THE EFFECTS OF ORAL CONTRACEPTIVES ON VASCULAR ENDOTHELIAL FUNCTION AS MEASURED BY FLOW MEDIATED DILATION AND SHEAR STRESS

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THE EFFECTS OF ORAL CONTRACEPTIVES ON VASCULAR ENDOTHELIAL FUNCTION AS MEASURED BY SALIVARY BIOMARKERS AND FLOW-MEDIATED DILATION

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ABSTRACT

Women's risk for heart disease sharply increases after menopause, which has been attributed to altered hormone levels after menopause. These hormones, estradiol and progesterone, are also found in oral contraceptives. Oral contraceptives' greatest area of influence on the cardiovascular system may be on the endothelium, a single layer of cells that forms the inner lining of every blood vessel. Endothelial function is quantified by flow-mediated dilation, which measures brachial artery dilation under shear stress. Salivary biomarkers estradiol, nitrate, progesterone, and testosterone indicate levels to compare with vascular endothelial function. The experimental group of women taking oral contraceptives had higher estradiol levels, but lower NO levels. However, the control group of women not taking oral contraceptives experienced greater percent change in FMD. While these findings do not correlate with existing literature, future research is needed to further explore oral contraceptives as they relate to female cardiovascular health.

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INTRODUCTION

Heart disease is the most common cause of death in American women, and women's risk for heart disease sharply increases after menopause (Schwandt, Coresh, Hindin, 2010). Much research has focused on the causes of this increased risk for heart disease in post-menopausal women. After menopause, the female body undergoes significant changes, including a decrease in circulating levels of hormones that are believed to have profound effects on the cardiovascular system. One of these hormones is serum estradiol, the most active naturally secreted estrogen hormone. Synthetic estradiol is also found in combination with synthetic progestin in combination oral contraceptives (Evans & Sutton, 2015).

The decrease in estradiol levels following menopause has been well studied, and the resulting increase in adverse effects on the cardiovascular system is widely accepted (Villablanca, Jayachandran, Banka, 2010). However, research is incomplete regarding the effects of oral contraceptives on the female cardiovascular system. Existing research focuses on the clinical reproductive and adverse effects of estrogen, such as thromboembolism risk. For example, one systematic review and meta-analysis from 2012 confirmed that oral contraceptives are associated with an increased risk for venous thromboembolism (Manzoli, De Vito, Marzuillo, Boccia, & Villari, 2012). The Nurses' Health Study II, a widely recognized and influential longitudinal study, examined the effects of oral contraceptives with regard to thromboembolism and breast cancer risks ("Key Contributions," n.d.). While these clinical effects are more easily recognizable, they fail to account for the pervasive impact of oral contraceptives on women's cardiovascular system. Oral contraceptives' greatest area of influence on the

cardiovascular system may be on the endothelium, a single layer of cells that forms the inner lining of every blood vessel (Deanfield, Halcox, & Rabelink, 2007).

BACKGROUND

Most studies focus on progesterone and estrogen's effects on the reproductive cycle of women. The focus of this study is endothelial function in women taking oral contraceptives during the different phases in their menstrual cycles. Results are compared with women not taking oral contraceptives, while men are used as a control group. All oral contraceptives in this study contained a combination of both estrogen and progesterone. In oral contraceptives, estrogen suppresses the release of follicle-stimulating hormone from the pituitary gland, while progesterone works in both the hypothalamus and the pituitary. In this way, progesterone suppresses luteinizing hormone, therefore suppressing ovulation and preventing pregnancy (Lehne, 2013). While prevention of pregnancy is the intended effect of oral contraceptives, these pills have latent effects on vascular endothelium.

Every blood vessel is lined with endothelial cells. These cells regulate vascular tone, platelet activity, leukocyte adhesion, and angiogenesis. Endothelium produces and releases several vasoactive molecules that can dilate or constrict the vessel in an attempt to increase tissue oxygen supply and respond to metabolic demand (Deanfield, Halcox, & Rabelink, 2007). Endothelial dysfunction facilitates pathogenesis and places a person at higher risk for cardiovascular disease (Vanhoutte, Shimokawa, Feletou, & Tang, 2016). Sex hormones, like progesterone, estrogen, and testosterone, contribute to endothelial function; their imbalance, therefore, can cause cardiovascular disease progression (Stachenfeld, 2016). Oral contraceptives alter the body's natural balance of sex hormones, so identifying their effects on endothelium may elucidate their long-term, systemic effects.

Endothelial cells, neurons, platelets, and neutrophils can contain the enzyme Nitric Oxide Synthase (NOS) which synthesizes Nitric Oxide (NO) in response to homeostatic stimuli. NO is a short-acting gas that diffuses to the vascular smooth muscle and initiates the process of relaxation, dilating the blood vessel, which increases its diameter. Thus, endothelial NOS, or eNOS, can produce NO to protect against maladaptive vasoconstriction and atherosclerosis (Moncada, Higgs, & Furchgott, 1997). In response to inflammatory stimuli, iNOS is a form of NOS induced by immunological stimuli or inflammation. NO synthesized by iNOS contributes to immunity in activated macrophages (Moncada, Higgs, & Furchgott, 1977). NO maintains vascular wall stability and inhibits inflammation and thrombosis (Deanfield, Halcox, & Rabelink, 2007). Still a third form, nNOS, functions in the brain, spinal cord, and peripheral nervous system (Moncada, Higgs, & Furchgott, 1977). A reduction in NO levels can cause endothelial dysfunction, which is correlated with hypertension, atherosclerosis, coronary syndrome, and other cardiovascular diseases. Researchers often describe NO as both a hallmark and predictor of endothelial dysfunction (Vanhoutte et al., 2016). Although NO is the hallmark gas, its half-life is short. NO levels are best indicated by measuring nitrite and nitrate levels (Moncada & Higgs, 1993).

Estrogen levels are indicated by levels of 17β-estradiol, the most active and most common naturally secreted endogenous estrogen (Choe, Khan-Dawood, & Dawood, 1983). Due to the body's quick metabolism of 17β-estradiol, ethinyl estradiol, an exogenous estrogen, is used in many oral contraceptives because it lasts longer in the body (Evans & Sutton, 2015). For both the male and female population, this hormone gradually increases throughout puberty. Before women reach menopause, estrogen levels

fluctuate throughout the different phases of the menstrual cycle. The first day of menstruation marks the beginning of this 28-day cycle and the beginning of the menstrual period, which can last 4-6 days. This phase has the lowest estrogen concentration. The highest secretion of estrogen occurs within the first 14 days before ovulation and constitutes the follicular phase. The period after ovulation marks the luteal phase, in which estrogen gradually decreases until next menstruation (Lowdermilk, Perry, & Alden, 2016).

Estradiol and NO are closely related. Estradiol binds to endothelial estrogen receptors, which stimulates endothelial cell proliferation and eNOS production, thereby increasing production of NO and relaxing vascular smooth muscle (Orshal & Khalil, 2004). In female guinea pigs, estrogen replacement has been found to increase endothelial NO production, thereby enhancing vascular sensitivity to vasodilators. Premenopausal women have higher levels of estrogen and thus, increased rates of NO production than men of the same age (Orshal & Khalil, 2004). This may account for premenopausal women's lower rates of cardiovascular disease.

In this study, cardiovascular endothelial function is measured by flow-mediated dilation (FMD) compared with shear stress. FMD and shear stress measurement entails monitoring blood vessel diameter and blood flow rate using ultrasound technology. Brachial artery diameter is measured once at rest, or baseline, and again after occlusion by a sphygmomanometric cuff. This assessment of blood flow and vessel dilation is used to quantify endothelial dysfunction and resulting cardiovascular disease (Corretti et al., 2002). See Figure 1. This study also is a replication and continuation of prior research regarding contraceptives and vascular endothelium (Tenney, 2016).

MATERIALS AND METHODS

PARTICIPANTS

A simple convenience sample of 10 young women and 5 young men were recruited from a private southern university. Inclusion criteria required that participants were between ages 19-24 years and had no history of heart disease or cardiovascular dysfunction. Exclusion criteria for participants included any diagnosed heart conditions or refusal to sign the informed consent. Participation was voluntary. The experimental group included the 5 women who were taking oral contraceptives (BC Females), whose results were compared with those of the 5 women who were not taking oral contraceptives. The 5 young men made up the control group. Ethinyl estradiol levels were consistently between 30 and 35 micrograms in all participants, but the levels of progesterone were varied. Data were collected from both groups of female participants at 3 different points in their menstrual cycles: once on day 1 of the menstrual cycle to correspond with the menses phase (M), once on day 11 ± 3 in the follicular phase (F), and once on day 21 ± 7 to represent the luteal phase (L). Men were studied only once and given a $25 Amazon gift card for participation. As an incentive to complete all data collection, female participants were offered a $25 Amazon gift card after the 3rd visit. All participants signed informed consent. The Institutional Review Board of Texas Christian University approved this study's protocol.

MATERIALS

Saliva samples were collected from each participant at each brachial artery measurement. The Salivary 17β-Estradiol Enzyme Immunoassay Kit (Salimetrics) was used to measure the amount of salivary estradiol in each saliva sample. The Salivary

Progesterone Enzyme Immunoassay Kit (Salimetrics) measured salivary progesterone in each sample, and salivary testosterone was measured by the Salivary Testosterone Enzyme Immunoassay Kit (Salimetrics). The Nitrate/Nitrate Colorimentric Assay Kit (Cayman Chemical Company) measured the salivary nitrate levels gathered from each participant.

The Siemens ACUSON Sequoia™ C512 Echocardiography System was used to capture ultrasound clips of the participants' brachial arteries and measure the shear stress of blood flow on the vessel endothelium. An L5 linear array transducer was used in all ultrasound clips. Experienced laboratory technicians trained the author prior to beginning data collection.

To measure the diameters of the brachial arteries and the shear stress, the author used Brachial Analyzer for Research, developed by Medical Imaging Applications LLC, in each frame of the ultrasound clips. Brachial Analyzer for Research provides an accurate, semiautomated measurement of the brachial artery diameter throughout an image sequence or clip. This program can record maximal blood vessel dilation and remove potential artifacts through analysis of multiple frames in sequence. The researcher provides the blood vessel wall borders to the first frame, and the program applies this data to the analysis of the whole sequence of frames. Each blood vessel diameter analysis is generated with a confidence index (Sonka, Liang, & Lauer, 2002). Brachial Analyzer for Researcher also calculates shear stress in cm/s based on the maximal blood flow during the captured clip.

STUDY PROTOCOL

All participants first provided informed consent, then a brief medical history, including height and weight, current medications, and menstrual history if applicable. All the saliva samples were properly labeled with participant number, date of collection, and menstrual phase, if applicable. Saliva samples were stored securely in a freezer in the research laboratory.

The subjects then lay down on a table, and the researcher used an ultrasound machine to measure the right brachial artery diameter of all participants. Participants were instructed to lie motionlessly until all data collection was complete. Brachial artery diameter was first measured by taking a 30-second baseline video clip. Shear stress was also captured at baseline by measuring blood flow at the center of the blood vessel. Next, the author inflated a blood pressure cuff on the right forearm to 200 mmHg for a duration of 5 minutes. At the end of the 5 minutes, the cuff pressure was released, restoring blood flow. Immediately after release, shear stress was captured again at the center of the brachial artery. Fifteen seconds after the cuff pressure was released, a 3-minute video clip was recorded. The author stored saliva samples, informed consent, medical history, and the ultrasound clips securely and gave each participant an ID number so that results were not associated with participant names.

ULTRASOUND ANALYSIS

The Brachial Analyzer program was used to analyze all ultrasound clips. The author defined the specific region of interest (ROI) on the brachial artery, in which the program would calculate a vessel diameter and confidence percentage for each frame to estimate accuracy. For any confidence percentage less than 70%, the author manually defined the vessel walls. Additionally, each frame was reviewed by the author, ensuring

complete accuracy. The baseline, or pre-cuff inflation, vessel diameters were compared with the vessel diameters after the blood pressure cuff was deflated. The author used Brachial Analyzer for research to measure and chart the differences between the two diameters. The shear stress before and after occlusion was also analyzed using Brachial Analyzer for Research by calculating measurements of both blood velocity and brachial artery diameter.

RESULTS

DEMOGRAPHICS

The male and female participants were all close in age to control for age-related cardiovascular and hormonal changes. The five males had a mean age of 21.8 ± 1.02 years (mean ± standard error of the mean [SEM]). The five females who were not using birth control had an average age of 21.2 ± 0.2 years. The five females using birth control had a mean age of 21.6 ± 0.25 years.

Another variable known to influence the cardiovascular system is body mass index (BMI). The group of women who were not taking birth control had a mean BMI of 23.2 ± 0.89 kg/m^2, while the participants taking birth control had a mean BMI of 22.3 ± 0.89 kg/m^2.There was no statistically significant difference in the mean BMIs of the two groups, as evidenced by p-values > .05. However, the male participant group had an average BMI of 27.44 ± 0.73 kg/m^2. Male average BMI was higher than that of both female groups. See Table 1.

For the experimental group of women taking oral contraceptives, all were taking combination oral pills on a 28-day regimen. One participant was taking Larin, a combination of 30 μg ethinyl estradiol and 1mg norethindrone acetate. The other 4 participants were taking Sprintec (250 μg progesterone, 35 μg ethinyl estradiol), Zenchent (400 μg norethindrone, 35 μg ethinyl estradiol), Ogestrel (30 μg ethinyl estradiol, 300 μg norgestrel), and Enskyce (150 μg desogestrel, 30 μg ethinyl estradiol).

SALIVARY ESTRADIOL LEVELS

The Enzyme Imunoassay Kit only measures endogenous 17β-estradiol and does not measure synthetic E_2 or ethinyl estradiol. For both groups of women, 17β-estradiol

levels were highest in the follicular phase. The females on birth control (BC Females) had higher levels of 17β-estradiol than the women who were not taking birth control (Females) at every phase in the menstrual cycle, respectively (M, F, L). With a p-value of 0.054, the differences between all groups' estrogen levels are not statistically significant. In their respective phases, the Females and BC Females did not have significantly different levels of estrogen in any phase. See Figure 1.

SALIVARY PROGESTERONE LEVELS

Progesterone levels, measured by the Progesterone Enzyme Immunoassay Kit (Salimetrics), gradually increased in each phase for women not taking birth control from menses to the luteal phase. Women in the control group had the lowest progesterone levels during menses and the highest progesterone during the luteal phase. However, females taking birth control had the lowest progesterone of all groups during menses and the highest overall progesterone levels during the follicular phase. Progesterone decreased for women in the experimental group in the luteal phase. With a p-value of 0.194, the variations in progesterone levels among the groups were not significant. See Figure 1.

SALIVARY TESTOSTERONE LEVELS

Testosterone levels measured by the Salivary Testosterone Enzyme Immunoassay Kit (Salimetrics) remained relatively constant for all participants regardless of menstrual phase. Since the p-value was 0.579, the difference between all groups' testosterone levels was not statistically significant. See Figure 2.

SALIVARY NITRATE LEVELS

NO levels were indicated by measurement of its metabolite, nitrate, using the Nitrate/Nitrite Colorimetric Assay Kit. In both groups of women, nitrate levels were highest in the follicular phase, consistent with the high 17β-estradiol levels during this phase. Females not taking birth control had salivary nitrate levels higher in each phase of the menstrual cycle than did women taking birth control in any phase. With a p-value of 0.119, the differences in nitrate levels between all groups were not statistically significant. In the respective phases, the Females and BC Females did not have significantly different levels of nitrate in any phase. See Figure 3.

FLOW-MEDIATED DILATION (FMD)

FMD measure vascular response and endothelial function related to varying levels of estradiol and nitrate. Figure 4 shows the percent change between the FMD of the blood vessel before occlusion and the FMD after occluded blood flow has returned. In this case, the p-value for the difference in FMD before occlusion was 0.0005, which was statistically significant. The p-value for all groups' difference in FMD after occlusion and release was 0.0003, which is also statistically significant. The percent change in FMD before and after occlusion was the highest overall in Females in the follicular phase with a 10% change. The FMD percent change was lowest overall in BC Females during their menstrual phase, at only a 4% change. In the respective phases of their cycles, the Females had greater FMD percent changes than the BC Females. When Females' and BC Females' results were compared in the respective menstrual phases, neither M, F, or L had a significant difference between the two groups before or after occlusion. Therefore, while the Male group's results deviated significantly from the two groups of women, the

women did not display significant differences in their FMD results between the two groups.

SHEAR STRESS

The BC Females during the M phase had the greatest shear stress percent change, 65.8%, out of any of the groups during any menstrual phase. The lowest percent change in shear stress was the Female group during L phase at 21.4%. However, when Females and BC Females are compared with each other in their respective menstrual phases, the two groups did not have significantly different results in any phase before or after vessel occlusion.

LIMITATIONS

All female arteries were viewed on ultrasound and analyzed by the author. Certain artifacts inherent in ultrasound imaging and analysis include "dropouts, false borders, noise, as well as vessel motion resulting either from true translation of the vessel or unwanted motion of the ultrasound probe" (Sonka et al., 2002).

In addition, NO levels collected from salivary samples may be inaccurate. While all salivary samples were placed in a freezer immediately upon collection, samples were allowed to thaw and refrozen before NO levels were measured with the Nitrate/Nitrite Colorimetric Assay Kit. This may have allowed bacteria in saliva to convert nitrate to nitrite and alter nitrite levels measured. These variables combined with the small sample size of only 5 participants per group could have distorted the results of this study.

DISCUSSION AND CONCLUSION

The results of the participants' demographics show no significant variation in age, but significantly higher average BMI in the Male participant group. BMI is shown to have an inverse relationship with flow-mediated dilation (Kirma et al., 2007). However, the male participants self-reported higher activity levels than did the women; none of the 5 men described their exercise level as "sedentary," whereas 3 of the 10 women reported they were "sedentary" (the other options were "moderate" and "athlete"). The difference in BMI for the males may be accounted for by increased muscle mass.

The women in the experimental group, BC Females, had higher 17β-estradiol levels than the women who were not taking oral contraceptives, Females, at every phase in the menstrual cycle. Although BC Females' estradiol levels were higher, p-values comparing levels in each phase (M, F, L) showed that the variations were not significant. However, although BC Females had higher estradiol levels, Females had higher nitrate levels during each phase.

Females had higher nitrate levels than BC Females during all 3 phases, and this was reflected in their endothelial function as measured by FMD percent change. Females' FMD percent change was notably greater than BC Females in both the menses and follicular phases. Females' FMD percent change was 50% greater than BC Females' in the menses phases, and 100% greater than BC Females' in the follicular phase. In the luteal phase, however, the two groups had equal FMD percent change. The FMD percent change of Males was equal to those of both the Females and BC Females.

The results of this study showed that BC Females had higher estradiol levels and lower FMD. This is inconsistent with prior findings in the literature that "as estradiol

levels increase, there is less likelihood that endothelial function will be blunted" (Luca et al., 2016). Due to limitations described above, this study should be replicated. In the future, this study should be replicated with a larger sample size of participants per group to ensure representative results. Salivary samples should not be allowed to thaw and refreeze before being tested. Newer ultrasound technology should be used to minimize artifact and maximize clarity.

The results and content of this study should be used to advance knowledge of oral contraceptives' cardiovascular effects. This study should also encourage further research on the subject. Continued research is vital to discovering the full scope of oral contraceptives' effects on the cardiovascular system and on the female body.

APPENDIX

Table 1

Participant Demographic Data

	Males (n=5)	Females (n=5)	BC Females (n=5)	P value
Age years (Mean±SEM)	21.8 ± 1.02	21.2 ± 0.2	21.6 ± 0.24	0.786

	Males (n=5)	Females (n=5)	BC Females (n=5)	P value
BMI kg/m^2 (Mean±SEM)	27.44 ± 0.73	23.2 ± 0.91	22.3 ± 0.89	0.984

	P value
BMI Males vs Females	0.012
BMI Females vs BC Females	0.849
BMI Males vs BC Females	0.003

Table 2

Endothelial Function

	Males	Females M	Females F	Females L	BC Females M	BC Females F	BC Females L
Shear Stress (%)	133.7	39	21.4	21.4	65.8	37.6	27.2
FMD (%)	8	6	10	8	4	5	8

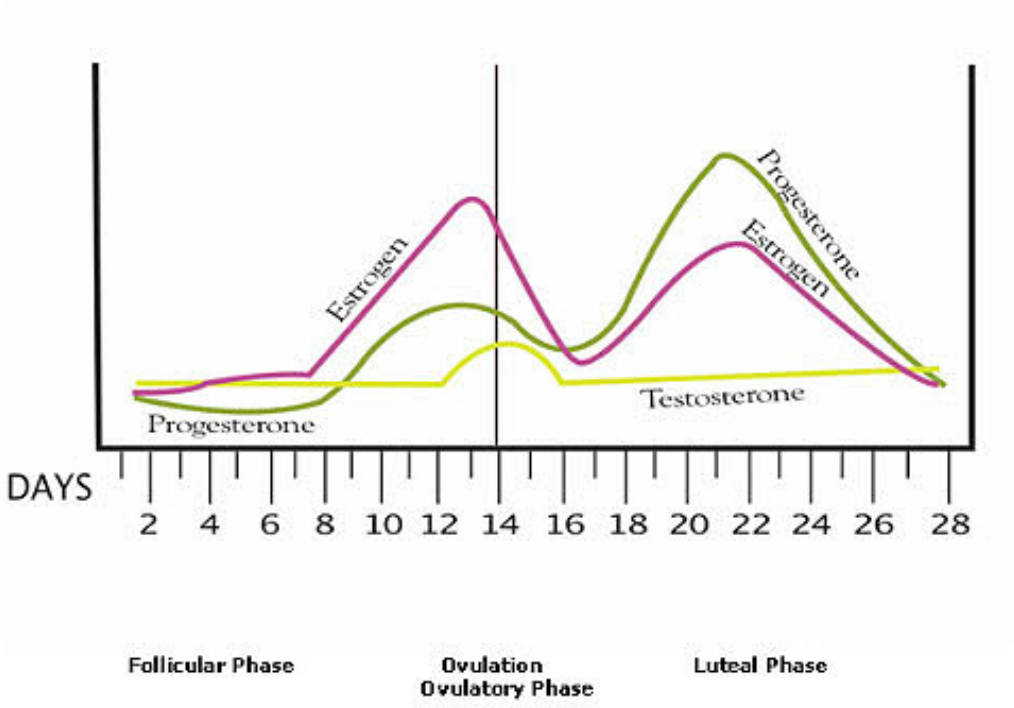

Figure 1. This graph shows the natural variations in estrogen, progesterone, and testosterone levels during a woman's 28-day menstrual cycle.

Hormone Imbalance, Menstrual Cycles & Hormone Testing. (2018). Retrieved March 08, 2018, from https://womeninbalance.org/about-hormone-imbalance/

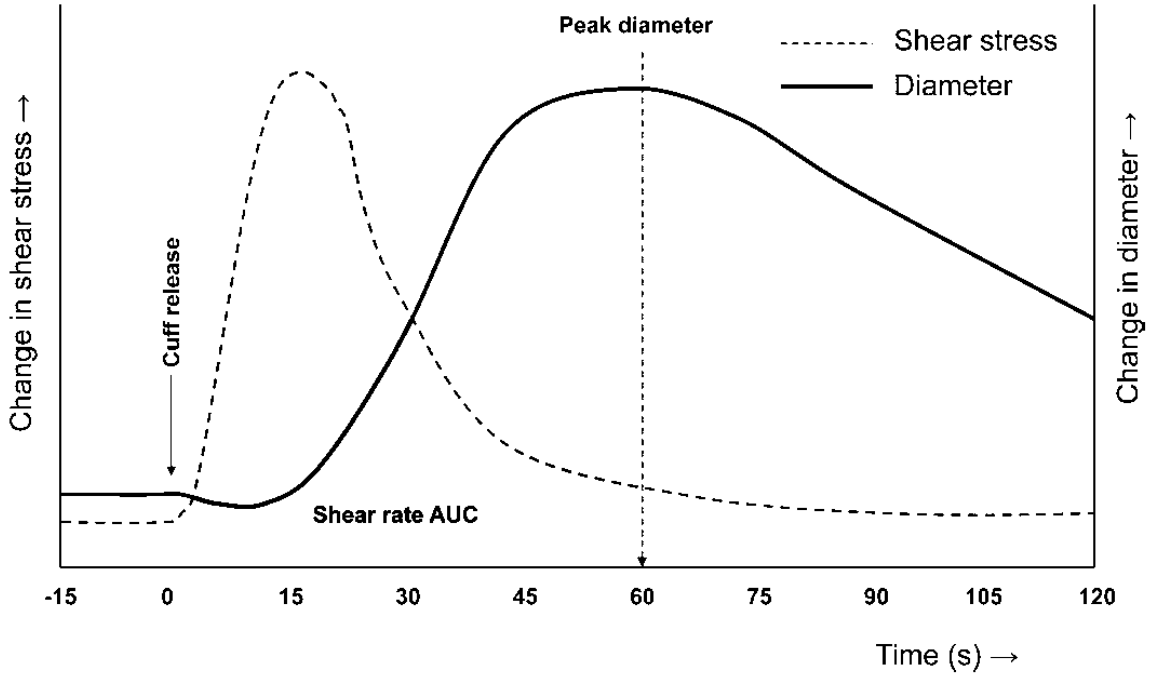

Figure 2. This graph depicts the relationship between shear stress and blood vessel diameter (Thiissen et al., 2011). The dotted line signifies the shear stress, the stress exerted on the blood vessel walls by blood flow. At the point of cuff release, the vessel is no longer occluded and shear stress increases until it peaks approximately 15 seconds later. The solid line represents the vessel diameter, which expands in response to increased shear stress. The blood vessel reaches maximum diameter approximately 1 minute after cuff release and the return of blood flow. While shear stress measures blood flow, FMD compares baseline blood vessel diameter with peak diameter after occlusion and release.

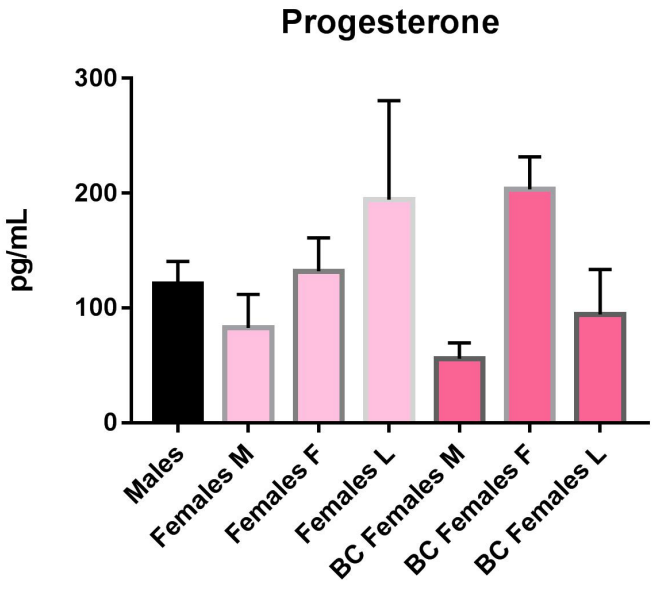

Figure 3. This graph shows average progesterone levels for all participant groups in pg/mL.

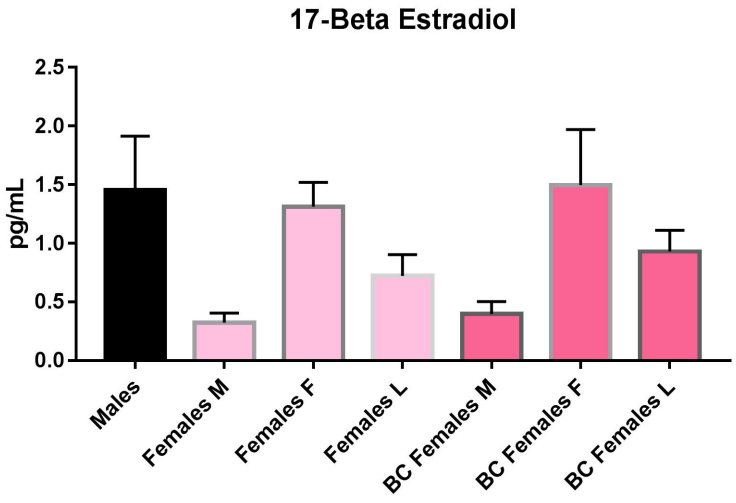

Figure 4. This graph shows average 17ß-estradiol levels for all participant groups in pg/mL.

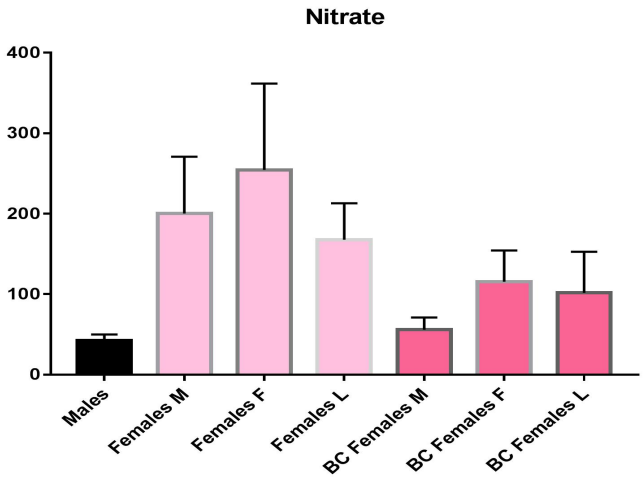

Figure 5. This graph shows average NO levels for all participant groups in pg/mL.

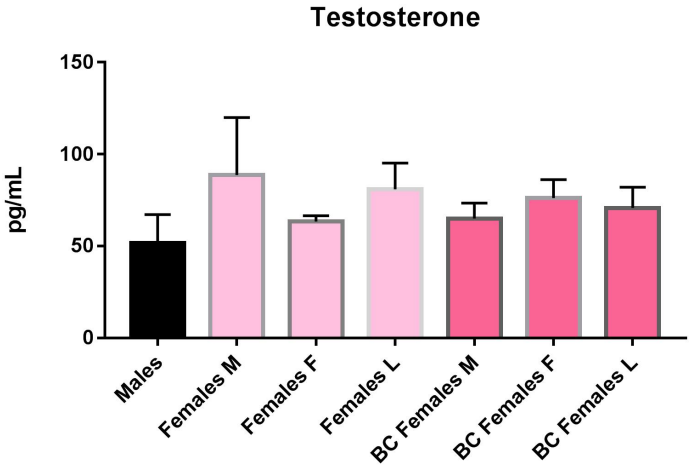

Figure 6. This graph shows average Testosterone levels for all participant groups in pg/mL.

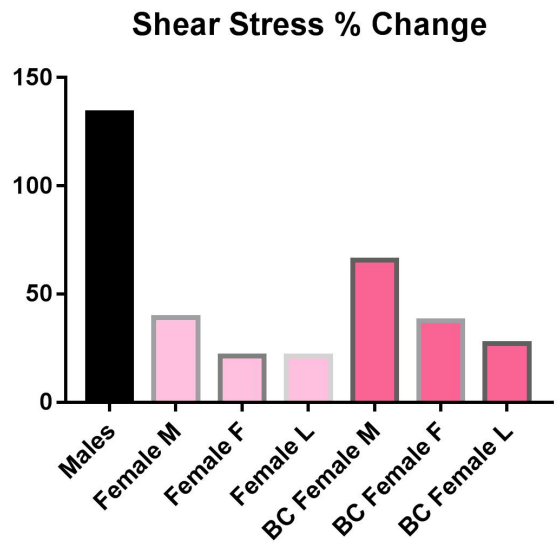

Figure 7. This graph shows aggregate percent change in shear stress before and after vessel occlusion for all participant groups.

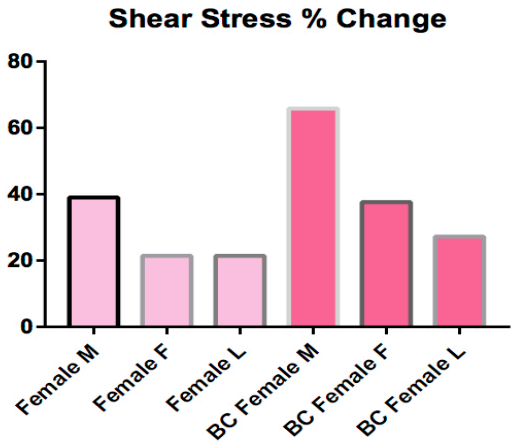

Figure 8. This graph shows aggregate percent change in shear stress before and after vessel occlusion for only the Female and BC Female participants.

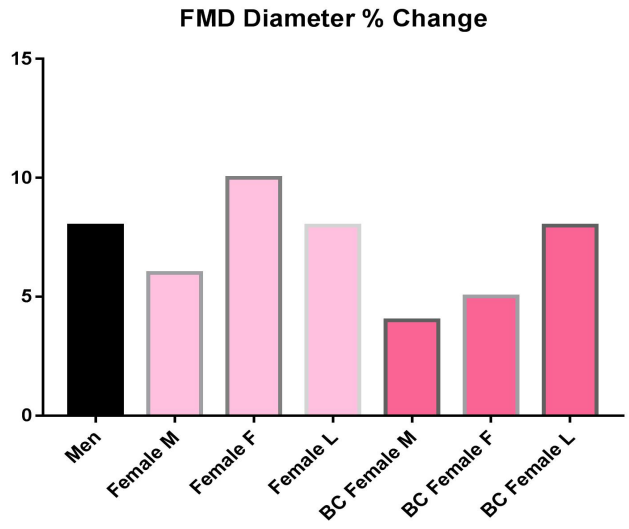

Figure 8. This graph shows aggregate percent change in FMD before and after vessel occlusion for all participant groups.

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