

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

by

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### Abstract

The role of oral contraceptives on the female cardiovascular system remains largely a mystery. However, it is widely accepted that estrogen plays a role in cardiovascular protection. Salivary biomarkers, namely estradiol and nitrate, allow for further understanding of estrogen and nitric oxide levels in young healthy females currently taking oral contraceptives. Flow-mediated dilation provides images of the dilation of the brachial artery under shear stress. The control group, those not taking oral contraceptives, was found to have higher levels of endogenous  $17\beta$ -estradiol. However, the experimental group, women taking birth control, experienced significantly greater blood vessel dilation than the control group. This finding has future influence in female cardiovascular health, vasoprotection, and the wider debate over the safety of oral contraceptives.

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## Introduction

According to a 2012 report by the Centers for Disease Control and Prevention, 62% of women ages 15-44 are using at least one method of contraception, 27% of whom are taking oral contraceptives (Jones, Mosher, & Daniels, 2012). Given a United States population of 315,000,000, this data translates into over 26,000,000 women using birth control. The impact of oral contraceptives on the cardiovascular system has long been established and widely accepted (Manzoli, De Vito, Marzuillo, Boccia, & Villari, 2012). However, much of the existing research has focused on adverse effects of the medication that can easily be recognized clinically, particularly venous thromboembolism. A systematic review discussed more than 50 studies on the long-term cardiovascular effects cause by years of contraception use (Manzoli et al., 2012). Yet there is a dearth of knowledge surrounding the day-to-day, subclinical effects that oral contraception has on the cardiovascular system of women, particularly the endothelium.

Endothelial cells are involved in nearly every process of the human body, but the depth of their influence continues to surface (Aird, 2015). Functional endothelial cells are involved in angiogenesis, the inflammatory response, hemostasis, and many other cardiovascular processes. Endothelial dysfunction can occur for a multitude of reasons including inflammation (Vanhoutte, Shimokawa, Feletou, & Tang, 2016). When inflammation occurs, reactive oxygen species are produced in the vasculature. These products inhibit nitric oxide (NO), which is a fundamental mediator of vasodilation. Therefore, inflammation can lead to vasoconstriction, precipitating atherosclerosis and other cardiovascular diseases (Vanhoutte, Shimokawa, Feletou, & Tang, 2016).

## Background

This study focuses on the differences in the endothelium between women taking oral contraceptives and women not taking oral contraceptives. The participants taking oral contraceptives take only those containing both estrogen and progesterone. The estrogen in these combined oral contraceptives suppresses the release of follicle-stimulating hormone from the pituitary gland. The progesterone, on the other hand, works in the hypothalamus and pituitary gland and suppresses luteinizing hormone, which triggers ovulation (Lehne, 2013). Because ovulation is inhibited by correctly administered contraception, resultant pregnancy is very unlikely.

The endothelium is a single-cell layer that lines every blood vessel in the cardiovascular system. It is a highly active organ, controlling and maintaining a multitude of bodily functions. Endothelial cells contribute to vasomotor tone control, cell and nutrient transport, blood vessel growth, and more (Aird, 2015). Endothelial cells also contribute to tissue oxygen supply, metabolic demand, and long-term organ perfusion (Deanfield, Halcox, & Rabelink, 2007). Because of the multitude of processes in which the endothelium is involved, identifying oral contraceptive's effect on the endothelium may highlight other endothelial influences throughout the body.

Nitric oxide synthase (NOS), a complex enzyme that acts in many bodily processes, comes from multiple sources and uses biological systems to synthesize Nitric Oxide (NO), a short-acting gas. In reaction to homeostatic stimuli, neurons, endothelial cells, platelets, and neutrophils produce small amounts of NO. Larger cells, such as macrophages and fibroblasts, also generate NO in response to inflammatory stimuli (Moncada, 1993). In the vascular system, NO is responsible for maintaining the vascular wall and inhibiting vascular inflammation and

thrombosis (Deanfield, Halcox, & Rabelink, 2007). Reduced levels of NO can alone lead to endothelial dysfunction, which is associated with hypertension, atherosclerosis, coronary syndrome, and many more disease processes (Vanhoutte, Shimokawa, Feletou, & Tang, 2016). Through a series of reactions, NO is eventually degraded into nitrite and nitrate in the body. However, because NO is a gas with a short half-life, the level is best measured by nitrite and nitrate (Moncada & Higgs, 1993).

Produced predominantly by the ovarian follicles, estradiol is the most active naturally secreted estrogen (Choe, Khan-Dawood, & Dawood, 1983). Estradiol levels gradually increase in both males and females throughout puberty. In premenopausal women, based on a 28-day cycle, estrogen is secreted most within the first 14 days before ovulation. This period of high estrogen secretion is known as the follicular phase. After ovulation, during the luteal phase, estrogen decreases, with its lowest concentration occurring during menstruation. Immediately after, estrogen levels begin to rise again until the next ovulation (Lowdermilk, Perry, & Alden, 2016).  $17\beta$ -estradiol is the most common endogenous estrogen (Choe, Khan-Dawood, & Dawood, 1983). However,  $17\beta$ -estradiol is quickly metabolized in the body, so it is not used in oral contraceptives. Thus, ethinyl estradiol, an exogenous estrogen, is the estrogen found in a variety of oral contraceptives (Evans & Sutton, 2015).

Estradiol's apparent vascular protective effects play an important role in vascular function and inflammation (Orshal & Khalil, 2004). Premenopausal women have a decreased rate of cardiovascular disease when compared to similarly aged men. Researchers believe that one factor in this statistic is estradiol's relationship with NO. Estradiol has been found to act as a NOS agonist, causing an upregulation of endothelial nitric oxide synthase (eNOS) mRNA. It is

clear that the total production of NO is greater in premenopausal women than men (Orshal & Khalil, 2004).

One way to quantify the cardiovascular endothelial function is by measuring flow-mediated dilation (FMD), an endothelium-dependence function that is used to assess cardiovascular disease caused by endothelial dysfunction (Corretti et al., 2002). This technique uses a sphygmomanometric cuff and ultrasound technology to monitor the dilation of a blood vessel, namely the brachial artery, under multiple conditions. See Figure 1.

### Materials And Methods

#### Participants

A simple convenience sample of eight young women was recruited from a private southern university. Inclusion criteria included non-smoking healthy subjects (i.e. no history of heart disease or cardiovascular dysfunction) ages 19 to 22 years. The experimental group included participants who had been regularly been taking estrogen-based oral contraceptives for at least one year. While the levels of progesterone varied between the participants, the levels of ethinyl estradiol were consistently between 20 and 30 micrograms. The control group had not been taking any form of contraceptives for one year or greater. The majority of the participants were in the luteal phase of the hypothalamic-pituitary cycle. Thus, they were past the peak of estrogen release that occurs on day 14 of the cycle (Lowdermilk, Perry, & Alden, 2016). Exclusion criteria included any diagnosed heart conditions and refusal to sign the informed consent. Participation was completely voluntary and participants could withdraw at any time without penalty. Students were not offered course credit and incentives were not given to participants. All subjects provided written informed consent before participation in the study.

The study protocol was approved by the Institutional Review Board of Texas Christian University.

### Materials

Each participant provided a saliva sample using the Oragene•RNA collection kit. The SalivaBio Oral Swab (SOS) was also used to collect a second saliva sample from each participant. The Salivary 17 $\beta$ -Estradiol Enzyme Immunoassay Kit (Salimetrics) was used to measure the amount of endogenous estradiol in the saliva samples. The Nitrate/Nitrate Colorimetric Assay Kit (Cayman Chemical Company) measured the amount of total nitrate from each participant.

The ultrasound clips of the brachial arteries were captured using the Siemens ACUSON Sequoia™ C512 Echocardiography System. In all clips, an L5 linear array transducer was used. The author was trained by experienced research laboratory technicians before data collection.

Brachial Analyzer for Research, developed by Medical Imaging Applications LLC, was utilized to measure the diameter of the brachial artery in each frame of each ultrasound clip. This program provides a semiautomated, accurate measurement of the blood vessel diameter throughout an image sequence. Because it is used for multiple frames one after another, Brachial Analyzer for Research is able to record maximal blood vessel dilation and remove potential artifacts. The researcher provides the near and far blood vessel wall borders on one frame, and the information is applied in the analysis of the whole sequence. A confidence index is generated with the analyses of all blood vessel diameters (Sonka, Liang, & Lauer, 2002).

### Study Protocol

After subjects provided written informed consent, a brief medical history, height and weight measurements, medication list, and menstrual history were obtained. During this time,

participants did not eat, drink, smoke, or use oral hygiene products for the ten minutes prior to saliva collection. Subjects then provided two saliva samples. First, the subjects filled the Oragene•RNA collection device to the fill line and the stabilizing liquid was released by closing the cap. Next the SOS was placed in the subject's mouth next to the cheek for one to two minutes to ensure full saturation. The collection swab was placed into the storage basket insert and the cap was secured onto the tube. All of the saliva samples were properly labeled and stored in a freezer in the research laboratory.

An ultrasound machine was then used to measure the right brachial artery diameter in recumbent subjects. Brachial artery diameter was measured first by recording a baseline video clip. Next, a blood pressure cuff was inflated on the right forearm to 180 mmHg for five minutes. The subjects were instructed to lay still and refrain from moving the arm until the data was collected. The cuff was then released and ultrasound video clips were recorded every 15 seconds for 3 minutes. Saliva samples, informed consent and medical history, and the ultrasound clips were kept under lock and key in the lab.

#### Ultrasound Analysis

All ultrasound clips were analyzed using the Brachial Analyzer program. The region of interest (ROI) was defined by the author, enabling the program to provide a diameter and confidence percentage for each frame of the clip. If the confidence percentage was less than 70%, the author manually defined the diameter of the artery, ensuring accuracy. The pre-cuff inflation artery diameters were compared to the diameters after the cuff was inflated and deflated. The differences were measured and charted using Brachial Analyzer for Research.

### Laboratory Analysis

The Salivary 17 $\beta$ -Estradiol Enzyme Immunoassay Kit quantitatively measures salivary estradiol. The reagent and plates were prepared with the desired number of strips in the strip holder. Twelve milliliters (mL) of HS Estradiol Assay Diluent was added to a disposable tube. Next, 100 microliters of standards, controls, and saliva samples were added to the appropriate wells. Fifteen microliters of the conjugate was then added to the 12 mL tube of HS Estradiol Assay Diluent in order to dilute the enzyme conjugate to 1:800. The adhesive cover was placed on the plate which was rotated for 5 minutes at 400 revolutions per minute (rpm) and then incubated at room temperature for 2 hours. Afterwards, the plate was washed four times with 1X wash buffer. Next, 200 microliters of TMB Substrate Solution was added to each well. The plate was placed back on the rotator for 5 minutes at 500 rpm, then covered and incubated at room temperature for 25 more minutes. Fifty microliters of Stop Solution was added to every well. For a last time, the plate was rotated for 3 minutes at 300 rpm until all of the wells turned yellow. The plate was then read at 450 nanometers (nm).

The Nitrate/Nitrite Colorimetric Assay Kit was used to measure total nitrate/nitrite concentration in a saliva sample. Two hundred microliters of Assay Buffer was added to the blank wells. Then up to 80 microliters of the sample was added to the wells. The final volume of each well was then adjusted to 80 microliters. Next, ten microliters of Enzyme Cofactor Mixture was dropped into each well, followed by ten microliters of Nitrate Reductase Mixture. The plate was then covered and incubated at room temperature for one hour. Afterward, 50 microliters of Griess Reagent R1 was added to the wells, immediately followed by Griess Reagent R2. The color was then allowed to develop for ten minutes at room temperature. Finally the absorbance was read at 540 nm.

## Results

### Demographics

The participants were all female: four control subjects not taking oral contraceptives and four experimental subjects currently taking estrogen-based oral contraceptives. Age and the body mass index (BMI) for both the control and experimental groups were not significant ( $p > 0.05$ ) (see Table 1).

All four of the control subjects were in the luteal phase of their hypothalamic-pituitary cycle. Therefore, their levels of endogenous estrogen were at the lowest points. The experimental subjects were in follicular phase meaning they had increased rates of endogenous estrogen secretion.

### Salivary Estradiol Levels

The control group was found to have higher levels of  $17\beta$ -estradiol than the experimental group. Overall estradiol levels are unknown because the Enzyme Immunoassay Kit only measures endogenous  $17\beta$ -estradiol. However, the difference between the two groups is not significant ( $p = 0.18$ ) (see Figure 2).

### Salivary Nitrate Levels

The Nitrate/Nitrite Colorimetric Assay Kit measures the end product of NOS – nitrate. The salivary nitrate levels among the experimental group were higher than those of the control group. The difference was not significant ( $p = 0.63$ ) (see Figure 3).

### Flow-Mediated Dilation (FMD)

The FMD figures demonstrate the percent change between the blood vessel diameter before occlusion and the peak blood vessel diameter after blood flow has returned. In this study, the difference in FMD results between the experimental and control group were significant ( $p =$

0.02). Because the same technician recorded and analyzed all of the ultrasound clips, the variables are equal for all participants and the percent change may be related to oral contraceptives and the corresponding levels of estradiol and nitrate (see Figure 4).

### Discussion

The control group, those not taking oral contraceptives, had insignificantly higher amounts of endogenous estrogen. However, these levels do not account for the exogenous ethinyl estradiol present in estrogen-based oral contraceptives. Therefore, based on the higher amounts of salivary nitrate in the experimental group, it can be concluded that the total estrogen levels are also higher in that group, despite the lower  $17\beta$ -estradiol levels. The exogenous estradiol from the oral contraceptives increases the total estrogen levels to higher than those in the control group. These results coincide with the FMD findings between the two groups. The significant difference in FMD is a result of the increased nitrate levels in the experimental group. In those who take oral contraceptives, the increased estrogen leads to increased nitrates, increasing the dilation of blood vessels under stress.

While estrogen-based oral contraceptives are not recommended for cardiovascular protection due to the high risk of thrombosis and other side effects over long-term use, it is important to recognize the positive effects these medications can have on blood vessels. With such a large population of females currently taking oral contraceptives, these findings impact millions of women.

### Limitations

The small sample size and homogenous subject group may limit these results to a certain population of young females. The subjects somewhat varied in their stage of the hypothalamic-pituitary cycle, possibly leading to differing  $17\beta$ -estradiol levels. Nitrate levels also may have

been influenced by the participants' diets the day before the saliva collection. The inability to directly measure ethinyl estradiol levels in the participants forced the authors to rely on roundabout estrogen measurement methods to conclude the results.

### Conclusion

There is a noticeable dearth of research surrounding the young healthy female population. With over 26,000,000 women currently taking oral contraceptives in the United States, more research is necessary to discover the vast effects of birth control on multiple body systems (Jones et al., 2012). Although estrogen levels in birth control are lower today than previously before, the risk of thrombosis still remains. However, there seems to be a positive impact on vascular response and dilation. The exogenous estrogen provided by oral contraceptives increases blood vessel dilation in response to shear stress. This increased vasodilation can prevent hypertension, decrease atherosclerosis, and inhibit a multitude of other cardiovascular diseases. Realizing that oral contraceptives may be vasoprotective for females, the newfound benefits must be weighted against the cons for each individual patient. This finding adds to the debate over the safety of oral contraceptives.

### Future Research

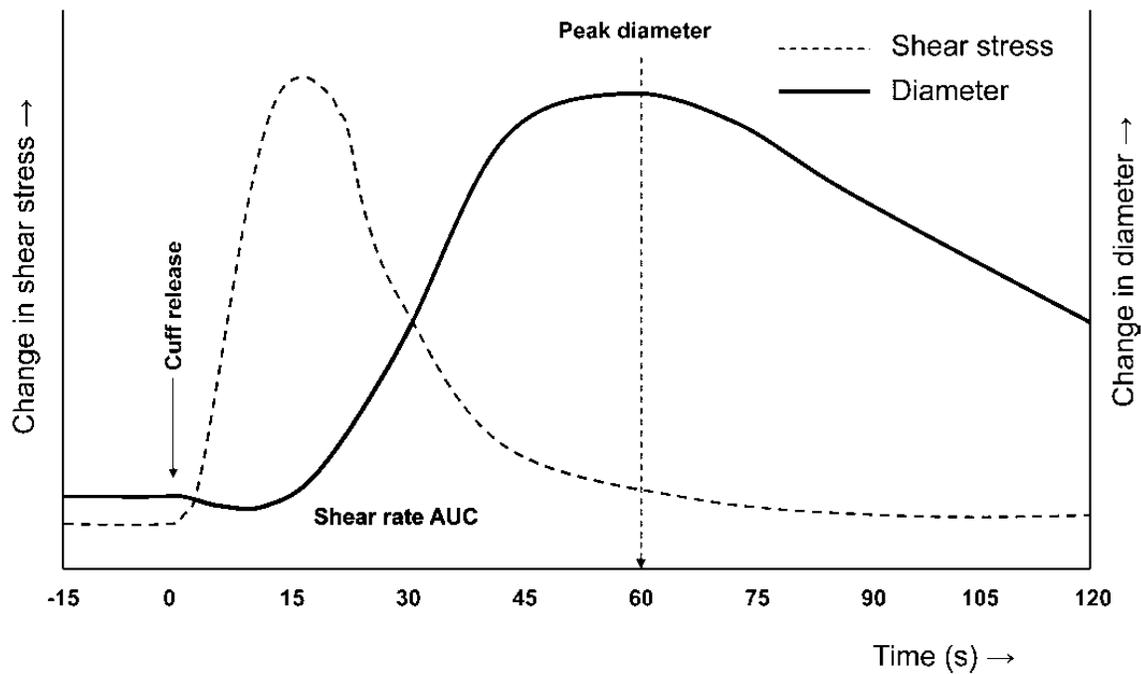
In the future, these results should be duplicated, but with a few slight changes to the methodology. The participants should provide saliva and FMD samples on days 1, 14, and 28 of their cycle. The influence of their cycles and the endogenous estrogen secreted during different phases can be measured more accurately and provide a more complete picture. Salivary estradiol and nitrate would continue to be measured with each of these samples. Although a homogenous group allows for results to be easily compared to each other among participants, a larger sample size would provide even more information.

Appendix

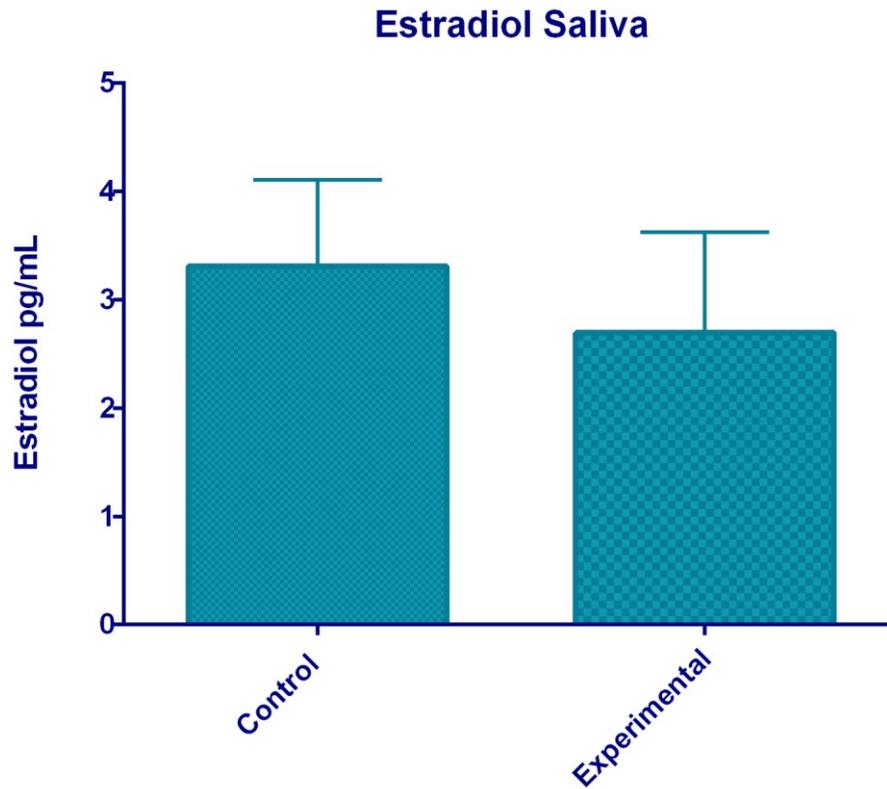
Table 1

*Participant Demographic Data*

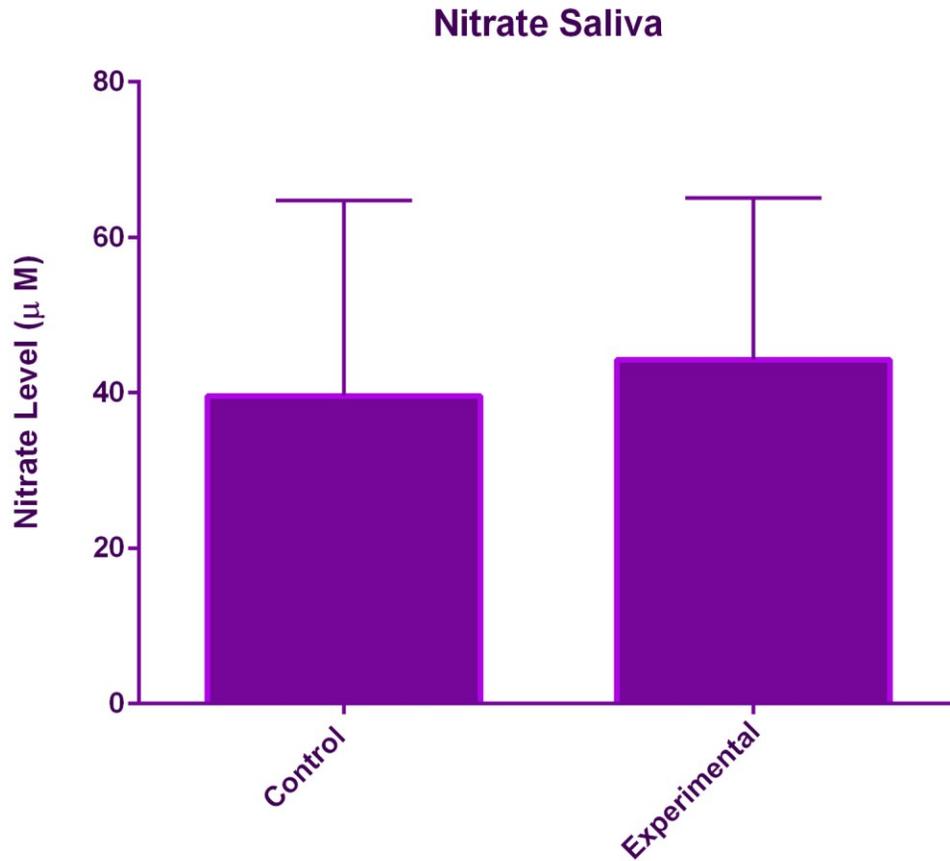
	Control (n=4)	Experimental (n=4)	<i>p</i> value	Significance
Age (years) (Mean±SEM)	21 ± 0	21.5 ± 0.29	0.13	Not significant
BMI (kg/m <sup>2</sup> ) (Mean±SEM)	22.5 ± 0.81	23.7 ± 1.7	0.55	Not significant



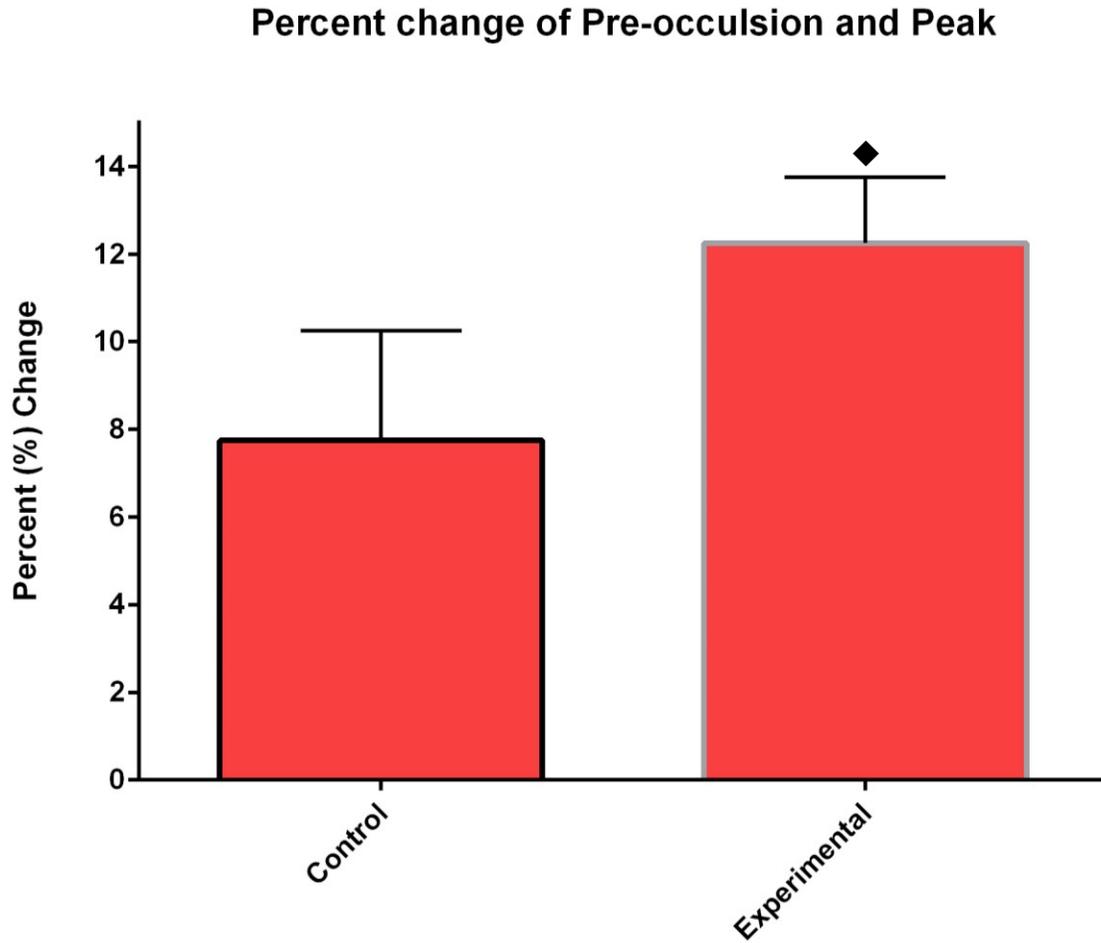
*Figure 1.* This image demonstrates the effect of shear stress on blood vessel diameter (Thijssen et al., 2011). The dotted line represents the shear stress exerted on the blood vessel by blood flow. When the cuff is released, the shear stress increases, peaking around 15 seconds after release. In response, the blood vessel diameter expands, as represented by the solid line. The blood vessel diameter peaks around 60 seconds after cuff release. FMD compares the blood vessel diameter before the cuff is inflated with its peak diameter after the release.



*Figure 2.* The endogenous estradiol levels from the saliva samples were compared between the control and experimental groups. The control group secreted more  $17\beta$ -estradiol than the experimental group taking oral contraceptives ( $p = 0.18$ ).



*Figure 3.* The salivary nitrate levels between the control and experimental groups were compared. The experimental group had higher concentrations of nitrate, the end product of NOS ( $p = 0.63$ )



*Figure 4.* The authors compared the percent change from the pre-occlusion blood vessel diameter to the diameter at the peak of dilation. Despite producing less estrogen, which acts like NOS, than the control group, the experimental group experienced significantly greater blood vessel dilation. (◆ □  $p \leq 0.02$ )

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