradiol; OCP = oral contraceptive pill; FMD = flow mediated dilation.

2.2.3.3 FMD Fluctuation over Phases of the Menstrual Cycle

Several studies have observed cyclic variation in FMD during the natural menstrual cycle, such that improvements in FMD have been reported in the late follicular phase, compared to the early follicular phase, in tandem with increases in natural estradiol levels (Table 3; Hashimoto *et al*, 1995; Kawano *et al*, 1996; Williams *et al*, 2001; Adkisson *et al*, 2010; Harris *et al*, 2012; Jarrard *et al*, 2015). However, the magnitude of improvement in FMD varies between studies, ranging in improvements of 14% to 188% during the LF phase, compared to the EF phase (Kawano *et al*, 1996; Williams *et al*, 2001).

Additionally, some studies (Hashimoto *et al*, 1995; Kawano *et al*, 1996; Harris *et al*, 2012), but not all (Williams *et al*, 2001; Adkisson *et al*, 2010), have observed maintained FMD improvement during the luteal phase, when both estrogen and progesterone levels are high. For example, a key study by Hashimoto *et al* (1995) assessed FMD in seventeen female participants (aged 25.1 ± 0.8 years) and seventeen age-matched males. These researchers found that FMD was the same in male and female participants during their early follicular phase (10.6 ± 0.8% and 11.2 ± 0.6%, respectively), but that FMD increased during the females' late follicular (18.2 ± 0.8%) and luteal (17.5 ± 0.7%) phases, in tandem with increases in estradiol levels. This was supported by a study by Kawano *et al* (1996), which assessed FMD in fifteen female participants (aged 29.6 ± 2.6 years), during the menses, follicular, and luteal phases. This study observed a similar increase in FMD from menses to follicular phases (4.9 ± 0.8% to 14.1 ± 0.9%), but less of an increase in the luteal phase (9.2 ± 1.1%).

However, some recent research has not observed improvements in FMD with menstrual cycle elevations in estradiol (Saxena *et al*, 2012; Rakobowchuk *et al*, 2013; Brandao *et al*, 2014; Luca *et al*, 2016; D'Urzo, 2017; Table 3). For example, Saxena *et al* (2012) reported FMD during the early follicular and early luteal (~2.2 ± 0.2 days from ovulation) phases of 20 premenopausal women (age 25.3 ± 0.9 years) and did not find a change in FMD with the menstrual cycle. The most likely explanation for this discrepancy in this study was that, as shown in Figure 1, estrogen levels fall off quickly post-ovulation and after ~2 days it is likely these researchers missed peak estrogen time point. This is evidenced by a smaller estradiol level during the early luteal phase, compared to other studies which have observed improvements in FMD (449 ± 64 pmol/L vs. 1362 ± 819 pmol/L, Harris *et al*, 2012).

Similarly, research by D'Urzo *et al* (2017) observed no difference in FMD between the early and late follicular phases, in twelve healthy, young female participants (aged 21 ± 2 years). However, it is likely that several of the participants in this study were not tested during their peak levels of estrogen, as 25% of participants exhibited lower than expected estradiol levels during the LF phase (Stricker *et al*, 2006; D'Urzo *et al*, 2017).

Finally, in a study by Rakobowchuk *et al* (2013), eight healthy women (aged 33 ± 10 years) took part in the study, examining FMD during the follicular, ovulatory, and luteal phases. While participants were tested during days 2-4 of their follicular phase, reported estrogen values were 445 ± 154 pmol/L, higher than estrogen levels reported in other studies which did observe differences in FMD with phase (Hashimoto *et al*, 1995; Williams *et al*, 2001; Adkisson *et al,* 2010; Harris *et al*, 2012). As a result, it is possible that increased estrogen during the early follicular phase masked any improvement that would have been observed with FMD.

In conclusion, some, but not all, studies provide evidence that FMD is enhanced during the menstrual cycle late follicular phase when estrogen levels are highest. This discrepancy in some study results may be due to some studies failing to align their FMD assessment with the peak in estrogen. However, other factors, including sensitivity to changes in estrogen may also be involved because the magnitude of change in FMD does not clearly correlate with the magnitude of change in estrogen (D'Urzo *et al*, 2017).

Table 3. A summary of studies that have examined the effects of natural menstrual cycle phase on FMD in premenopausal women.

Citation	Population & Age	Study Design	FMD	Estradiol	Mechanisms
Hashimoto *et al*, 1995	n = 17 premenopausal women & age-matched males Age: 25.1 ± 0.8 years	FMD assessed during EF, LF, L phases	↑ FMD during LF and L; FMD same in men & women during EF	↑ E2 during LF and L; same levels women EF and men	None.
Kawano *et al*, 1996 (Abstract)	n = 15F Age: 29.6 ± 2.6 years	FMD assessed EF, LF, L	↑ FMD during LF and L (highest at LF)	↑ E2 during LF and L (highest LF)	↑ Nitrite-Nitrate in LF; lowest in EF *Conclusion: Estimate of NO bioavailability increases with increased E2.*
Williams *et al*, 2001	n = 15F Age: 22.8 ± 0.7 years	FMD assessed EF, LF, EL, LL	↑ FMD from EF to LF ↓ FMD to EL; recover at LL	↑ E2 throughout LF, EL, LL	None.
Adkisson *et al*, 2010	n = 23F Age: 19 ±1 years	FMD assessed EF, LF, EL, LL	↑ FMD during LF only	↑ E2 during LF only	↑ NO_x (Nitrate/Nitrite) during LF *Conclusion: Estimate of NO bioavailability increases with increased E2.*
Harris *et al*, 2012	n = 25 (10M/15F) AgeM: 23 ± 0.9 years AgeF: 20 ± 0.5 years	FMD assessed during EF, LF, L for females; males studied once as control	↑ FMD during LF and L, compared to EF and men	↑ E2 during F and L	None.

Citation	Population & Age	Study Design	FMD	Estradiol	Mechanisms
Saxena *et al*, 2012	n = 20F Age: 25.3 ± 0.9 years	FMD assessed during EF and EL	↔ FMD during cycle	↑ E2 from EF to EL	None.
Rakobowchuk *et al*, 2013	n = 8F Age: 33 ± 10 years	FMD assessed during EF, LF, L	↔ FMD during cycle	↑ E2 from EF to LF	None.
Brandao *et al*, 2014	n = 21F Age: 24.76 ± 5.5 years	FMD assessed EF, LF, EL, LL	↔ FMD for EF to LF (non-significant increase) ↓ FMD to EL; recover at LL	Not reported.	None.
Jarrard *et al*, 2015	n = 6F with diabetes n = 15F control	FMD assessed EF and LF	↑ FMD from EF to LF in control participants ↔ FMD for EF to LF in participants with diabetes	Not reported.	+Antioxidant cocktail with participants with diabetes allowed for increase in FMD during LF. *Conclusion: Interaction between diabetes, estrogen, and FMD may be a result of oxidative stress*
Luca *et al*, 2016	n = 10F Age: 22.9 ± 1.0 years	FMD assessed EF, LF	↔ FMD for EF to LF	↑ E2 from EF to LF	None.

Table 3. Literature review of studies exploring the impact of estrogen on endothelium-dependent vasodilation assessed by RH-FMD, in premenopausal participants with natural menstrual cycles, in chronological order. M: menstrual; F: follicular; O: ovulation; L: luteal; EF: early follicular; LF: late follicular; EL: early luteal; LL: late luteal; FMD = flow mediated dilation.

2.2.4 Mechanism of the Impact of Estrogen on FMD

2.2.4.1 eNOS Phosphorylation and Expression

Animal and in vitro research on endothelial cells has demonstrated that estradiol will bind to estrogen receptors (ER), specifically ERα, on the endothelium (Figure 2). This results in enhanced non-genomic eNOS activation via protein kinase phosphorylation and enhanced production of NO (Caulin-Glaser *et al*, 1997; Lantin-Hermoso *et al*, 1997; Chen *et al*, 1999; Chambliss & Shaul, 2002; Darblade *et al*, 2002; Novensa *et al*, 2011). In vitro research has determined that ERα expression is enhanced in endothelial cells during periods of high estrogen exposure (Ihionkhan *et al*, 2002; Stirone *et al*, 2003). In humans, researchers have observed that ERα receptor expression is enhanced during the LF phase of the menstrual cycle of premenopausal women and decreased in postmenopausal women (Gavin *et al*, 2009). Furthermore, a strong relationship has been observed between ERα receptor expression and FMD (Gavin *et al*, 2009). Through its genomic effects, estrogen is also capable of increasing eNOS gene expression (Chambliss & Shaul, 2002). Taken together this indicates that estrogen upregulates eNOS and ERα receptor expression, while estrogen binding to ERα receptors increases eNOS phosphorylation, thereby improving NO bioavailability and FMD (Figure 2).

2.2.4.2 Antioxidant Effects.

Several animal and in vivo studies have observed antioxidant effects associated with increased estradiol (Arnal *et al*, 1996; Stehlow *et al*, 2003; Sitrone *et al*, 2005; Novensa *et al*, 2011; Haas *et al*, 2012). For example, a study by Arnal *et al* (1996) observed a dose-dependent decrease in superoxide anion (O_2^-) production in bovine aortic endothelial cells when treated with estradiol. Treatment with estradiol in a female mouse model has also demonstrated a decreased formation of superoxide anions (Novensa *et al*, 2011). Another ROS, hydrogen peroxide (H_2O_2), was decreased in the vessels of ovariectomized female rats, following estradiol treatment (Stirone *et al*, 2005). These studies provide preliminary evidence for the effects of estradiol treatment on decreasing ROS presence (Figure 2).

Additionally, the detoxification of ROS, such as superoxide anions and hydrogen peroxide, is catalyzed by several enzymes - superoxide dismutase and glutathione peroxidase, for example. Researchers have observed that estradiol treatment in ovariectomized female rats increases superoxide dismutase expression and activity in a concentration dependent manner (Stehlow *et al*, 2003; Stirone *et al*, 2005), and ovariectomized rats with estrogen deficiency experienced a downregulation of superoxide dismutase (Stehlow *et al*, 2003). Finally, in premenopausal women, glutathione peroxidase activity was heightened during the late follicular and early luteal phases, when estradiol is highest, compared to the early follicular phase (Massafra *et al*, 2000).

Overall, these results indicate that estrogen is capable of decreasing ROS presence and upregulating antioxidants. Given that NO reacts with superoxide anions to form peroxinitrite (NO_3^-), decreasing ROS would increase NO bioavailability (Figure 2). This may contribute to the enhanced endothelial function observed during periods of high estrogen.

2.3 Impact of Acute Hyperglycemia on Endothelial Function

Acute hyperglycemia, a transient increase in blood glucose levels, may have a detrimental effect on endothelial function assessed by FMD. This has been well established by the majority of studies using oral glucose tolerance tests (OGTT; 75g glucose) or test meals with high carbohydrate content (Akabari *et al*, 1998; Kawano *et al*, 1999; Title *et al*, 2000; Ceriello *et al*, 2002; Zhu *et al*, 2007; Baynard *et al*, 2009; Ceriello *et al*, 2011; Mah *et al*; 2011; Watanabe *et al*, 2011; Grassi *et al*; 2012; Suzuki *et al*, 2012; Nakayama *et al*, 2013; Mah *et al*, 2013; Wang *et al*, 2013; Greyling *et al*, 2015; Walsh *et al*, 2016), in the last two decades (Table 4). However not all studies have reported a negative effect (Bagg *et al*, 2000; Siafarikas *et al*, 2004; Dengel *et al*, 2007; Weiss *et al*, 2008; Xiang *et al*, 2008; Table 5). As summarized by Loader *et al* (2015) in a recent systematic review and meta-analysis, while it is generally agreed that acute hyperglycemia leads to a transient decrease in FMD in normoglycemic participants, there is considerable variability in the degree of impairment (18% to 64% FMD impairment) and time-course of impairment (1h-3h post-ingestion). The following section aims to 1) summarize key studies exploring the impact of acute hyperglycemia on FMD in participants with healthy glycemic control, 2) provide a potential explanation for contradicting findings, and 3) offer a summary of the evidence regarding mechanisms which may underlie the impact of acute hyperglycemia on the FMD response.

2.3.1 Evidence of Impaired FMD with Hyperglycemia

One of the first studies examining the impact of acute hyperglycemia on FMD was conducted by Kawano *et al* (1999). Participants with normal glucose tolerance, impaired glucose tolerance, and patients with type 2 diabetes mellitus underwent an oral glucose tolerance test (75 g of glucose), assessing brachial artery FMD before and 1h and 2h following ingestion. In the normal tolerance group (n=17, age: 52.6 ± 1.8 years), FMD decreased by ~44% 1h post-ingestion, returning to baseline levels after 2h (Pre: 7.53 ± 0.40%, 1h Post: 4.24 ± 0.28%, 2h Post: 6.35 ± 0.40%). Furthermore, a negative correlation was observed between the degree of hyperglycemia and %FMD assessed 1h and 2h post ingestion ($r = -0.61$, $p < 0.01$; Kawano *et al*, 1999).

Results in similarly aged participants with normal glucose tolerance have since been confirmed by Ceriello *et al*, (2002), Baynard *et al*, (2009), Ceriello *et al*, (2011), Nakayama *et al*, (2013), and Greyling *et al*, (2015) as depicted in Table 4. In these studies of middle aged participants (aged 44 – 57), FMD was consistently lower at 1h post glucose ingestion, returning to baseline measures at 2h post, where reported.

Soon after the work of Kawano *et al* (1999), Title *et al* (2000) conducted a study using the same techniques, but in a younger population (age: 25.5 ± 3.1 years) of males and females. These researchers found a similar ~43% impairment in FMD, but this was not observed until 2h post-ingestion. A hyperglycemia induced impairment in young, healthy participants has been confirmed in several studies Zhu *et al*, (2007), Mah *et al*, (2011), Watanabe *et al*, (2011), Grassi *et al*, (2012), Suzuki *et al*, (2012), Mah *et al*, (2013), and Walsh *et al* (2016), as depicted in Table 4 In these studies of younger people (22 - 33 years), FMD impairment varied from 18% to 36%, with impairments observed from 30min post-ingestion to 2h post-ingestion, with the lowest point usually at 1h post-ingestion, if multiple time points were reported.

Table 4. A summary of studies that have examined acute hyperglycemia induced impairments in FMD.

Citation	Population & Age	Study Design	FMD	Glucose/Insulin	Mechanistic Insight
Akabari *et al*, 1998	n = 20, 15M/5F Age: 32.3 [23-49]	FMD assessed pre- and 1h post 75g OGTT	↓ FMD 1h post	↑ Glucose @ 30 & 60 post	None.
Kawano *et al*, 1999	n = 17, 11M/6F Age: 52.6 ± 1.8 years	FMD assessed pre- and 1h, 2h post 75g OGTT	↓ FMD 1h post; returned to baseline, 2h post.	↑ Glucose & Insulin @ 1h, 2h post	↑ TBARS 1h post + correlation TBARS & glucose *Conclusion: Hyperglycemia increases oxidative stress indicator TBARS.*
Title *et al*, 2000	n = 10, 6M/4F Age: 25.5 ± 3.1 years	FMD assessed pre- and hourly to 4h post 75g OGTT	↓ FMD 2hpost; returned to baseline, 4h post.	↑ Glucose & Insulin @ 1h post	↔ Vitamin C/E attenuated decline with hyperglycemia *Conclusion: Antioxidants Vitamin C/E attenuate FMD decline with hyperglycemia.*
Ceriello *et al*, 2002	n = 20 12M/8F Age: 53.5 ± 2.5 years	FMD assessed pre- and 1h, 2h post 75g OGTT	↓ FMD 1h post; returned to baseline, 2h post.	↑ Glucose @ 1h post	↑ Nitrotyrosine (NT) @ 1h post *Conclusion: Hyperglycemia increases oxidative stress indicator NT.*
Zhu *et al*, 2007	n = 11M Age: 22.6 ± 2.3 years	FMD assessed pre- and hourly to 4h post 75g OGTT	↓ FMD 2hpost; returned to baseline, 4h post.	↑ Glucose @ 1h post; ↑ Insulin @ 1h, 2h post	↔ Malondialdehyde (MDA) *Conclusion: Hyperglycemia does not alter oxidative stress indicator MDA.*

Citation	Population & Age	Study Design	FMD	Glucose/Insulin	Mechanisms
Baynard *et al*, 2009	n = 13, obese M/F (ratio not reported) Age: 53 ± 1 years	FMD assessed 1h post meal: Ensure (80g carbohydrate, 12g fat 18g protein)	↓ FMD 1h post	↑ Glucose & Insulin @ 1h post	None.
Ceriello *et al*, 2011	n = 12 (6M/6F) Age: 50.5 ± 2.5 years	FMD assessed 1h, 2h post OGTT and test meal: 26.8g protein, 50.4g fat, 90g carbohydrates	↓ FMD 1h post; returned to baseline, 2h post in OGTT ↔ FMD in test meal	↑ Glucose @ 1h post OGTT ↔ Glucose @ 1h post-test meal ↑ Insulin @ 1h, 2h post for OGTT and test meal	↑ Nitrotyrosine (NT) @ 1h post for OGTT, not test meal *Conclusion: Hyperglycemia (OGTT, not test meal) increases oxidative stress indicator NT.*
Mah *et al*, 2011	n = 16 males Age: 21.6 ± 0.8 years	FMD assessed 30, 60, 90min post-OGTT or 75g fructose	↓ FMD 30, 60, 90 post- OGTT; lowest at 60 ↔ FMD in fructose	↑ Glucose @ 30, 60, 90, 120 in glucose; 30, 60 in fructose ↑ Insulin at 30, 60, 90, 120, 150 for both	↑ MDA 30, 60, 90, 120 in glucose; 30, 60, 90 in fructose ↓ Arginine in both groups, all time points *Conclusion: Hyperglycemia increases oxidative stress indicator MDA; decreases bioavailability of NO precursor arginine.*
Watanabe *et al*, 2011	n = 14 OGTT (8M/6F), Age: 33.4 ± 11.9 years n = 11 control (7M/4F), Age: 33.4 ± 8.4 years	FMD assessed 60, 120, 180 post-OGTT or placebo	↓ FMD 60, 120, 180 post-OGTT ↔ FMD in placebo	↑ Glucose @ 30, 60, 120 in OGTT ↑ Insulin at 30, 60, 120 in OGTT	None.

Citation	Population & Age	Study Design	FMD	Glucose/Insulin	Mechanisms
Grassi *et al*, 2012	n = 12 (5M/7F) Age: 28.2 ± 2.7 years	FMD assessed 1h, 2h, 3h post-OGTT; with white chocolate (WC) or dark chocolate (WC) 3day lead-in	↓ FMD 1h post-OGTT in WC ↔ FMD post-OGTT in DC	Not reported.	↑ Oxidative stress marker serum 8-iso PGF(2a) in WC ↔ serum 8-iso PGF(2a) in DC *Conclusion: Hyperglycemia increases oxidative stress indicator serum 8-iso PGF(2a) in WC, but not DC (antioxidant).*
Suzuki *et al*, 2012	n = 37, 19M/18F Age: 31.8 ± 9.2 years Three groups: test meal (n=12), oral glucose load (n=14), control (n=11)	FMD assessed 1h, 2h post-OGTT or test meal (CalorieMate energy supplement - 40.7g carbohydrate, 22.2g fat)	↓ FMD 1h, 2h post-OGTT ↓ FMD 1h post-test meal ↔ FMD in placebo	↑ Glucose @ 30, 60, 120 in OGTT only ↑ Insulin at 30, 60, 120 in OGTT only	None.
Nakayama *et al*, 2013	n = 23 M Age: 44 ± 10 years	FMD assessed 1h post-sugar drink (Maltose 75g + 0.45g citric acid in 300mL carbonated water)	↓ FMD 1h post-drink	Not reported	↔ FMD with Onion extract containing ~51mg quercetin (daily) for 30days *Conclusion: Antioxidant quercetin attenuates FMD decline with hyperglycemia.*

Citation	Population & Age	Study Design	FMD	Glucose/Insulin	Mechanisms
Mah *et al*, 2013	n = 15M Age: 21.8 ± 0.8 years	FMD assessed 30, 60, 90 post-OGTT	↓ FMD 30, 60, 90 post-OGTT; peak at 60	↑ Glucose and Insulin to peak at 30, return to baseline by 150min	↑ MDA 30, 60, 90, 120 ↔ FMD with y-tocepherol supplementation *Conclusion: Hyperglycemia increases oxidative stress indicator MDA; antioxidant y-tocepherol attenuates FMD impairment.*
Wang *et al*, 2013	n = 33 (12M/21F) Age: 52.62 ± 8.51 years	FMD assessed 2h post-OGTT	↓ FMD 2h post	↑ Glucose 2h post	↓ NO (Nitrate-Nitrogen), SOD post-ingestion ↑ MDA, endothelin-1 post-ingestion *Conclusion: Hyperglycemia increases oxidative stress and decreases NO availability.*
Greyling *et al*, 2015	n = 10M Age: 57 ± 9 years	FMD assessed 1h post-OGTT	↓ FMD 1h post	↑ Glucose 1h post	↔ FMD with heating of arm (↑ Shear rate) *Conclusion: Hyperglycemia impairment attenuated by increased shear stress via heating.*

Citation	Population & Age	Study Design	FMD	Glucose/Insulin	Mechanisms
Walsh *et al*, 2016	n = 12 (7M/5F) Age: 26 ± 2.5 years	FMD assessed 1h, 2h post-OGTT	↓ FMD 1h, 2h post	↑ Glucose & Insulin 30, 60, 90 post; peak at 30	↔ FMD with TUDCA ingestion. *Conclusion: Hyperglycemia impairment attenuated by antioxidant TUDCA ingestion.*

Table 4. Literature review of studies involving healthy participants with endothelium-dependent vasodilation assessed by RH-FMD, which observed impairments in FMD following an OGTT, in chronological order. M = male; F = female; FMD = flow mediated dilation; OGTT = oral glucose tolerance test.

2.3.2 Conflicting Evidence: No Impairment of FMD with Hyperglycemia

Despite ample evidence of an impairment in FMD during acute hyperglycemia, five studies have reported a lack of impairment (Bagg *et al*, 2000; Siafarikas *et al*, 2004; Dengel *et al*, 2007; Weiss *et al*, 2008; Xiang *et al*, 2008; Table 5). This may be due to a variety of factors. For example, the content of the test meal including potential antioxidants (Weiss *et al*, 2008) or administering the test meal using infusion (Bagg *et al*, 2000) may have impacted the results. Additionally, a study by Siafarikas *et al* (2004) observed no impairment in FMD following hyperglycemia, but reported a much lower magnitude of increase in glucose levels compared to other studies. Given the negative correlation between glucose and FMD observed by Kawano *et al* (1999), a lack of a robust increase in blood glucose may be the reason for this observed lack of FMD impairment.

Similarly, population may play a role in the lack of FMD impairment with hyperglycemia. For example, children may respond differently to hyperglycemia. A study by Dengel *et al* (2007) found an improvement in FMD 2h post-glucose ingestion. The authors speculate that acute impairments in endothelial function may require repeated hyperglycemia 'insults' over the aging process before the endothelium is negatively affected by an acute glucose stimulus. Additionally, studies by Weiss *et al* (2008) and Siafarikas *et al* (2004) included women without controlling for menstrual cycle. It is possible that null results were due to a protective effect of estrogen on the impact of acute hyperglycemia (Zhu *et al*, 2007).

Table 5. A summary of studies that have not observed acute hyperglycemia induced impairments in FMD.

Citation	Population & Age	Study Design	FMD	Glucose/Insulin	Mechanisms
Bagg *et al*, 2000	n = 10, 8M/2F Age: Median: 24 [19-35]	FMD assessed 1h post- infusion of 10% dextrose or saline (control)	↔ FMD with dextrose or saline infusion	↑ Glucose and insulin 1h post with dextrose, not saline	None.
Siafarikas *et al*, 2004	n=32 OGTT (11M/21F) n=7 Control (4M/3F) Age: 21.0 ± 1.8 years	FMD assessed 1h, 2h post-OGTT	↔ FMD with OGTT and control	↑ Glucose and insulin 1h, 2h post in OGTT group	None.
Dengel *et al*, 2007	n = 15 normal weight children (7M/8F) Age: 11.3 ± 0.4 years	FMD assessed 1h, 2h post- 1.75g/kg bodyweight, to a max of 75g	↑ FMD over time (until 120min)	↑ Glucose and insulin AUC increased; highest at 30min	None.
Weiss *et al*, 2008	n = 13 (5M/8F) Age: 48 ± 17 years	FMD assessed 1h post-meal: Snickers bar and lemon-lime Sprite (14g fat, 101g glucose, 28g fructose, 38g sucrose, 5g protein)	↔ FMD 1h post	↑ Glucose and insulin 1h post	↓ TBARS post *Conclusion: Hyperglycemia decreased indicator of oxidative stress TBARS.*

Citation	**Population & Age**	**Study Design**	**FMD**	**Glucose/Insulin**	**Mechanisms**
Xiang *et al*, 2008	n = 26 (14M/12F) Age: 50 ± 6 years	FMD assessed 1h, 2h post-OGTT	↔ FMD 1h, 2h post	↑ Glucose and insulin 1h, 2h post	↑ TBARS trend 1h post (not significant) ↔Nitrite/Nitrate *Conclusion: Hyperglycemia increased indicator of oxidative stress TBARS.*

Table 5. Literature review of studies involving healthy participants with endothelium-dependent vasodilation assessed by RH-FMD, which did not observed impairments in FMD following an OGTT, in chronological order. M = male; F = female; FMD = flow mediated dilation; OGTT = oral glucose tolerance test.

2.3.3 Mechanisms of FMD Impairment with Hyperglycemia

There is some evidence that acute hyperglycemia may decrease the bioavailability of endothelial-derived NO, resulting in endothelial dysfunction observed post-ingestion in most studies (Figure 2; Giugliano *et al*, 1997; Du *et al, 2001;* Wang *et al*, 2013). The most prevalent explanation for a decrease in NO bioavailability is increased production of reactive oxygen species (ROS), such as peroxinitrite and superoxide anion, which react with NO (Tesfamariam & Cohen, 1992; Cosentino *et al*, 1997; Konukoglu *et al*, 1997; Koska *et al*, 1997; Du *et al*, 1999). This hypothesis is supported by several findings (see Table 4 and 5, "Mechanisms" column). For example, Kawano *et al* (1999) observed an increase in thiobarbituric acid reactive substances (TBARS; a measure of ROS via lipid oxidation), post-glucose ingestion, coincident with the decrease in FMD. Furthermore, there was a positive correlation between TBARS and plasma glucose levels ($r = 0.62$, $p < 0.001$) and a negative correlation between TBARS and %FMD ($r = -0.58$, $p < 0.01$).

This finding is supported by Ceriello *et al* (2002) and Ceriello *et al* (2011) who found increases in nitrotyrosine, an indirect measure of peroxynitrite, at 1h post-glucose ingestion. This elevation corresponded with an impairment in FMD at 1h post-ingestion. Furthermore, Mah *et al* (2011) and Mah *et al* (2013) observed increases in malondialdehyde (MDA), an end product in lipid oxidation by ROS, at the same time points as impairments in FMD were observed. Additionally, there was a negative relationship between FMD AUC and plasma MDA AUC ($r = -0.80$), such that increases in MDA correlated with decreases in FMD (Mah *et al*, 2011; Mah *et al*, 2013). Finally, Wang *et al* (2013) found that NO and superoxide dismutase (SOD) were significantly decreased and MDA was significantly increased after glucose ingestion. SOD is an enzyme responsible for catalyzing the conversion of a superoxide radical into more stable ROS:

O_2 and hydrogen peroxide. A decrease in SOD following hyperglycemia may be responsible for the increase in ROS as less SOD is available to stabilize superoxide radicals (Wang et al, 2013).

Administration of antioxidants is another technique used to determine if oxidative stress contributes to the impairment observed with acute hyperglycemia (Tesfamariam & Cohen, 1992). For example, Title *et al* (2000) reported that the hyperglycemia-induced impairment of FMD was abolished with the administration of Vitamins C and E. This finding of an attenuation of hyperglycemia-induced impairments in FMD following antioxidant supplementation is consistent with other studies using different antioxidants including dark chocolate (Grassi *et al*, 2012), alpha-tocopherol and ascorbic acid (Sheikh-Ali *et al*, 2009), onion extract/quercetin (Nakayama *et al*, 2013), and y-tocopherol (Mah *et al*, 2013).

2.4 The Putative Interaction Between Estrogen and Hyperglycemia

The prior sections have reviewed evidence indicating that FMD is enhanced during the LF phase, but is acutely impaired with acute hyperglycemia. However, it is unknown how the impact of acute hyperglycemia on FMD interacts with menstrual cycle phase and associated fluctuating estrogen levels (EF vs LF phase). Given the research described above, acute hyperglycemia impairs FMD potentially via increasing oxidative stress (ROS; Figure 2). Since estrogen can function as an antioxidant, it is hypothesized that the hyperglycemia induced impairment in FMD will be attenuated during the late follicular (LF) phase when estradiol levels are elevated.

In support of this hypothesis, recent in vivo evidence by Haas *et al* (2012) explored the effects of hyperglycemia on superoxide generation, with and without the presence of estradiol. Haas *et al* (2012) found that hyperglycemia at supraphysiological levels (27.6 mmol/L) elevated superoxide anion production, which was then reduced significantly in the presence of estradiol.

This has been further supported by animal research in diabetic male rats, which observed amelioration of elevated glucose, insulin, and ROS levels during hyperglycemia when rats received estradiol supplementation (Hamden *et al*, 2008; Ahmed & Hassanein, 2012).

Although no study to date has explored the effects of hyperglycemia on FMD with varying estrogen levels in humans, some research on other acute insults may provide insight. For example, a recent study by Luca *et al* (2016) evoked ischemia and reperfusion (IR) injury during two phases of the menstrual cycle: EF and LF. IR blunted FMD during the EF phase, but this negative effect was abolished during the LF phase. Furthermore, they found a relationship between estradiol and IR-related dysfunction. However, a study by Harris *et al* (2012), which explored the impact of a high fat meal on FMD during the EF, LF, and LL phases of the menstrual cycle, was not consistent with the findings by Luca *et al* (2016). Instead, these researchers found that in contrast to men, who experienced impaired FMD following a high fat meal, the meal did not result in decreased FMD in women during any phase despite fluctuation in estradiol. The researchers concluded that regardless of menstrual phase, women were protected against the negative impact of a high fat meal, observed in age-matched males.

Taken together, this research suggests that estrogen exerts a protective influence against insults to endothelial function, although this protection may (Luca *et al*, 2016) or may not (Harris *et al*, 2012) be influenced by menstrual cycle fluctuation in estradiol. Both IR injury (Gao *et al*, 2008; Loukogeorgakis *et al*, 2010), a high fat meal (Bae *et al*, 2001; Tsai *et* al, 2004; Berry *et al*, 2008; Wallace *et al*, 2010), induced FMD impairment are thought to involve ROS, and protection from these insults via estrogen may involve estrogen's action as an antioxidant (Figure 2; Arnal *et al*, 1996; Stehlow *et al*, 2003; Sitrone *et al*, 2005; Novensa *et al*, 2011; Haas *et al*, 2012).

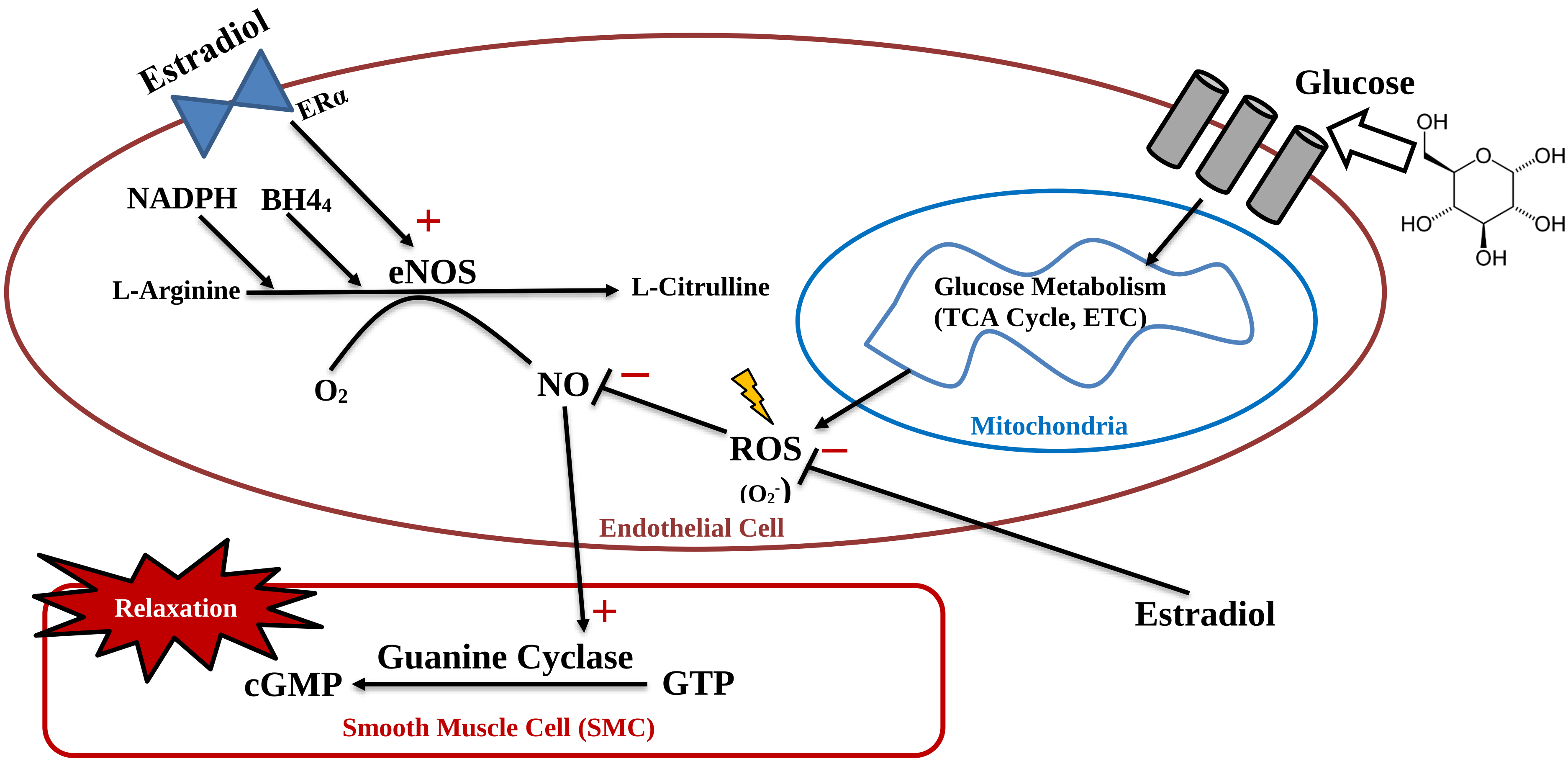

Figure 2. Schematic of hypothesized mechanisms underlying interaction between hyperglycemia and estradiol. Nitric oxide (NO) is produced by the conversion of L-Arginine to L-Citrulline, using oxygen (O_2), catalyzed by the enzyme endothelial nitric oxide synthase (eNOS; Palmer et al, 1988). NO activates guanine cyclase (GC) in smooth muscle cells (SMCs) to convert guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP), which, through a series of steps, results in relaxation of SMCs and subsequent vasodilation of the artery (FMD; Moncada et al, 1991). During acute hyperglycemia, glucose enters the endothelial cell and proceeds to the mitochondria for cellular respiration, producing energy and reactive oxygen species (ROS) byproducts (e.g. O_2^-; Tesfamariam & Cohen, 1992; Yu et al, 2006). ROS decrease the bioavailability of NO (Giugliano *et al*, 1997), reducing activation of GC and relaxation of SMCs. Increased estradiol levels (e.g. during the late follicular phase of the menstrual cycle) increases NO bioavailability through upregulation of eNOS (Caulin-Glaser et al, 1997; Chambliss & Shaul, 2002) and scavenging ROS via its antioxidant capacity (Sitrone *et al*, 2005; Haas *et al*, 2012), which partially or fully restores SMC relaxation and artery vasodilation.

2.5 Summary and Conclusions

In summary, this literature review has examined the separate roles of estrogen and acute hyperglycemia on endothelial function as assessed by FMD. This review has also provided evidence of a potential mechanism by which estrogen may offer protection against the effects of acute hyperglycemia when assessing endothelial function.

Based on the research provided in this review, there was an evident need for the proposed study, exploring the FMD response to acute hyperglycemia in premenopausal women during two phases of the natural menstrual cycle. No study to date had examined the impact of acute hyperglycemia on FMD in premenopausal women alone, and no study had compared this impact on FMD during phases of the menstrual cycle. Furthermore, a recent meta-analysis exploring the impact of meal consumption on FMD calls for the need for more research in premenopausal women, exploring sex differences and the impact of menstrual phase on the vascular effects of meal consumption (Thom *et al*, 2016).

Overall, given the reasons stated above, the study detailed in Chapter 3 provided novel and important contributions to the literature that surrounds acute hyperglycemia, estrogen fluctuations with the menstrual cycle, and FMD.

Chapter 3: Menstrual phase influences the acute hyperglycemia-induced impairment in brachial artery flow-mediated dilation.

The following thesis chapter has been formatted in accordance with the guidelines of the American Journal of Physiology: Heart and Circulatory Physiology.

Title: The influence of menstrual phase on acute hyperglycemia-induced impairment in brachial artery flow-mediated dilation.

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Abbreviations: EF, early follicular; LF, late follicular; $EF_{Control}$, early follicular water ingestion control visit; $EF_{Glucose}$, early follicular glucose ingestion visit; $LF_{Glucose}$, late follicular glucose ingestion visit; HR, heart rate; MAP, mean arterial pressure; FMD, flow-mediated dilation; SR, shear rate; SR 30sAUC, shear rate area under the curve 30 seconds post-deflation; ROS, reactive oxygen species; LH: luteinizing hormone; NO: nitric oxide.

Abstract (250/250 words)

Acute hyperglycemia transiently impairs endothelial function in healthy males when assessed via flow-mediated dilation (FMD). However, research in female participants is lacking and the impact of menstrual phase (early follicular (EF); late follicular (LF)) on vulnerability to acute hyperglycemia-induced endothelial dysfunction is unknown. Seventeen healthy, naturally menstruating women (21 ± 1 years [mean ± SD]) participated in three visits. During two visits ($EF_{Glucose}$; $LF_{Glucose}$), brachial artery FMD was assessed before and 60-, 90-, and 120-min after an oral glucose challenge (75g glucose). During an additional EF visit, participants ingested 300mL of water ($EF_{Control}$). Blood samples were taken to assess blood glucose, insulin, estradiol and progesterone. Glucose and insulin increased 30min post-glucose ingestion, with no difference between visits ($p < 0.001$). FMD did not change in $EF_{Control}$ ($p = 1.00$) but was reduced 90min post-glucose ingestion during $EF_{Glucose}$ (Pre: 8.7 ± 2.9%, Post90: 6.2 ± 2.8%, $p = 0.001$; Cohen's d = 0.864). FMD-impairment during $LF_{Glucose}$ was not significant (Pre: 8.4 ± 2.4%, Post120: 6.8 ± 2.7, $p = 0.089$; d = 0.649). A direct comparison of EF and $LF_{Glucose}$ delta-FMD did not reach significance ($p=0.117$) revealing a small effect of phase (d = 0.431) on vulnerability to hyperglycemia-induced FMD impairment. These results suggest that cyclic elevation in estrogen during the menstrual cycle provides a small degree of protection from acute hyperglycemia-induced endothelial dysfunction. To fully characterize the impact of acute stimuli on vascular function in premenopausal women, it is important to examine responses across multiple phases of the menstrual cycle.

Keywords: endothelial function, FMD, glucose regulation, vascular function, estrogen

New & Noteworthy (70/75 words)

This is the first study to examine the impact of acute hyperglycemia on endothelial function [flow-mediated dilation (FMD)] in premenopausal women across the menstrual cycle. FMD was impaired 90 min following glucose ingestion in the early follicular (EF) phase, however this impairment did not reach significance the late follicular (LF) phase. Overall the results suggest that the LF phase confers a small degree of protection from acute hyperglycemia-induced endothelial dysfunction.

3.1 Introduction

Ingestion of a substantial glucose load, either in the form of a meal or an isolated bolus of glucose (e.g. oral glucose tolerance test dose of 75g) results in a transient hyperglycemia that is exacerbated in the presence of metabolic disease (Kawano *et al*, 1999). Studies performed predominantly in men have typically shown that acute hyperglycemia transiently impairs endothelial function as assessed by flow-mediated dilation (FMD; Zhu *et al*, 2007; Mah *et al*, 2011; Wanatabe *et al*, 2011; Grassi *et al*, 2012; Suzuki *et al*, 2012; Mah *et al*, 2013; Walsh *et al*, 2016). This hyperglycemia-induced FMD impairment is observed in young healthy adults, as well as those with impaired glucose tolerance (Kawano *et* al, 1999; Loader *et al*, 2015), with the greatest reduction in FMD typically observed 1-2hrs following ingestion of the glucose load (Loader *et al*, 2015). These transient periods of endothelial dysfunction may become clinically important if they accumulate to result in a more chronic loss of vascular function with repeated exposures to hyperglycemia (Kawano *et al*, 1999).

In contrast to the negative impact of hyperglycemia, estrogen has well-established protective effects on endothelial function. For example, elevated synthetic estrogen levels appear to improve FMD in post-menopausal women on estrogen hormone replacement therapy (Liberman *et al*, 1994; Hashimoto *et al*, 2002; Sherwood *et al*, 2007), and in premenopausal women on oral contraceptive pills (Meendering *et al*, 2010; Thompson *et al*, 2011). Likewise, it has often been shown that FMD is augmented during the late follicular phase of the natural menstrual cycle, when estrogen (estradiol) levels are elevated (Hashimoto *et al*, 1995; Kawano *et al*, 1996; Adkisson *et al*, 2010; Harris *et al*, 2012). No study to date has examined the impact of acute hyperglycemia on endothelial function in women exclusively. It is therefore unclear how acute hyperglycemia influences FMD in premenopausal women and how cyclic estrogen fluctuation with the menstrual cycle impacts vulnerability to acute hyperglycemia-induced endothelial dysfunction.

Mechanistically, acute hyperglycemia appears to increase reactive oxygen species (ROS), which react with nitric oxide (NO) to decrease its bioavailability, thus reducing FMD (Tesfamariam & Cohen, 1992; Cosentino *et al*, 1997; Konukoglu *et al*, 1997; Koska *et al*, 1997; Kawano *et al*, 1999; Green *et al*, 2014). Estrogen may confer protection against the impact of hyperglycemia through its antioxidant effects (Hamden *et al*, 2008; Ahmed & Hassanein, 2012; Haas *et al*, 2012; Harris *et al*, 2012; Luca *et al*, 2016). This is supported by cell culture studies reporting that hyperglycemia-induced increases in ROS are suppressed by the introduction of estrogen (Hamden *et al*, 2008; Ahmed & Hassanein, 2012; Haas *et al*, 2012). Additionally, when estradiol levels are high in the late follicular phase, premenopausal women appear to be protected against FMD impairment with a ROS insult created with ischemia reperfusion injury (Gao *et al*, 2008; Loukogeorgakis *et al*, 2010; Luca *et al*, 2016). Taken together this evidence suggests that premenopausal women may be protected against hyperglycemia-induced impairment in FMD during cyclic elevations in estrogen.

The purpose of this study was to determine the impact of menstrual phase [early follicular (EF) vs. late follicular (LF) phases] on the FMD response to acute hyperglycemia. We hypothesized that FMD would be impaired in response to hyperglycemia during the EF phase when estradiol levels are low, but that this impairment would be attenuated during the LF phase when estradiol levels are high. Investigating the FMD response to acute hyperglycemia in a previously understudied population of premenopausal women is important to (i) characterize their response to acute hyperglycemia; and (ii) explore the role of estrogen in preventing the pathogenesis of hyperglycemia-induced vascular dysfunction.

3.2 Materials and Methods

3.2.1 Ethical Approval

All experimental procedures were approved by the Queen's University Health Sciences Research Ethics Board, which conforms to the standards set by the Declaration of Helsinki (with the exception that this study was not registered in a database). Prior to participation, volunteers provided written consent on forms approved by the same Board.

3.2.2 Participants and Screening Visit

17 healthy, non-smoking, recreationally active, women, between 20-29 years of age, recruited from the Queen's University and Kingston community completed this study. Prior to commencing the study, 26 volunteers came to the laboratory for a screening visit to become familiar with the laboratory environment and to assess their eligibility for participation. Volunteers were asked to complete a medical screening questionnaire, which included questions regarding their menstrual cycle history and pregnancy. Volunteers who reported cardiovascular or metabolic diseases, or taking medications, including oral contraceptives pills (n=2), were excluded. Volunteers were also excluded if their menstrual cycles were irregular (1 missed cycle in last 6 months). Height and weight were measured to determine body mass index (BMI), and volunteers were excluded if their BMI was greater than 30 kg/m^2. Blood pressure was assessed, characterized by the average of five measurements with an automated blood pressure assessment device (BpTRU Medical Devices, Coquitlam, BC). Volunteers were excluded if they were hypo- (<90/60 mmHg) or hypertensive (>140/90 mmHg). Volunteers were also excluded if self-reported physical activity exceeded three hours of structured moderate-to-vigorous activity per week (n=1). Volunteers were also familiarized with the study protocol and a brachial artery image and velocity signal were acquired to ensure that a strong signal and clear image could be

detected (n=3). Finally, volunteers were excluded if they were unable to complete one or more experimental visits, due to scheduling challenges (n=3).

3.2.3 Experimental Design

Participants attended three experimental visits ($EF_{Control}$, $EF_{Glucose}$ and $LF_{Glucose}$; Figure 3). Two visits occurred during the EF phase (days 2-6 following the onset of menses), and these visits were separated by approximately 48h. The third visit occurred during the LF phase when estradiol levels are typically at their highest level. The participant cycle length was estimated as the average length of their two previous menstrual cycles. In order to coincide with the LF phase, the third visit was scheduled 16 days prior to the anticipated last day of their cycle (Williams *et al*, 2001). Given that the luteal phase is on average 14 days from the time of ovulation to the next onset of menses, this placed the third visit ($LF_{Glucose}$) 2 days prior to the calculated ovulation date for each participant (Lenton *et al*, 1984; Reed and Carr, 2015). The LF phase was confirmed through use of a standard ovulation kit, with the $LF_{Glucose}$ visit occurring on or before the date of a positive ovulation test (Clearblue Easy Ovulation Predictor Kit). Ovulation was identified in 15/17 participants.

Participants were instructed to abstain from food consumption for 12h, to avoid alcohol and caffeine for 24h and to avoid exercise for 48h prior to each experimental visit. Participants were asked to consume the same meal prior to fasting before each experimental visit. Participants came to the laboratory in the morning (6am-10am), at the same time for each visit within participants, to control for diurnal variation in FMD (Ringqvist *et al*, 1999). Upon arrival, they were asked to lay supine with their arms extended for a 30min rest period (Figure 3). During the rest period, a blood draw was taken to assess fasting blood glucose and viscosity (all visits) as well as insulin, estradiol and progesterone ($EF_{Glucose}$ and $LF_{Glucose}$ visits only). This was followed by one standard reactive hyperemia FMD test. In the $EF_{Glucose}$ and $LF_{Glucose}$ visits participants then consumed an oral glucose challenge (75g glucose in a 300mL solution; Glucodex, Rougier). A second venous blood draw was taken 30min post-ingestion to assess blood glucose, insulin, and whole blood viscosity. In the $EF_{Control}$ visit, participants consumed an equivalent amount of water (300 mL; Pure Life, Nestle), and only blood glucose and whole blood viscosity were assessed were assessed 30min post-ingestion. FMD tests were then performed at 60min, 90min, and 120min post-ingestion in all visits.

3.2.4 Experimental Procedures

Heart Rate (HR) and Blood Pressure (BP). A three-lead electrocardiogram (ECG) was used to measure HR in beats per minute (bpm). This signal was recorded continuously in the program LabChart (ADInstruments, Colorado Springs, CO, USA). BP was characterized by the average of two measurements with an automated BP assessment device (BpTRU Medical Devices, Coquitlam, BC) before each FMD test.

Brachial Arterial Diameter & Velocity Measurements. Brachial artery diameter was measured using 2D ultrasound in B-mode (12MHz, Vivid i2 Medical Systems, Mississauga, Canada), using an intonation angle of 68°, as previously described (Pyke *et al*, 2008). Ultrasound images were recorded with a VGA to USB frame-grabber (Epiphan Systems Inc., Ottawa, Canada), and saved as avi files on a separate computer using Camtasia Studio (TechSmith, Okemos, MI, USA), as previously described (Jazuli & Pyke, 2011). Brachial artery blood velocity was measured using Doppler ultrasound (4MHz, Vivid i2 GE Medical Systems, Mississauga, Canada). The Doppler shift frequency spectrum was analyzed using a Multigon 500P TCD (Multigon Industries, Yonkers, NY, USA) and sampled continuously using Powerlab

(ADInstruments, Colorado Springs, CO, USA). The velocity signal was stored via LabChart for later analysis.

Reactive Hyperemia FMD. FMD trials were performed according to established guidelines (Thijssen *et al*, 2011a). A pneumatic cuff was placed around the left forearm, distal to the location of the ultrasound probe. Brachial artery diameter and blood velocity measurements were recorded during 1 min of baseline prior to cuff inflation to 250 mmHg for 5min. Diameter and velocity measurements were recorded in the last minute of cuff inflation and for 3min following cuff release.

3.2.5 Data Analysis

Blood Glucose and Hormone Analysis. Blood glucose, insulin, 17β-estradiol and progesterone were analyzed in the CORE laboratory at Kingston General Hospital. All samples were same-day tested. Plasma insulin levels were assessed using a chemiluminescent microparticle immunoassay and glucose levels using a glucose assay, per manufacturer instructions (ARCHITECT Systems 16000, Abott Laboratories, Abott Park, IL, USA). Estradiol levels were determined using the Beckman Coulter UniCel Dxl 800 Access Immunoassay system, with a detection limit of <100 pmol/L. Progesterone levels were determined using a using a chemiluminescent microparticle immunoassay, per manufacturer instructions, with a detection limit of <1.0 nmol/L.

Whole Blood Viscosity Analysis. Whole blood viscosity was measured in duplicate, and the average of the two values is reported. Analysis was performed within 15 minutes of blood sample acquisition at a shear rate of 225 s^{-1} at 37°C with a cone-and-plate viscometer (Brookfield DV-II+ Pro, Brookfield AMETEK, USA).

Insulin Resistance Index (HOMA-IR). Insulin resistance was estimated using the homeostatic model assessment for insulin resistance (HOMA-IR), using the following calculation: fasting insulin (uU/L) x fasting glucose (nmol/L)/22.5 (Matthews et al, 1985).

HR and MAP. Resting HR is reported as the one-minute average recorded during the baseline minute of each FMD test. Resting MAP is reported as the average of two BP assessments prior to each FMD test, using the following formula: MAP = [systolic blood pressure + 2(diastolic blood pressure)]/3.

Brachial Artery Diameter & Blood Velocity Analysis. Diameter analysis was completed by a single researcher (J.S.W.) blinded to FMD trials (pre-ingestion, 60min post-ingestion, 90min post-ingestion, 120min post-ingestion) and to visit [experimental visits 1 ($EF_{Control}$), 2 ($EF_{Glucose}$) and 3 ($LF_{Glucose}$)]. Artery diameter was analyzed using automated edge-detection software (Encoder FMD and Bloodflow v3.0.3, Reed Electronics, OK, United Kingdom) as described previously (Jazuli & Pyke, 2011). Diameter values were compiled into three-second time bins for FMD and shear rate analysis. Blood velocity was analyzed in three-second average time bins using LabChart software (AD Instruments, CO, USA), as described previously (Pyke *et al*, 2008).

Shear Rate (SR). Given that whole blood viscosity levels were unchanged across visits and time (results below), shear rate was used to estimate shear stress. SR was calculated using the time aligned three-second average blood velocity and diameter time bins, using the following equation: mean blood velocity/artery diameter. The SR stimulus was evaluated as an area-under-the-curve (SR-AUC) in the 30s following cuff-release (SR 30sAUC; Pyke and Tschakovsky, 2007; D'Urzo et al, 2018). Baseline SR is reported as the average shear rate in the 1 minute prior to cuff inflation.

Flow-mediated dilation (FMD). FMD is reported as both an absolute change (absFMD) and as a percent change (%FMD) in diameter. AbsFMD is reported as the difference in millimeters between the baseline minute and the peak three-second average diameter bin, post-cuff release. %FMD was calculated as the %difference between these values.

3.2.6 Statistical Analysis

All analysis was performed using SPSS, Version 24 (IBM, Chicago, IL, USA). All data are reported as mean ± standard deviation (SD), and statistical significance was set as $p < 0.05$. A linear mixed model (compound symmetry co-variance structure) with factors time (pre-ingestion, 60min post-ingestion, 90min post-ingestion, 120min post-ingestion) and visit [experimental visits 1 ($EF_{Control}$), 2 ($EF_{Glucose}$) and 3($LF_{Glucose}$)] was used to examine %FMD, AbsFMD, SR baseline and SR 30sAUC, HR, and MAP. To further explore phase differences in the impact of acute hyperglycemia on %FMD, a paired t-test was used to compare the delta %FMD impairment at the nadir in the $EF_{Glucose}$ and $LF_{Glucose}$ visits (90min and 120min, respectively). A linear mixed model (compound symmetry co-variance structure) with factors time (pre-ingestion, 30min post-ingestion) and visit [experimental visits 1 ($EF_{Control}$), 2 ($EF_{Glucose}$) and 3($LF_{Glucose}$)] was used to examine blood glucose and blood viscosity. The same test was used to examine blood insulin levels with the exception that the $EF_{Control}$ visit was not included as insulin was not assessed. Paired t-tests were used to compare HOMA-IR measures between the $EF_{Glucose}$ and $LF_{Glucose}$ visits. Due to a large proportion of values falling below the detectable limits for the estradiol and progesterone assays in one or more visits, these data are described without accompanying statistical analysis. For some variables, the full n of 17 was unavailable due to technical issues; therefore, the n included in the analysis for each variable is provided in the table or figure legend. Participants with more than one missing assessment were excluded from the analysis of that variable.

3.3 Results

3.3.1 Participant Characteristics

Participants were 21 ± 1 years old with a BMI of 22.2 ± 3.5 kg/m^2. The average menstrual cycle length was 30 ± 5 days (range: 21 – 42 days), with the average ovulation day detected on cycle day 16 ± 3 (range: 10 – 22). The average number of days from the LF visit to ovulation was 3 ± 2 days (range: 0 – 8).

3.3.2 Blood Analysis: Hormones, Glucose, Insulin, Viscosity

Blood samples for estradiol and progesterone were obtained and analyzed in 13 of 17 participants. In 11/13 participants during the $EF_{Glucose}$ visit and 2/13 participants during $LF_{Glucose}$ visit, estradiol levels fell beneath the detectable limit of the assay (100 pmol/L). In the remaining 11 participants in the $LF_{Glucose}$ visit, estradiol concentration was well above 100 pmol/L, indicating a substantial increase in circulating estradiol in the LF phase (Table 6). In 8/13 participants during the $EF_{Glucose}$ visit and 10/13 participants during the $LF_{Glucose}$ visit, progesterone levels fell beneath the detectable limit of the assay (1 nmol/L). Overall blood progesterone levels were low and appeared to change little from the $EF_{Glucose}$ to the $LF_{Glucose}$ visit.

HOMA-IR measures were unaltered from the $EF_{Glucose}$ to the $LF_{Glucose}$ visit ($p = 0.650$; Table 6). Blood glucose levels remained unaltered across time during the $EF_{Control}$ but were significantly elevated 30min post-ingestion during the $EF_{Glucose}$ and $LF_{Glucose}$ visits, with no differences between visits ($p < 0.001$; Figure 4A). Similarly, blood insulin levels were significantly elevated 30min post-ingestion during the $EF_{Glucose}$ and $LF_{Glucose}$ visits, with no differences between visits ($p < 0.001$; Table 6). Whole blood viscosity was unchanged across visits and time (Figure 4B).

3.3.3 Baseline Hemodynamic Variables (HR, MAP)

Baseline HR was significantly elevated (~6 bpm) during the $EF_{Glucose}$ and $LF_{Glucose}$ visits following the ingestion of glucose ($p < 0.001$, Pre vs all Post; Table 7), but was unaltered during the $EF_{Control}$ visit (Table 7). Baseline MAP was significantly elevated (~3 mmHg) at Post120 vs. Pre (main effect of time, $p = 0.019$), with no differences between visits (Table 7).

3.3.4 Baseline SR and SR Stimulus (SR 30sAUC)

Baseline SR was significantly lower at Post90 and Post120 vs Pre ($p = 0.037$ and $p = 0.003$, respectively), with no differences between visits (Table 7). The SR stimulus (30sAUC) remained unaltered across visits and time (Table 7).

3.3.5 Baseline Diameter and FMD

Baseline diameter remained unaltered across visits and time (Table 7). The interaction between visit and time for %FMD approached significance ($p = 0.054$). Exploring this near-significant interaction, the $EF_{Control}$ visit Pre %FMD was significantly lower than the $EF_{Glucose}$ and $LF_{Glucose}$ visit Pre %FMD ($p = 0.002$ and $p = 0.009$, respectively; Figure 5A). There were no significant differences in %FMD over time during the $EF_{Control}$ visit (Figure 5A). During the $EF_{Glucose}$ visit, %FMD was significantly lower at Post90 compared to Pre ($p = 0.001$; Cohen's d =0.864) and Post60 ($p = 0.023$, Cohen's d = 0.631; Figure 5A). During the $LF_{Glucose}$ visit, while no significant differences over time were detected, there was a trend for lower %FMD at Post120 vs Pre (p = 0.089, Cohen's d = 0.649; Figure 5A). Delta %FMD at the nadir time point was not significantly different between the $EF_{Glucose}$ and $LF_{Glucose}$ visits ($EF_{Glucose}$ visit (Post90): -2.5 ± 2.6%; $LF_{Glucose}$ visit (Post120): -1.7 ± 2.5%, p = 0.117, Cohen's d = 0.431; Figure 5B). AbsFMD results were similar to %FMD (Table 7).

3.4 Discussion

This study is the first to explore the impact of menstrual phase (EF vs LF phase) on the FMD response to acute hyperglycemia. In line with our hypothesis, acute hyperglycemia significantly impaired FMD during the EF phase, while impairment did not reach significance in the LF phase. Comparing the delta FMD between phases suggests that the LF phase confers a small protective effect against hyperglycemia induced FMD impairment (d=0.431). These results were observed in the absence of phase differences in glucose handling or the shear stress stimulus. Protection during the LF phase may be due to the impact of elevated estradiol and these findings indicate that it is important to assess the impact of acute stimuli on endothelial function during multiple phases of the menstrual cycle in order to fully characterize responses in premenopausal women.

Impact of acute hyperglycemia on FMD

The present study demonstrates an acute hyperglycemia-induced impairment in FMD during the EF phase at 90min post-ingestion (Figure 5A). This impairment agrees with studies in men which have consistently reported FMD impairment between 30min-2h following ingestion of a glucose load (Loader *et al*, 2015). The magnitude of impairment observed in this study was ~26% at 90min post-ingestion (Cohen's d = 0.864, large effect size), consistent with observations in similar studies of young participants (22-33 years; predominantly men) without metabolic disease (-18% to -36% impairment; Zhu *et al*, 2007; Mah *et al*, 2011; Wanatabe *et al*, 2011; Grassi *et al*, 2012; Suzuki *et al*, 2012; Mah *et al*, 2013; Walsh *et al*, 2016). In contrast with our observations in the EF phase, in the LF phase the reduction in FMD post-glucose ingestion failed to reach significance, suggesting an attenuation in the degree of impairment (at its nadir: Post120; p = 0.089 vs. Pre; Cohen's d = 0.649, moderate effect size; Figure 5A). However, when directly comparing the magnitude of FMD impairment between the $EF_{Glucose}$ and $LF_{Glucose}$ visits, the difference did not reach significance (Delta % FMD p = 0.117; Cohen's d = 0.431, small effect size; Figure 5B). This suggests that 45 participants would be required to detect the phase difference in delta FMD as significant (alpha 0.05; power 0.8). Taken altogether, these data suggest that during the LF phase there is a small reduction in the vulnerability to hyperglycemia induced FMD impairment.

Previous studies that have included women have not controlled for the potential impact of the menstrual cycle and this may have impacted their conclusions (Siafarikas *et al*, 2004; Weiss *et al*, 2008; Watanabe *et al*, 2011; Grassi *et al*, 2012; Suzuki *et al*, 2012). For example, studies by Siafarikas and colleagues (2004) and Weiss and colleagues (2008) demonstrated a lack of FMD impairment following hyperglycemia in mixed samples of healthy young men and women. However, female participants may have been in their LF phase during testing and this may have attenuated the hyperglycemia-induced impairment, contributing to the negative findings at the group level. To further support this, the only mixed sex study that controlled for phase (females tested during the EF phase (7M/5F, age: 26 ± 3 years)), found impairment in FMD at 60 and 120min post-glucose ingestion (Walsh *et al*, 2016).

Impact of menstrual phase on FMD

In the present study, menstrual phase did not impact baseline FMD, demonstrated by the similar pre-glucose ingestion FMD in the $EF_{Glucose}$ and $LF_{Glucose}$ visits (Figure 5A). This is inconsistent with the dominant understanding that FMD is elevated in the LF phase (Hashimoto *et al*, 1995; Kawano *et al*, 1996; Williams *et al*, 2001; Adkisson *et al*, 2010; Thijssen et al, 2011a; Harris *et al*, 2012). In contrast to seminal investigations, and in agreement with the current findings, the majority of recent studies have not detected changes in FMD across the menstrual cycle in healthy young women (Saxena *et al*, 2012; Rakobowchuck *et al*, 2013; Luca *et al*, 2016; D'Urzo *et al*, 2017; Shenouda *et al*, 2018). Elevated FMD during the LF phase is typically attributed to the impact of elevated estradiol levels on increasing NO bioavailability (Cicinelli et al, 1996; Adkisson et al, 2010). Studies may not always capture participants at their peak level of estradiol during LF phase assessments, and it is possible that variation in circulating estradiol concentration contributes to inter-study variability in the impact of phase on FMD.

While the estradiol levels in the LF phase of the current study were relatively low ((~290 pmol/L, Table 6; despite proximity to ovulation (~3 days prior)) overall, studies that have identified elevated FMD in the LF phase relative to the EF phase have reported similar LF estradiol levels to studies reporting no impact of phase on FMD (elevated FMD: ~560 pmol/L (Hashimoto *et al*, 1995; Kawano *et al*, 1996; Williams *et al*, 2001; Adkisson *et al*, 2010; Harris *et al*, 2012) vs. no change in FMD: ~580 pmol/L (Saxena *et al*, 2012; Rakobowchuck *et al*, 2013; Luca *et al*, 2016; D'Urzo *et al*, 2017; Shenouda *et al*, 2018)). Further, studies to date, including recent studies by our group (D'Urzo et al, 2017; Shenouda et al, 2018; current study, data not shown) have failed to identify a relationship between circulating estradiol levels and FMD. Taken together, this suggests that insufficient elevation in estradiol during the LF phase FMD assessment does not fully explain the absence of phase differences in FMD. Estradiol exerts its impact on endothelial cells via binding to the estradiol receptor alpha (Caulin-Glaser et al, 1997). It is possible that downstream variability in estradiol receptor alpha expression across the menstrual cycle is more important to the impact of phase on FMD than variability in circulating estradiol per se (Gavin et al, 2009; Shenouda et al, 2018).

An unexpected result from this study was that the $EF_{Control}$ visit pre-ingestion FMD (day 3 ± 1 of EF phase) was lower than the pre-ingestion FMD observed in the $EF_{Glucose}$ visit (day 5 ± 1 of EF phase) and the $LF_{Glucose}$ visit (Figure 5A). Given that mental stress can result in FMD impairment (Ghiadoni *et al*, 2000), it is possible that some participants experienced a blunted FMD during the first visit (always $EF_{Control}$) due to stress induced by the novel laboratory experience, including a phlebotomy procedure. The level of stress may have abated in the $EF_{Glucose}$ visit (always the second visit), given greater familiarity with the procedures and laboratory environment. We did not measure cortisol levels or sympathetic nervous activity, which may contribute to stress-induced FMD impairment (Poitras and Pyke, 2013). However, pre-ingestion baseline HR and MAP were not elevated in $EF_{Control}$ relative to $EF_{Glucose}$ (Table 7), suggesting that if participants were 'stressed' in their first visit, it did not manifest as a hemodynamic response. Another possibility is that there were physiologically-relevant differences in estradiol levels in the two EF visits (~days 3 and 5 of the cycle; Schliep et al, 2014), and a higher level of estradiol later in the EF phase ($EF_{Glucose}$ visit) may have had a positive influence on FMD. However, estradiol levels were not assessed in the $EF_{Control}$ visit and were below our assay detection limit of 100pmol/L in the majority of participants in the $EF_{Glucose}$ visit. Recent unpublished work by our group suggests that FMD is stable over 3 visits within the

EF phase however further research regarding the potential for systematic variability in FMD over the 7 days of the EF phase may be needed.

Putative mechanisms responsible for menstrual phase differences in the impact of acute hyperglycemia on FMD

The small effect of phase on vulnerability to acute hyperglycemia-induced FMD impairment could be related to glucose handling, however, blood glucose and insulin levels did not differ between the $EF_{Glucose}$ and $LF_{Glucose}$ visits (Table 6, Figure 4A). In contrast, and as expected, estradiol levels were higher in the LF phase (rose above the assay minimum detection limit; Table 6). Although we were not able to assess a relationship between phase changes in estrogen and the impact of hyperglycemia on FMD, it is possible that estrogen may have influenced the hyperglycemia-endothelial function interaction (Cicinelli et al, 1996; Adkisson et al, 2010).

Acute hyperglycemia-induced impairment in FMD appears to be mediated by an increase in ROS, which may decrease NO bioavailability (Tesfamariam & Cohen, 1992; Konukoglu *et al*, 1997; Koska *et al*, 1997; Kawano *et al*, 1999). However, elevated estradiol levels may provide protection against ROS, through estrogen's antioxidant effects (Hamden *et al*, 2008; Ahmed & Hassanein, 2012; Haas *et al*, 2012). This mechanism is supported by cell culture studies, which report a suppression of the hyperglycemia-induced increase in ROS with the introduction of estrogen (Hamden *et al*, 2008; Ahmed & Hassanein, 2012; Haas *et al*, 2012). Furthermore, while this is the first study in humans to explore the impact of acute hyperglycemia on FMD in different phases of the menstrual cycle, a recent study by Luca et al (2016) demonstrated protection of FMD from another acute ROS insult (ischemia reperfusion injury) in the LF phase. Taken together, this suggests that during the LF phase, estrogen may have exerted a protective influence against a ROS insult evoked by acute hyperglycemia; however, further research is necessary to confirm this mechanistic hypothesis.

Methodological Considerations

While the within visit analysis only identified a significant impairment in FMD in the EF and not the LF phase, we were underpowered to detect a significant phase difference with a direct comparison of delta FMD. Future studies that aim to explore what appears to be a subtle impact of menstrual phase on hyperglycemia induced FMD impairment would benefit from recruiting a larger sample. The present study did not include a water ingestion control visit in the LF phase, due to the difficulty in scheduling two visits within the short period of substantially elevated estradiol levels prior to ovulation (Owen *et al*, 1975; Shenouda *et al*, 2018). Similar studies exploring menstrual phase differences in FMD with acute interventions have also omitted a control visit during the LF phase (high fat meal: Harris *et al*, 2012; ischemia reperfusion: Luca *et al*, 2016). However, the EF phase control visit indicates that FMD remains stable over ~2.5 hours while resting in the laboratory in the absence of glucose ingestion (Figure 5A). With respect to blood analysis, the minimum detectable limits in the estradiol and progesterone assays contributed to a lack of resolution when detecting low levels of circulating hormones (Table 6). It is possible that small but important changes in circulating hormones across visits were missed. Additionally, we did not include an assessment of endothelial-independent FMD as an indicator of smooth muscle function; as a result, we cannot conclusively determine whether impaired FMD in the early follicular phase during hyperglycemia is due to endothelial dysfunction or smooth muscle dysfunction. However, smooth muscle function appears to be preserved during hyperglycemia (Loader et al, 2015) and across the menstrual cycle (Shenouda et al, 2018). Finally, we did not assess levels of NO or ROS, and thus cannot make conclusions regarding a mechanistic role of these factors in what appears to be a small effect of phase on vulnerability to hyperglycemia-induced FMD impairment. Future research is needed to explore the potential for a protective antioxidant effect of estrogen in the LF phase.

Conclusion

Acute hyperglycemia impaired endothelial function in premenopausal women during the EF phase of the menstrual cycle, consistent with previous research in men (Loader *et al*, 2015). However, the brachial artery exhibited a small degree of protection against this impairment when estradiol levels were elevated in the LF phase. This research indicates the importance of assessing the vascular impact of acute stimuli during multiple phases of the menstrual cycle. Future research is needed to examine the impact of acute hyperglycemia on endothelial function in female populations with different exposure to estrogen including those prescribed synthetic hormones in the form of oral contraceptive pills, or hormone replacement therapy.

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Table 6. Blood analysis: estradiol, progesterone, HOMA-IR, and insulin during EF and LF phase glucose ingestion visits.

	$EF_{Glucose}$		$LF_{Glucose}$		p-values
	Pre-Glucose	**Post-Glucose**	**Pre-Glucose**	**Post-Glucose**	
Estradiol (pmol/L) n=13	n=11: <100 n=2: 108, 166		n=2: <100 n=11: 293 ± 247		
Progesterone (nmol/L) n=13	n=8: <1.0 n=5: 1.5 ± 0.5		n=10: <1.0 n=3: 1.5 ± 0.5		
HOMA-IR n=13	1.7 ± 1.0		1.6 ± 0.4		p = 0.650
Insulin (pmol/L) n=8	62 ± 34	543 ± 240	48 ± 11	626 ± 341	**T p < 0.001** V p = 0.577 T*V p = 0.434

Values are mean ± SD. Paired t-test p value is provided for HOMA-IR. P-values for insulin reflect the main effects and interactions from the linear mixed model. T: Time. V: Visit. EF: early follicular phase. LF: late follicular phase. Significant findings are bolded.

Table 7. Hemodynamic & FMD variables during the three visits.

		HR (bpm) n=17	MAP (mmHg) n=10	Baseline SR (s^{-1}) n=17	SR 30sAUC (s^{-1}) n=16	Baseline Diameter (cm) n=17	AbsFMD (cm) n=17
$EF_{Control}$	**Pre**	62.3 ± 6.8	73.7 ± 7.8	17.6 ± 10.1	4070 ± 1725	0.285 ± 0.029	0.019 ± 0.006
	Post60	62.4 ± 7.5	75.9 ± 7.1	15.9 ± 7.9	4125 ± 1213	0.283 ± 0.026	0.020 ± 0.007
	Post90	63.7 ± 8.3	73.9 ± 6.2	15.7 ± 7.9[#]	4039 ± 1208	0.283 ± 0.029	0.019 ± 0.007
	Post120	64.0 ± 7.3	76.5 ± 6.8^	13.9 ± 7.4[#]	3873 ± 1236	0.281 ± 0.025	0.019 ± 0.006
$EF_{Glucose}$	**Pre**	62.0 ± 8.7	74.0 ± 7.8	23.6 ± 19.2	4334 ± 1298	0.284 ± 0.027	0.024 ± 0.006
	Post60	67.1 ± 9.3*	74.8 ± 6.8	19.0 ± 10.1	4010 ± 1475	0.281 ± 0.027	0.023 ± 0.008
	Post90	67.6 ± 8.1*	75.4 ± 8.4	16.8 ± 9.5[#]	3759 ± 1274	0.286 ± 0.026	0.018 ± 0.008[$]
	Post120	68.4 ± 8.2*	74.2 ± 4.6^	15.2 ± 7.6[#]	3985 ± 1268	0.285 ± 0.028	0.022 ± 0.009
$LF_{Glucose}$	**Pre**	61.5 ± 6.7	71.3 ± 5.4	21.7 ± 15.6	4086 ± 1329	0.286 ± 0.025	0.024 ± 0.007
	Post60	66.2 ± 7.7*	73.0 ± 6.3	17.5 ± 8.4	4173 ± 1180	0.278 ± 0.027	0.021 ± 0.008
	Post90	67.4 ± 7.6*	75.6 ± 5.2	15.4 ± 10.2[#]	3914 ± 1487	0.286 ± 0.027	0.020 ± 0.007
	Post120	69.2 ± 8.7*	76.6 ± 5.2^	13.1 ± 4.5[#]	3491 ± 925	0.286 ± 0.027	0.019 ± 0.008
p-values		**T p < 0.001** **V p < 0.001** **T*V p = 0.044**	**T p = 0.026** V p = 0.495 T*V p = 0.148	**T p = 0.004** V p = 0.146 T*V p = 0.857	T p = 0.478 V p = 0.926 T*V p = 0.928	T p = 0.589 V p = 0.072 T*V p = 0.836	**T p = 0.007** **V p = 0.014** T*V p = 0.066

Values are mean ± SD. p-value denotes main effects and interactions of factors Time (T), and visit (V). Significant main effects and interactions are bolded. Pre = Pre-ingestion, Post60 = 60min post-ingestion, Post90 = 90min post-ingestion, Post120 = 120min post-ingestion. SR = shear rate. AUC = area under the curve. HR = heart rate. MAP = mean arterial pressure. AbsFMD = absolute FMD. EF = early follicular phase; LF = late follicular phase. Post hoc analysis exploring the interaction between visit and time for HR revealed that HR was significantly higher than Pre in all post glucose ingestion time points (*). Post hoc analysis exploring the main effect of time for MAP revealed that MAP during Post120 was significantly higher than Pre (^). Post hoc analysis exploring the main effect of time for baseline SR revealed baseline SR was lower Post90 and Post120 vs. Pre (#). Post hoc analysis exploring the near significant interaction between visit and time for AbsFMD revealed that AbsFMD was lower Post90 vs Pre during the $EF_{Glucose}$ visit only ($).

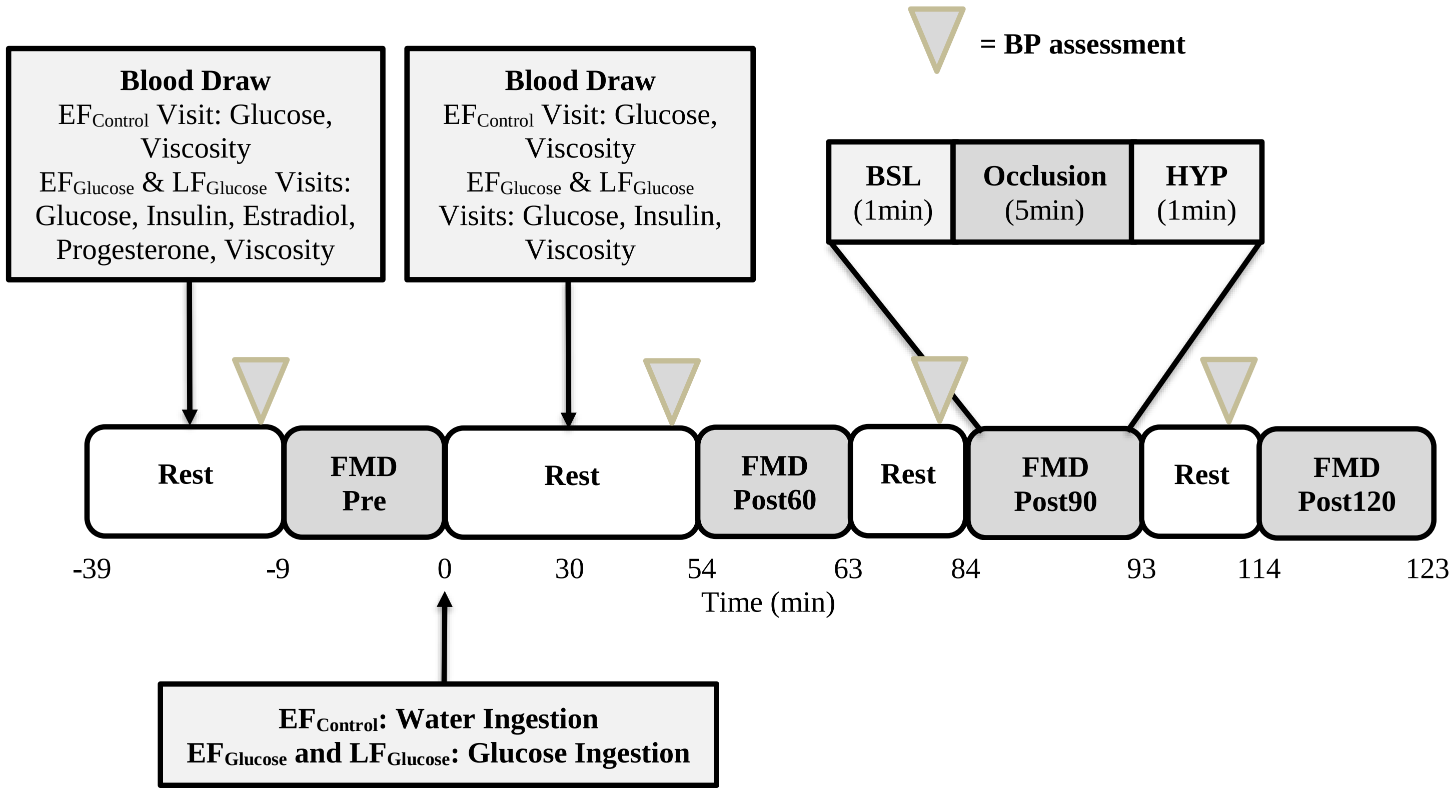


Figure 3. Experimental visit timeline. $EF_{Control}$ and $EF_{Glucose}$ visits occurred during the early follicular phase, whereas the $LF_{Glucose}$ visit occurred during the late follicular phase of the menstrual cycle. FMD: reactive hyperemia flow-mediated dilation; Pre = Pre-ingestion, Post60 = 60min Post-ingestion, Post90 = 90min Post-ingestion, Post120 = 120min Post-ingestion; BSL: baseline; HYP: hyperemia; BP = blood pressure assessment.

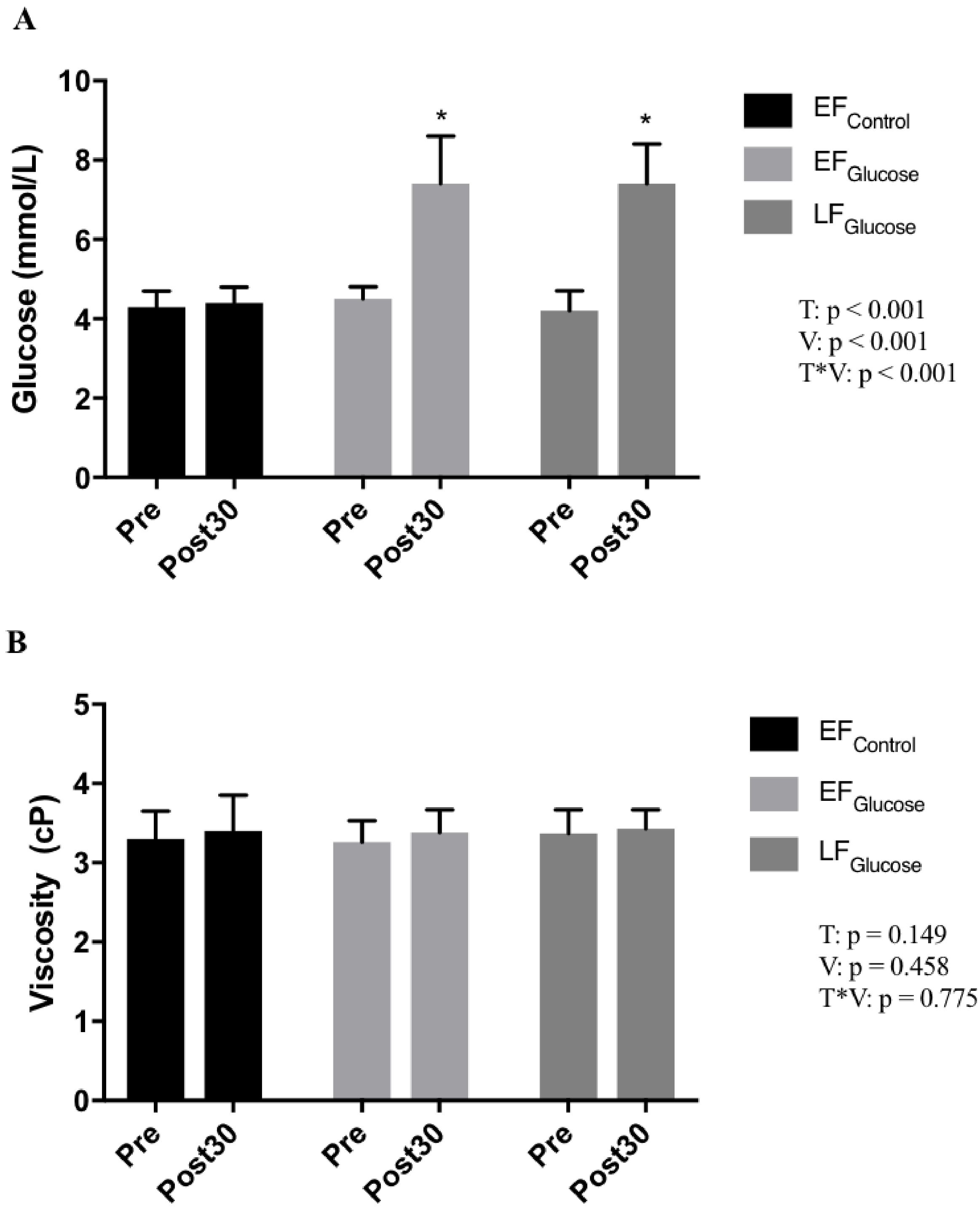
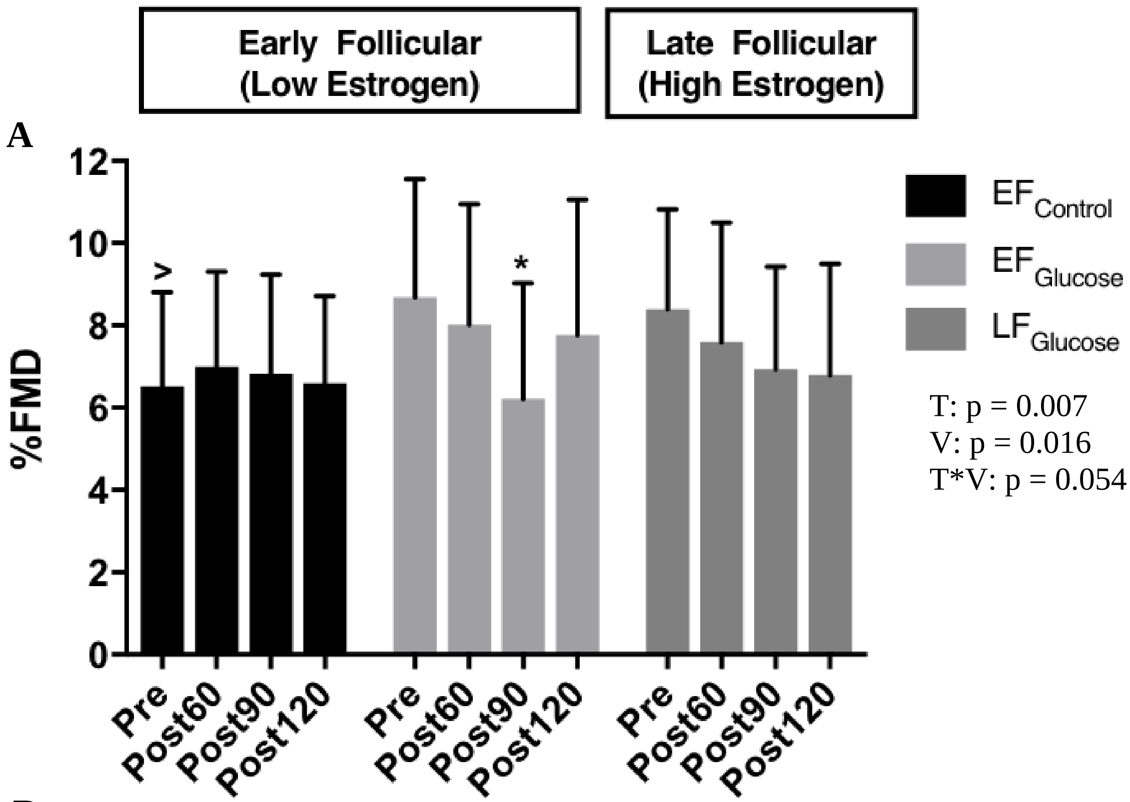


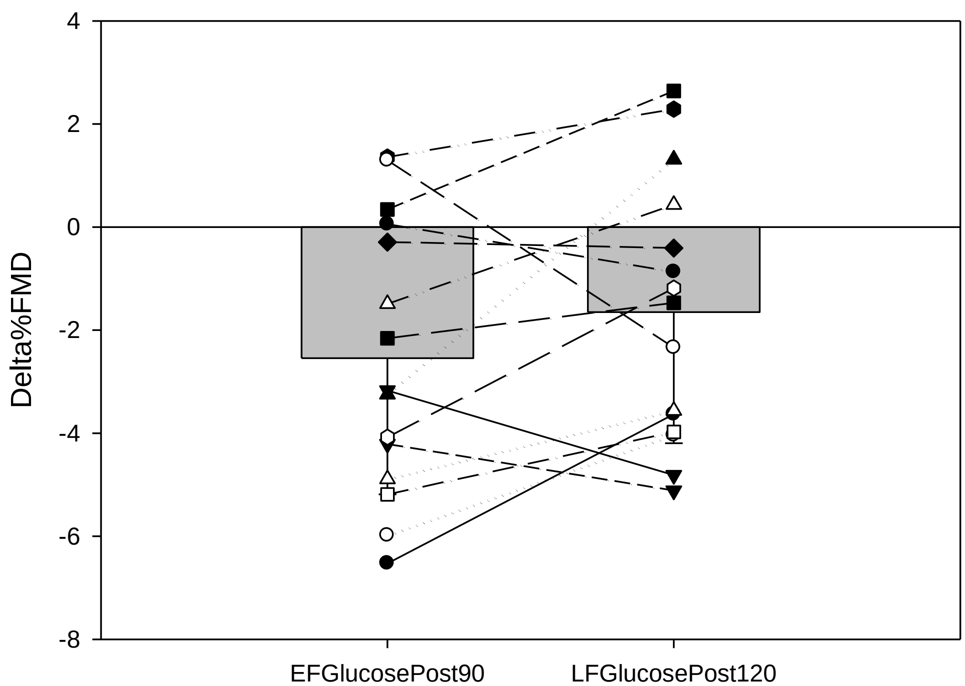
Figure 4. Blood glucose (A) and viscosity (B) Pre- and Post-ingestion during the three visits. n=9 for both variables. EF: early follicular; LF: late follicular; T: Time; V: Visit. Pre: Pre-ingestion; 30Post: 30min Post-ingestion; cP = centipoise. *significant increase from Pre, within the $EF_{Glucose}$ and $LF_{Glucose}$ visits ($p < 0.001$). Error bars reflect the SD.

A



Early Follicular
(Low Estrogen)
Late Follicular
(High Estrogen)
%FMD
12
10
8
6
4
2
0
>
*
Pre
Post60
Post90
Post120
Pre
Post60
Post90
Post120
Pre
Post60
Post90
Post120
EF Control
EF Glucose
LF Glucose
T: p = 0.007
V: p = 0.016
T*V: p = 0.054

B



Delta%FMD
4
2
0
-2
-4
-6
-8
EFGlucosePost90
LFGlucosePost120

Figure 5. (A) %FMD and (B) Pre to post-hyperglycemia delta %FMD. n=17 (A), n=15 (B); 2 participants were missing Post120 trials in the LF phase. Panel B: the delta is presented for the trial with the largest post-hyperglycemia decrease in FMD in each phase (Post90 and Post120 respectively). Symbols and lines reflect individual responses and the bars depict the group means. FMD: flow-mediated dilation; EF: early follicular; LF: late follicular; Pre: Pre-ingestion; Post60: 60min Post-ingestion; Post90: 90min Post-ingestion; Post120: 120min Post-ingestion; T: time; V: visit. *significantly lower than the $EF_{Glucose}$ visit Pre ($p = 0.001$) and Post60 ($p = 0.023$) >significantly lower than the $EF_{Glucose}$ visit Pre ($p = 0.002$) and $LF_{Glucose}$ visit Pre ($p = 0.009$).

Chapter 4: General Discussion

On average, Canadians consume 110g of sugar - the equivalent of 26 teaspoons - every day, with half of this consumption coming from added processed sugars (55g; Bowman et al, 2008). In the context of this thesis research, a 75g bolus of glucose is the equivalent of some familiar desserts or beverages, comprised mainly of added processed sugars. For example, a large soda pop at the movie theatres contains 64-88g of sugar, a Starbucks “Java Chip Frappuccino” contains 72g of sugar, and a regular size McDonalds “M&Ms McFlurry” contains 86g of sugar (“Starbucks Nutrition Information”; “McDonalds Canada Nutrition Facts”; “Cineplex Nutrition Information”). Previous research in men has indicated that the elevation in blood glucose (acute hyperglycemia) that occurs following ingestion of these large doses of sugar results in transient endothelial dysfunction (Loader et al, 2015). Importantly, chronic endothelial dysfunction is a precursor to the development of cardiovascular disease (Brevetti et al, 2003; Matsuzawa et al, 2015) and it is possible that frequent periods of dysfunction following ingestion of large doses of sugar may accumulate to clinically relevant endothelial dysfunction (Kawano et al, 1999). Given the positive relationship between chronically elevated blood glucose levels (hyperglycemia) and increased cardiovascular disease risk (Sarwar et al, 2010), exploring the impact of sugar intake on cardiovascular disease and its precursors is of importance.

Interestingly, one population that appears to have the lowest prevalence rate for cardiovascular disease is premenopausal women (Jousilahti et al, 1999). Elevations in estradiol levels throughout the menstrual cycle may confer protection against the impact of acute insults to the endothelium lining the arteries (high-fat meals: Harris et al, 2012; ischemia-reperfusion injury: Luca et al, 2016), contributing to prevention of chronic endothelial dysfunction. Given the prevalence of sugar consumption in the Canadian diet, examining the impact of acute hyperglycemia on endothelial function in a previously understudied population of premenopausal women is novel and important.

The study outlined in Chapter 3 examined the impact of acute hyperglycemia on FMD during the early follicular (EF) and late follicular (LF) phases of the menstrual cycle. This study found that FMD was impaired 90min post glucose-ingestion during the EF phase; however, this impairment was not detected in the LF phase. Although delta FMD analysis did not reveal differences in FMD impairment between phases, it is possible we were underpowered to detect the small degree of protection provided by the LF phase. The findings of this study provide the first evidence that estradiol fluctuations with the menstrual cycle may confer a small degree of protection to arteries against the impact of elevated blood glucose levels. It is possible that this contributes to the overall cardioprotection observed in premenopausal women (Jousilahti et al, 1999). The following sections of this chapter will discuss the methodological considerations of the study in Chapter 3 and review proposed directions for future research.

4.1 Methodological Considerations

4.1.1 Control Visit

A strength of the study in Chapter 3 was the inclusion of a time-control visit ($EF_{Control}$) in the experimental design. The $EF_{Control}$ visit was the first experimental visit participants took part in where participants became re-familiarized with the experimental protocol, from the screening visit, and consumed an equivalent volume of water (300mL) instead of the glucose beverage. The inclusion of the $EF_{Control}$ visit was important to identify the impact of time on FMD; any differences observed during the two glucose visits ($EF_{Glucose}$ and $LF_{Glucose}$) could then be attributed to the hyperglycemia intervention and not due to prolonged rest (~3hrs). Additionally, the majority of studies examining the impact of hyperglycemia on FMD have not included a time-based control to rule out the impact of time on FMD (Loader et al, 2015); as a result, this was of particular importance to include in testing this novel population of premenopausal women. Given that there were no differences in FMD over time during the $EF_{Control}$ visit, this reinforces the interpretation that the decline in FMD observed during the $EF_{Glucose}$ and $LF_{Glucose}$ visits was due to acute hyperglycemia and not the result of time.

However, a limitation of the study design was that the order of the $EF_{Control}$ and $EF_{Glucose}$ visits was not counterbalanced. While there was initial intention to counterbalance, with later participants in data collection participating in the $EF_{Glucose}$ visit first, this did not occur due to an oversight in planning. As a result, we cannot make clear conclusions regarding the reason for the unexpected finding that baseline FMD was lower in the first EF Visit ($EF_{Control}$) versus the second EF visit ($EF_{Glucose}$). Counterbalancing the two EF visits would have allowed us to explore how the visit order versus days since menses onset impacted FMD.

As identified in the study discussion in Chapter 3, we speculate that the differences in Pre trials from these visits may be the result of either: (1) blunting of FMD in $EF_{Control}$ as a result of a novel laboratory environment and/or phlebotomy procedure evoking a stress response, or (2) enhancement of FMD in $EF_{Glucose}$ as a result of slightly elevated estradiol levels during the “late” EF phase. However, failing to support the second possibility, recent research from our group indicates that FMD remains constant throughout three visits in the EF phase (Tremblay and Pyke (in review)). This is in agreement with current vascular guidelines for assessment of premenopausal women, which suggest that FMD should remain constant across the first 7 days of the menstrual cycle (Thijssen et al, 2011a). As a result, further research is necessary exploring the first speculation: the impact of acute laboratory stress (e.g. phlebotomy procedure) on FMD.

4.1.2 Whole Blood Viscosity Measurements

Another strength of the study in Chapter 3 was the inclusion of whole blood viscosity measurements. Shear stress is the stimulus for FMD, determined by the integration of three variables: whole blood viscosity, blood velocity, and vessel diameter using the calculation: shear stress = ((4*whole blood viscosity*blood velocity)/vessel diameter) (Padilla et al, 2008). However, in circumstances in which whole blood viscosity is not expected to change, shear rate can be used as an estimate for shear stress, using the following calculation: shear rate = (blood velocity/vessel diameter). Indeed, using shear rate to estimate the shear stress stimulus for FMD is a widely adopted approach, and considered appropriate when studying young, healthy participants (Padilla et al, 2008; Thijjsen et al, 2011a).

However, some studies have found that whole blood viscosity changes with the menstrual cycle (Larrson et al, 1989; Agoreyo and Okorie, 2016) and with acute hyperglycemia (Cinar et al, 2001; Irace et al, 2014). As a result, we elected to include measurements of whole blood viscosity in the study design. If differences were found across menstrual cycle phase or with acute hyperglycemia, whole blood viscosity measures would have been necessary to integrate into the characterization of the stimulus for FMD (Papaioannou and Stefanadis, 2005; Pyke and Tschakovsky, 2005). Due to difficulties in blood sampling (see “4.1.5 Challenges with Venipuncture”) we were only able to perform whole blood viscosity analysis in a subset of participants (n=9). However, this subset analysis indicated that whole blood viscosity was not impacted by acute hyperglycemia or menstrual cycle phase, and therefore we elected to use shear rate as the estimate for shear stress, with minimal concern regarding systematic error introduced by an effect of the experimental conditions on whole blood viscosity.

4.1.3 Assessment during Late Follicular Phase

An additional strength of this study was the method employed to align the $LF_{Glucose}$ visit with the elevation in estradiol levels, which occurs just prior to ovulation. Given the assumption that estradiol levels are predicted to be highest 12-14 days following the onset of menses, the majority of studies have placed the LF phase visit assessing FMD on a fixed date within this range (e.g. day 13; Adkisson et al, 2010; Harris et al, 2012; Rakobowchuk et al, 2013; Brandao et al, 2014; Luca et al, 2016; D'Urzo et al, 2017). However, a critique of this method is that this assessment may miss the peak estradiol levels of many participants, given the considerable potential for inter-individual variability in menstrual cycle length and ovulation day (Cole et al, 2009).

In contrast, a technique first introduced by Williams and colleagues (2001) differs from the aforementioned technique in that it recognizes the importance of determining the LF phase visit based on individual menstrual cycle length to account for individual variability and better predict the surge in estradiol levels (Cole et al, 2009). This technique is informed by the observation that the majority of variability in cycle length occurs in the follicular phase, rather than the luteal phase of the menstrual cycle (Reed and Carr, 2015). As recommended by Williams et al. (2001), the day of testing was determined by taking the average length of at least two prior cycles, and then subtracting the relatively stable 14-day luteal phase in order to estimate ovulation day. We then performed testing two days in advance of that to attempt to align the $LF_{Glucose}$ visit with the pre-ovulation estradiol surge (Lenton et al, 1984; Williams et al, 2001; Reed and Carr, 2015).

In the study in Chapter 3, we utilized this calculation to theoretically improve accuracy in assessing the LF phase. We then utilized ovulation testing and blood hormone analysis to determine the $LF_{Glucose}$ visit estradiol levels and proximity to ovulation. However, while the calculation aimed to have testing occur within 2 days of ovulation, our actual group average was 3 ± 2 days from ovulation (range: 0 to 8 days prior), with at least two participants not experiencing detectably elevated estradiol levels. However, while this approach was imperfect, utilizing the fixed method (e.g. day 13 following menses onset) to schedule the $LF_{Glucose}$ visit would have resulted in greater variability in the time to ovulation: ranging from 9 days prior to ovulation to 3 days following ovulation (12-day range). Taken together, while the calculated method proposed by Williams et al (2001) has theoretical strength with some ability to decrease variability present in the fixed day technique, further research is necessary to refine a method of determining the LF phase visit to capture peak estradiol levels.

4.1.4 Challenges with Recruitment

The original intent for the study presented in Chapter 3 was to explore the impact of acute hyperglycemia on FMD in participants who are prescribed synthetic estrogen supplementation through 3rd and 4th generation oral contraceptive pills (OCPs; Torgrimson *et al,* 2007; Meendering *et al,* 2009; Meendering *et al,* 2010; Thompson *et al,* 2011). Recent studies have found that the active pill phase of 3rd and 4th generation, but not 2nd generation, OCPs enhances FMD compared to the placebo phase (Meendering *et al,* 2009, Thompson *et al,* 2011). It was hypothesized that the FMD enhancement observed in 3rd and 4th generation, but not 2nd generation, OCPs was the result of different synthetic progesterone derivatives added to the synthetic estrogen (Meendering *et al,* 2009). Specifically, the 2nd generation progesterone derivative levonogestrel appears to counteract the enhancement synthetic estrogen provides for FMD (Torgrimson *et al,* 2007); an effect which is absent in 3rd and 4th generation OCPs (Meendering *et al,* 2009). As a result, in order to examine the potential protection that the OCP active phase may provide to the endothelium against the impact of an acute hyperglycemia insult, only 3rd and 4th generation OCP users were to be recruited.

After screening ~60 participants for inclusion criteria over the course of approximately one month of recruitment efforts, only 4 participants qualified for taking 3rd or 4th generation OCPs, as most participants were prescribed 2nd generation OCPs. As a result, we changed the primary focus of the study to explore the impact of menstrual cycle phase on vulnerability to acute hyperglycemia. However, unlike OCPs, which result in a stable elevation in synthetic estrogen for 21 days out of a 28-day cycle, estradiol levels fluctuate across a natural cycle, with peak levels occurring immediately prior to ovulation (see Figure 1, Chapter 2). As a result, it was essential to track the menstrual cycle over at least two cycles to aid in the assessment of FMD close to the peak in estradiol during the LF visit phase, as indicated previously (Williams et al, 2001; Adkisson et al, 2010; D'Urzo et al, 2017). This tracking required considerable time and patience; however, surprisingly, 16/17 participants were already accustomed to tracking their cycle using a cell phone application – usually the "Period Tracker" app. While the app was not used to track cycle lengths for this study, it was useful in communicating with participants near the start of their next predicted menstrual cycle and anticipating when scheduling for experimental visits would occur to ensure that supplies and lab personnel were available. As a result, this sped up the process of planning data collections, allowing 17 participants to complete the study. This was greater than the 7 participants that we anticipated would be required to detect an impairment in FMD of 2% with hyperglycemia (based on research in young men (Zhu et al, 2007; Watanabe et al, 2011; Grassi et al, 2012; Walsh et al, 2016)), and only slightly below our conservative target of 20 women.

4.1.5 Challenges with Venipuncture

At the start of data collection, it was apparent that performing venipuncture on female participants would be more challenging than originally anticipated; in particular, attempting to complete two blood draws in one visit proved to be a challenge. As a result, two techniques to improve blood draw acquisition were pursued: first, a more experienced phlebotomist (Alyssa Fenuta) was brought on to support the study; and second, screening of participants for venous access (visual and tactile assessment) occurred during the screening visit. Despite the early struggles with blood draws, we acquired blood estradiol and progesterone levels in 13/17 participants, insulin levels in 10/17 participants, and glucose and viscosity measures in 9/17 participants. Although not ideal, blood analysis in a subset of participants allowed us to identify how hormones, glucose handling, and viscosity measures were impacted by the experimental conditions.

4.2 Future Directions

4.2.1 Exploration of Underlying Mechanisms

A limitation of the study presented in Chapter 3 was that the potential mechanisms which may be underlying the results were not explored fully. As previously discussed in Chapter 2, acute hyperglycemia results in increases in endothelial reactive oxygen species (ROS), which decreases NO bioavailability, ultimately decreasing FMD (Figure 2; Tesfamariam & Cohen, 1992; Cosentino *et al*, 1997; Konukoglu *et al*, 1997; Koska *et al,* 1997; Kawano *et al*, 1999; Green *et al*, 2014). Estradiol, on the other hand, increases NO bioavailability via increasing eNOS expression and through its antioxidant capacity to scavenge ROS (Figure 2; Hamden *et al*, 2008; Ahmed & Hassanein, 2012; Haas *et al,* 2012; Harris *et al*, 2012; Luca *et al*, 2016).

With regards to NO bioavailability assessment, there is limited research exploring the separate impacts of menstrual phase and acute hyperglycemia on NO bioavailability. Research by Adkisson and colleagues (2010) found increases in estimates of systemic NO concurrent with increases in estradiol levels during the LF phase. However, research by Kawano and colleagues (1999) was unable to detect differences in the same estimates of systemic NO bioavailability following a hyperglycemia challenge. While both researchers measured estimates of NO bioavailability, namely nitrate and nitrite, the methods utilized were different (Adkisson et al, 2010: enzyme-linked immunosorbent assay; Kawano et al, 1999: autoanalyzer method based on the Griess reaction). Given these unexpected results by Kawano and colleagues (1999), researchers hypothesized that the method used may not have been sensitive enough to detect the estimates of NO bioavailability (nitrate, nitrite) from the oxidized products of the interaction between NO and hyperglycemia-induced ROS. Taken altogether, while differences in NO bioavailability were detected across phases (Adkisson et al, 2010), it is unknown if the same assay would have been sensitive enough to detect transient differences with acute hyperglycemia. Additionally, a limitation of this method is that assessments represent only estimates of systemic NO bioavailability and may not be a true reflection of local endothelial cell NO bioavailability.

With regards to ROS assessment, to our knowledge, no research has been conducted exploring phasic differences in ROS detected either at the endothelial cell level or systemically via venous blood sampling in humans. However, research in endothelial cell cultures has shown decreased ROS production with elevated estradiol levels (Wagner et al, 2001; Yen et al, 2001). Additionally, several studies have demonstrated increased ROS species with hyperglycemia, including malondialdehyde (MDA; Mah et al, 2011; Mah et al, 2013; Wang et al, 2013), nitrotyrosine (NT; Ceriello et al, 2002; Ceriello et al, 2011), ROS marker 8-iso-PGF(2α) (Grassi et al, 2012), and thiobarbituric acid reactive substances (TBARS; Kawano et al, 1999). There was uncertainty as to whether or not ROS assays would have been sensitive enough to detect hyperglycemia-induced ROS differences across phases, given the variability that exists for several of the ROS assessment methods (Dikalov et al, 2007). Similar to limitations with NO assessment, all ROS measures were taken from systemic blood plasma, rather than assessments at the endothelial cell level.

While assessments of ROS and NO were initially explored, we concluded that there was uncertainty as to whether or not current techniques to assess NO bioavailability and ROS would be sensitive enough to detect both transient differences in response to acute hyperglycemia *and* differences across phases of the menstrual cycle. Additionally, current methods assess NO and ROS systemically rather than at the local endothelial cell level, limiting our ability to make conclusions regarding the influence of hyperglycemia on endothelial cell ROS or NO production across phases. More invasive techniques, such as endovascular biopsy of the radial artery or a superficial forearm vein, may be necessary to assess underlying mechanisms involved in NO bioavailability, such as eNOS expression and nitrotyrosine formation (a ROS molecule), at the local vasculature (Colombo et al, 2001; Fadini and Avogaro, 2010; Onat et al, 2011).

4.2.2 Extension to Additional Populations

Approximately 16% of premenopausal women aged 15-49 in Canada, with approximately 30% of young women (15-29), are prescribed OCPs; and it is predicted that approximately 80% of women will use OCPs at some point in their lifetime (Rotermann et al, 2015). However, the impact of acute hyperglycemia on endothelial function in women prescribed synthetic hormones is unknown, and requires future study. Given the results of the study presented in Chapter 3, we would expect to see acute hyperglycemia-induced impairments in FMD during the placebo phase of OCP, similar to that of the EF phase of the menstrual cycle when estradiol levels are low. Likewise, we would expect to see a small degree of protection from impairments in FMD, during the active phase of OCP, similar to the LF phase of the menstrual cycle when estradiol levels are elevated.

Furthermore, exploring the impact of synthetic estrogen hormone replacement therapy (HRT) in postmenopausal women would also be of interest as an extension to this research. We would expect to see acute-hyperglycemia induced impairments in FMD pre-HRT, similar to that of the EF phase of the menstrual cycle. Further, similar to the LF phase in the study in Chapter 3, we would expect to see a small degree of protection from impairments in FMD post-HRT. This research extension would contribute valuably to further our understanding of the benefits associated with HRT and endothelial function (Liberman et al, 1994; Harvey et al, 2005; Moreau et al, 2013).

Finally, exploring how progressively heightened levels of estrogen during each trimester of pregnancy (Schock et al, 2016) impact vulnerability to hyperglycemia-induced impairments in FMD is a natural extension to this research. It is possible that steadily increasing estrogen levels across trimesters may further protect arteries from the impact of acute hyperglycemia on FMD.

4.2.3 Methodological Extension: Additional Arteries, FMD in response to sustained increases in shear stress

There is evidence that arteries in the upper and lower limbs respond differently to acute stimuli; therefore, the brachial artery may not fully represent the systemic impact of acute stimuli on endothelial function (Thijssen et al, 2011b). Additionally, the lower limbs are more vulnerable to atherosclerosis and peripheral artery disease development, as the progression of these conditions are more common in the lower limbs compared to the upper limbs (McDermott et al, 2000; Sanada et al, 2005; Tindera et al, 2011). As a result, extending the methods of this study to explore the response to hyperglycemia in the vasculature of the lower limbs (e.g. popliteal, femoral arteries) would provide a more holistic view of the systemic impact of hyperglycemia on endothelial function. Additionally, given the hypothesis that acute susceptibility to hyperglycemia-induced endothelial dysfunction may lead to more chronic impairment (Kawano et al, 1999), identifying arteries at greatest risk for dysfunction is of importance.

Finally, the dominant method for assessing FMD is the use of the reactive hyperemia FMD (RH-FMD) test, which produces a transient shear stress stimulus; this was the method used in the study presented in Chapter 3. Another method for assessing FMD, uses a sustained increase in shear stress to elicit FMD, often through handgrip exercise or prolonged skin heating (sustained stimulus FMD (SS-FMD) (Tremblay and Pyke, 2018). Exploring this method is relevant as several day-to-day activities, such as exercise, increase shear stress in a sustained manner. Studies have identified that RH-FMD and SS-FMD may respond differently to acute stimuli and disease states, such as acute mental stress (Szijgyarto et al, 2012), or in patients with Type 1 diabetes (Bellien et al, 2010) or obesity (Slattery et al, 2016). Furthermore, while RH-FMD is primarily mediated by NO-dependent pathways (Joannides et al, 1995), SS-FMD may be mediated by a more complex combination of both NO-dependent and -independent pathways, thus requiring further research to explore potential differences (Mullen et al, 2001; Bellien et al, 2010; Tremblay and Pyke, 2018). Understanding differences which may exist in the impact of acute hyperglycemia on RH-FMD versus SS-FMD may aid to again more holistically examine the impact of hyperglycemia on endothelial function.

4.3 Summary and Conclusions

Canadians consume 110g of sugar on average per day (Bowman et al, 2008), resulting in transient periods of acute hyperglycemia. Previous studies in predominantly male populations have observed that acute hyperglycemia impairs endothelial function, as assessed by FMD (Loader et al, 2015), which may contribute to clinically relevant dysfunction over time (Kawano et al, 1999). Exploring the vulnerability to these periods of acute hyperglycemia in understudied populations, such as women, is of importance.

This was the first study conducted in premenopausal women to observe impairment in FMD during the EF phase of the menstrual cycle, similar to that of predominantly male studies (Loader et al, 2015). There appeared to be a small degree of protection against hyperglycemia-induced impairment in FMD during the LF phase. Additionally, contrary to previous research which has found enhanced FMD in the LF phase, the study presented in Chapter 3 did not find any change in FMD prior to ingestion of glucose, across menstrual cycle phases. Beneficial effects of estrogen on the vascular endothelium, namely its ability to increase NO bioavailability (Du et al, 2008) and its antioxidant effects (Hamden et al, 2008; Ahmed & Hassanein, 2012; Haas et al, 2012), may have attenuated some of the vulnerability to acute hyperglycemia without resulting in enhancement of baseline FMD.

The results presented in Chapter 3 suggest that assessing the impact of a vascular insult (e.g. acute hyperglycemia, mental stress, exercise, injury or inflammation etc.) in only one phase of the menstrual cycle may be inadequate to fully capture the vascular consequences in premenopausal women. Further studies investigating the mechanisms underlying the protective effect of estrogen against acute hyperglycemia-induced dysfunction, and other acute insults to the endothelium, is needed.

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Appendix A: Research Ethics Board Approval

QUEEN'S UNIVERSITY HEALTH SCIENCES & AFFILIATED TEACHING HOSPITALS RESEARCH ETHICS BOARD (HSREB)

HSREB Amendment Acknowledgment/Ethics Clearance

October 26, 2017

Dr. Kyra Pyke
School of Kinesiology and Health Studies
Queen's University

ROMEO/TRAQ: #6010275
Department Code: PHE-134-13
Study Title: Protocol title: The role of acute phosphate supplementation on endothelium dependent and independent vasodilation. NSERC grant title: The role of shear stress and sympathetic nervous activity in the regulation of arterial function and structure.
Review Type: Delegated
Date Ethics Clearance Issued: October 26, 2017

Dear Dr. Pyke,

The Queen's University Health Sciences & Affiliated Teaching Hospitals Research Ethics Board (HSREB) has reviewed the amendment application and granted ethics approval/acknowledgement for the following:

- Request for approval to explore the impact of cyclic changes in estrogen on the endothelial response to hyperglycemia in women with natural menstrual cycles (not on OCPs).
- Expansion of your inclusion criteria to include women who are recreationally active (rather than sedentary).
- Elimination of one of the visits: instead, there will be two visits during the low estrogen phase and only one during the high estrogen phase.
- Addition of the use of ovulation kits.
- Addition of assessment of blood viscosity to the measures of blood being taken
- Addition of Josh Tremblay, PhD student who will be measuring blood viscosity from an additional 5 ml draw (total draw each visit <50ml).
- CORE certificate – J. Tremblay
- Updated Medical Screening Form
- Facebook Recruitment Script – Non-OCP Women
- Online Website Advertisement
- Recruitment Poster
- Classroom Recruitment Script
- Updated Meal Log Sheet
- Information/Consent Form – Version 15 – 01/10/2017

Yours sincerely,

Albert F. Clark.
Chair, Health Sciences Research Ethics Board

Appendix B: Consent Form

School of Kinesiology and Health Studies
Queen's University

Kyra E. Pyke, Ph.D., Principal Investigator
Study performed in Room 400D, School of Kinesiology and Health Studies
Tel: 613-533-6000 x. 79631
Fax: 613-533-2009
E-Mail: pykek@queensu.ca

CONSENT FORM
FOR RESEARCH PROJECT ENTITLED:

The role of acute glucose supplementation on endothelium-dependent vasodilation

This is an important form. Please read it carefully. It tells you what you need to know about this study. If you agree to take part in this research study, you need to sign this form. Your signature means that you have been told about the study and what the risks are. Your signature on this form also means that you want to take part in this study.

Purpose of the Study:

You are being invited to participate in a research study directed by Dr. Kyra Pyke to evaluate the impact of glucose (sugar found in food) intake on the function of the main artery of the upper arm (brachial artery). Dr. Pyke or a student investigator will read through this consent form with you and describe the procedures in detail and answer any questions you may have. This study has been reviewed for ethical compliance by the Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board.

The purpose of the study is to identify whether acute exposure to elevated blood glucose levels temporarily impairs vasodilation (vessel widening) in response to increases in blood flow. This branch of the study is examining this in two phases of the menstrual cycle.

Benefits For You:
none

Description of Experiment and Risks:

What will happen? During this study, you will take part in the specific experimental procedures outlined below.

HEART RATE MEASUREMENTS:
Heart rate is continuously monitored by an electrocardiogram (ECG) through 3 spot electrodes on the skin surface or via a chest strap. The electrodes are placed on the chest and abdomen and they can detect the electrical activity that makes your heart beat.

RISKS: This procedure is entirely safe. In a very small group of individuals, a skin rash might occur from the adhesive on the electrodes. There is no way of knowing this ahead of time. The rash, if it develops, will resolve itself within a day or so. Avoid scratching the rash and keep clean.

BLOOD PRESSURE MEASUREMENTS:
A small cuff is fit around your finger. This cuff inflates to pressures that match the blood pressure in your finger, so you feel the cuff pulsing with your heartbeat. It shines infrared light through your finger to measure changes in the size of your finger with each heartbeat. An upper arm cuff will be placed on the right arm and will also be inflated periodically. This procedure is similar to what is experienced when blood pressure is taken at the doctor's office.

RISKS: This technique is non-invasive and poses no risk.

LIMB BLOOD FLOW AND BLOOD VESSEL DIAMETER MEASUREMENTS:
The blood flowing through your brachial (above the elbow) arteries can be detected, and your artery size measured using Doppler and imaging ultrasound. A probe will be placed on the skin over your artery and the investigator will control adjustments in its position by hand. High frequency sound (ultrasound) will penetrate your skin. The returning sound provides information on blood vessel size and blood flow.
RISKS: This technique is non-invasive and poses no risk.

FOREARM OCCLUSION:
A blood pressure cuff will be secured below your elbow on your left arm. This cuff will be inflated to 250mmHg for 5 min to limit blood flow into your forearm. You may feel a strong pressure and some mild tingling with cuff inflation but it should only be mildly uncomfortable. If there is pain, immediately notify the investigator and the cuff will be deflated and repositioned. Upon cuff release there will be a large rush of blood into your forearm. This may feel warm and you may experience mild tingling but no discomfort.
RISKS: This technique is non-invasive and poses no risk.

GLUCOSE SUPPLEMENT:
The glucose supplement (75g of glucose in solution) will be obtained from the Kingston General Hospital Pharmacy and administered orally in liquid form. For comparison there

are 39g of sugar in a can of coca cola. This dose of glucose will cause your blood glucose levels to rise.

RISKS: Consuming this dose of sugar does not pose a significant risk in heathy individuals (no diabetes or any other metabolic disease).

VENOUS BLOOD DRAW:
To measure blood glucose, insulin, evidence of levels of highly reactive molecules, and molecules that react with them (Reactive oxygen species, antioxidant status), estrogen and progesterone levels, and blood viscosity, a blood sample will be taken from a vein at your elbow or in your hand following standard venepuncture technique. In order to make the vein easy to identify, a non-latex tourniquet will be briefly applied to your upper arm. A needle will be inserted into the identified vein to collect the sample. Once the sample is collected the needle will be removed and a bandage will be applied.

RISKS: Brief discomfort will be associated with the needle prick itself and a modest amount of soreness may persist at the site for 1-2 days. As with any break in the skin there is risk of infection although it is very small in this case. The skin will be cleaned prior to the venipuncture and a bandaid will be applied immediately following collection of blood. Some bleeding may occur under the skin resulting in the formation of a bruise. Application of pressure directly following sample collection will minimize this risk. Puncturing a blood vessel increases the risk of clot formation, but his is very rare with this type of venous sampling.

ARTERIAL PRESSURE WAVE MEASUREMENTS: A small pencil like pressure transducer will be placed over your pulse on the side of your neck, on your femoral artery pulse in your upper thigh and on the top or side of your foot. These recordings will be used to measure how quickly the pressure wave created by your beating heart travels through your arteries. This tells us about the stiffness of your arteries. These measurements will be performed periodically while you rest.
RISKS: *This technique is non-invasive and poses no risk.*

7-DAY PHYSICAL ACTIVITY RECALL: This is a questionnaire that will ask you to report your physical activity levels over the past 7 days.
RISKS: This poses no risk

OVULATION TESTS: Following the second experimental visit, participants will be given 7 ovulation tests (ClearBlue Ovulatory Kits). Participants will be asked to record details of their last menstrual period and based on the start date of their last period, the experimenter will determine when the participant should start ovulation testing, during the second experimental visit. The participant will be given a start date and they will use on ovulation test each day for seven days to help determine what day of their cycle they typically ovulate. Their ovulatory day corresponds with the high estrogen phase of the menstrual cycle and thus will represent the high estrogen experimental visit (visit #3).

For each ovulation test, the participant will be required to remove the protective cap from the test and hold the absorbent tip in their urine stream for ten seconds. Once complete, the participant will replace the cap and lay the test on a flat surface with the reading window facing up (as indicated in the manufacturers instructions provided). Within three minutes the test results will appear in the reading window, with a positive result indicating high likelihood of ovulation. Once ovulation is confirmed, participants will be instructed to send an e-mail to the lead student investigator noting only the date, with no personal information regarding their test results, to protect participant privacy. From there, the high estrogen phase experimental visit (visit #3) will be booked.

How long will it take?

Screening visit #1: On an initial visit you will be asked to sit down and have your blood pressure, height and weight measured. You will also be asked to fill out a medical screening form and answer some questions about physical activity. These questions and measures will allow us to determine whether or not you meet the study inclusion and exclusion criteria. Next, you will lie down while we will use ultrasound to get an image of the artery in your upper arm to make sure that we can get clear pictures. <u>This visit will take approximately 20-30 min.</u>

Experimental Visit #1, 2, & 3

Visit 1 and 2 will occur during the menstrual (low estrogen) phase of the menstrual cycle between days 3 and 6, 48h apart. Visit 3 will occur during the ovulation (high estrogen) phase of the menstrual cycle, typically between days 12 and 15, scheduled based on the ovulation kit results previously described. In the low estrogen phase visits (1 & 2), on one visit the glucose supplement will be consumed and on the other visit an equal volume of water (~300 ml) will be consumed.
These visits will take a maximum of 3 hours each. While lying down and resting, you will be instrumented for heart rate, blood pressure and blood flow (ultrasound) measurements. During this rest period, we will also make the arterial pressure wave measurements. After a 20 min rest period you will undergo one trial of 5-minute cuff inflation and release on the forearm, while we measure the blood flow in your upper arm (brachial artery). You will then consume the glucose supplement or the equivalent volume of water. You will then rest for approximately 1h and will then perform 2 - 4 more trials of 5-minute cuff inflation and release on the forearm, while we measure the blood flow in your upper arm (brachial artery). We will also assess arterial stiffness periodically (4-5 times) during rest periods between trials. A blood draw will be taken before and after ingesting the glucose supplement/water.

Talking and Movements:

Talking or moving during the times that we are taking measurements will cause variations in the measurements we are making. If you have any discomfort, please let us know immediately and we can temporarily break from data collection. However, if everything is comfortable, please maintain a very quiet posture. Even very slight movements interfere with our experiments.

Special Instructions:

Participants are asked to not exercise for 48h, or drink alcohol or caffeine during the 24 hours prior to the study. Also, we ask that you do not consume any food during the 12 hours preceding the experiments. We ask that you consume the same pre-study meal for each experimental visit and will provide you with a meal log to record that. You should empty your bladder immediately prior to starting the test. When the study is finished, we will have you sit in the laboratory for a short time to allow you to readjust to the upright posture. These precautions should be enough to prevent any sensations of dizziness upon standing. Please be aware that sensations of dizziness are not normal and you should let us know if you experience any discomfort before you leave the laboratory. It is also important that you maintain your pre-study physical activity levels and diet throughout this study.

Attached Medical Screening Form:

This questionnaire asks some simple questions about your health and any medical conditions. This information is used to guide us with your entry into the study. Current health problems indicated on this form, which are related to cardiovascular or metabolic diseases, exclude you from the study.

Safety Precautions:

Safety precautions for the study will include the following:

- ❑ Before entering the study you will be screened using a medical screening form. You will not be able to enter the study if anything is found which indicates that it is dangerous for you to participate.

- ❑ We will monitor your heart rate during testing, and you will be sitting or laying on your back. These precautions allow us to quickly identify if you are experiencing an unusual response and it is expected that simply stopping the experimental manipulation will allow you to quickly recover.

Confidentiality:

All information obtained during the course of the study is strictly confidential and will not be released in a form traceable to you, except to you and your personal physician upon your request. <u>Your consent form and any personal health information reported on the health questionnaire</u> will be kept in locked files which are available only to the investigators and research assistants who will perform statistical analysis of the data. There is a possibility that your data file, including identifying information, may be inspected by officials from the Health Protection Branch in Canada in the course of carrying out regular government functions. Also, the Queen's University Health Sciences Research Ethics Board may review the records as part of their research audit responsibilities. The study results will be used as anonymous data for scientific publications and presentations, or for the education of students in the School of Kinesiology and Health Studies at Queen's University.

Study Compensation

You will receive a gift certificate for $30 for each experimental visit attended to thank you for participation.

Freedom to Withdraw from the Study

Your participation in this study is voluntary. You may refuse to participate or you may discontinue participation at any time during the duration of the study without penalty and without affecting your future medical care or academic evaluation.

Blood Analysis Follow-Up

We are not a clinical laboratory and the principal investigator Dr. Pyke is not a physician. We use the data collected to answer our research questions and do not examine it with respect to its significance for your personal health. We also do not have the credentials to diagnose medical conditions. If you request to be informed regarding the results of specific tests, they will be provided if possible, but we cannot provide an interpretation of any health significance. If you have any concern regarding the parameters evaluated we recommend that you seek medical consultation.

For the glucose, insulin, estrogen, and progesterone levels in your blood, the analysis will be done in the Kingston General Hospital lab after your participation. It is possible that you will be unnecessarily worried about a finding that is outside of published norms. Please indicate if you would still like to be contacted to receive a copy of these results.

Please indicate if you would still like to be contacted. YES NO (please circle)

This contact information should be valid for at least 12 weeks following participation.

If YES above: I would like to receive this information via:

- ☐ Email (provide email address): ____

OR

- ☐ Mail (provide mailing address):

 ____

 ____

 ____

Participant Statement and Signature Section
I have read and understand the consent form for this study. I have had the purposes, procedures and technical language of this study explained to me. I have been given sufficient time to consider the above information and to seek advice if I choose to do so. I have had the opportunity to ask questions which have been answered to my satisfaction. I am voluntarily signing this form. I will receive a copy of this consent form for my information.

If at any time I have further questions, problems or adverse events, I will contact:

Jennifer Williams (Student Investigator)
jenny.williams@queensu.ca
Room 401 D, SKHS 28 Division St.
Queen's University, Kingston, ON, K7L 3N6
Tel: (613) 533-6000, ext, 79377

Kyra E. Pyke, Ph.D. (Principal Investigator)
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Room 301C, SKHS 28 Division
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Jean Cote, Ph.D. (School of Kinesiology and Health Studies Director)
Room 206, KHS
Queen's University, Kingston, ON, K7L 3N6
Tel: (613) 533-6601

If I have any questions concerning research participant's rights, I will contact:
Dr. Albert F. Clark, Chair of the Queen's University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board
Office of Research Services
Fleming Hall, Jemmett Wing 301
Queen's University, Kingston, ON, K7L 3N6
Tel: 1-844-535-2988

By signing this consent form, I am indicating that I agree to participate in this study.

______________________	______________________
Participant Signature	Signature of person obtaining consent
______________________	______________________
Participant Name (please print)	Name of person obtaining consent (please print)
______________________	______________________
Date (day/month/year)	Date (day/month/year)

Appendix C: Medical Questionnaire for Research Study

School of Kinesiology and Health Studies

MEDICAL QUESTIONNAIRE FOR RESEARCH STUDY

The role of acute glucose supplementation on endothelium-dependent vasodilation.

Faculty Investigator:

Kyra E. Pyke, PhD, School of Kinesiology and Health Studies

Student Investigator:

Jennifer Williams

To the study participant: Please answer all questions in sections 1 and 2 of this form.

SECTION 1: PERSONAL DATA (please print)

Year of Birth: ____

SECTION 2: MEDICAL HISTORY

1. Do you currently smoke? ________ **yes/no** (**If yes**, please stop completing the form and return it to the student investigator)

   If previous smoker date of last cigarette________________ (year, month)

2. Please answer the following questions regarding your menstrual cycle history:

   a) Are you currently taking oral contraceptive pills?

   ____ Yes ______ No (**If yes** please stop completing form and return to student investigator)

   b) Have you given birth in the last 12 months?

   ____ Yes ______ No

   c) How old were you when you first menstruated?

   _______________ years old

   d) Are you currently having regular menstrual cycles?

   ____ Yes ______ No

   e) Currently, what is the average duration of your menstrual cycle (A full cycle goes from the start of menstrual flow [menses] to the start of the next menstrual flow [menses])? The average cycle length is 28 days.

   _____ days.

   f) How many days do you typically experience menstrual flow each cycle? Please check the correct response below:

   ____ 0 days
   ____ 1 day
   ____ 2 days
   ____ 3 days
   ____ 4 days
   ____ 5+ days

g) Please estimate the number of menstrual cycles you have had in the past 12 months:

______ (number) menstrual cycles

h) Have you had any of the following procedures?
______ Hysterectomy
______ Oophorectomy (removal of the ovaries)

3. Do you have, or have you ever had, problems with any of the following?

	Yes	No
i. Heart or blood vessels	___	___

(these might include but are not limited to: heart attack, stroke, heart murmur angina, coronary artery disease, high blood pressure, high cholesterol, congenital heart disease, any heart operation, bleeding or clotting disorders)

	Yes	No
ii. Kidney	___	___
iii. Nerves or brain	___	___
iv. Breathing or lungs	___	___
v. Hormones, thyroid, or diabetes	___	___
vi. Muscles, joints, or bones	___	___

vi. Other (please list) ____

4. Please list the diagnosis or/or briefly describe any problems identified in #3

____

____

____

____

____

____

5. Are you presently taking any medications? If yes, please list.

____

____

____

____

6. Do you have any allergies to medications, adhesive tape, latex, sweeteners etc.?

____

____

7. Have you previously had an adverse reaction to a blood draw? (e.g. fainting, severe anxiety)
   Yes No

I acknowledge that the study investigators completed this form according to my specifications; this information is true to the best of my knowledge.

____________________________	____________________________
Participant Name	Participant Signature

Date (dd/mm/yyyy): _______________