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The Effects of Menstrual Phase on the Response of Cutaneous Microvasculature

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THE EFFECTS OF MENSTRUAL PHASE ON THE RESPONSE OF CUTANEOUS MICROVASCULATURE

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Abstract

Estrogen is well-known to be protective against cardiovascular disease in women. In addition to improving lipid metabolism, it also decreases vascular resistance and enhances vascular reflexes, thereby improving vasomotor stability and increasing the arterial capacity for dilatation. Laser Doppler flowmetry (LDF) has demonstrated these changes in research trials, and is emerging as having potential application in many clinical and surgical situations. In this study, our aim was to examine the impact of estrogen upon baseline blood flow as well as the response to vasodilatory interventions and to further evaluate the utility of laser Doppler as a clinical non-invasive measurement of blood flow in such contexts. We compared blood flow in the forehead cutaneous microvasculature of women during both high and low estrogen states of their menstrual cycle, and compared this to the flow in male subjects. To evaluate differences in vascular reactivity, we subjected the microvasculature to two challenges: the cutaneous application of nitroglycerin to the site of the probe; and transient occlusion of flow to evince a hyperemic response. Furthermore, to investigate the reproducibility of laser Doppler data, we examined both temporal and spatial variability, and used each subject as his/her own control. We found significant spatial variability in the LDF measure of baseline flow rates. Temporal variability was also seen within subjects, but was decreased by using median baseline values. Hormone state in females did not significantly affect baseline flow, response to topical nitroglycerin, or hyperemic response to occlusive

pressure. In males, the difference between session 1 and session 2 LDF readings was not significant.

Although LDF has potential clinical applications, the clinical scenarios and patient populations must be further defined. Furthermore, the most practical technique with consistent reproducibility must be developed.

Acknowledgements

To my parents Don and Nancy for their unwavering support and my husband Ehab for his vigorous encouragement. And to the rest of my family for their happily received distractions.

My great thanks to Dr. David Silverman for his tireless discussions and debates of methodology, techniques, and interpretation, and to Robert Schonberger for his invaluable assistance with data and statistical analysis.

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And finally to Yale University School of Medicine I would like to express my gratitude for providing me with the great privilege of entering such a community as this, in which intellectual growth is always fostered and personal development applauded. My desire is to achieve the great standard impressed upon me by the many professors and practitioners of the Yale medical community.

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Introduction

Estrogen affects many tissues within the body, made evident with the changes that occur following menopause. Well-recognized are the effects upon fertility, sexual function, and bone density, but the hot flashes and flushing during menopause may have been an early hint of the interaction of estrogen with cardiovascular function. In fertile women, cyclic alterations in arterial pressure and blood flow parameters have been noted [1-3]. It is now known that the variability in hormone levels throughout a woman's life has significant effects upon vascular function.

Considering the significant impact of estrogen upon the endometrial vasculature throughout the menstrual cycle, it should not be surprising that the hormone has more widespread consequence upon cardiovascular function. In fact, the protective effects of estrogen upon cardiovascular health are now well recognized [4-7]. Heart disease has been the malady of men, and indeed its risk in women is much lower [2]. Soon after menopause, however, this risk matches that of men [8]. The rate of cardiovascular disease in women is rising, and it has become the leading cause of death in women in the United States [9]. Hormone replacement therapy can lower their risk by 50%, although improvement in lipid metabolism can only account for 25-50% of this reduction [10,11].The additional improvement in cardiovascular function may be attributed to enhanced vessel reflexes, flow rates, and reduction in vascular resistance [12].

Recent studies have documented this suspected influence of estrogen upon global cardiovascular function. Women have been noted to have lower skin perfusion than men[13]. During the high estrogen luteal phase of the menstrual cycle, women have lower diastolic blood pressure [1]. Endothelial-dependent vasodilation of the brachial

artery is greater in the follicular and luteal phase compared to the menstrual phase [14]. Exogenous estrogen treatment of menopausal women has been found to decrease their coronary and peripheral vascular resistance. In women suffering from angina or migraines, estrogen treatment may provide symptomatic relief [15].

Estrogen-induced vasoactivity

Although the precise mechanism of action has not yet been defined, estrogen has been found to affect vascular function both through endothelial-dependent and endothelial independent actions. The overall effect of estrogen is to improve vasomotor tone and stability, to increase vessel diameter, and to augment the capacity of vessels to dilate, thereby reducing vascular resistance. Its presence has been shown to influence the production, release, or metabolism of many vasoactive substances, including nitric oxide (NO), prostacyclin, endothelin, calcium ions, and monoamine neurotransmitters [15,16]. With both acute administration and long-term therapy, exogenous estrogen has been found to augment the vasodilatory effect of acetylcholine and corticotropin-releasing hormone [17]. In vascular samples from various tissues, estrogen receptors have been identified on endothelial and vascular smooth muscle cells, in both the nuclear compartment and cytosol as well as upon the cellular membrane [6,12,15]. While the nuclear receptors mediate genomic estrogenergic effects, binding of the extra-nuclear receptors may initiate the rapid effects on vasomotor function seen with acute estrogen administration [15]. These receptors have been found to be upregulated by the presence of estrogen and down-regulated by some progestins [16,18,19].

Perhaps the most significant mediator of the estrogenergic vasoregulation is nitric oxide. Its tonic release is an important determinant of vascular smooth muscle tone; acute

local production of NO occurs in response to various signals. Acetylcholine induces the endothelium to release NO, which has a vasodilatory effect strong enough to overwhelm the vascular smooth muscle contractile response to ACh. Estrogen is known to stimulate the release of NO and enhances the response of the endothelial cells to ACh [7,16]. Women with atherosclerosis experienced coronary artery vasoconstriction in response to ACh, but infusion of 17ß-estradiol resulted in vasodilation within 20 minutes [20]. In human endothelial cell cultures, administration of estrogen at physiological concentrations alone caused a rapid increase in NO production. This response was coupled with an increased concentration of cGMP, signifying that this estrogen-associated increase in NO production was non-genomic. Furthermore, blocking estrogen receptors inhibited the response [21]. In women, basal NO decreases following menopause, although hormone replacement therapy (HRT) can restore premenopausal levels [16,22]. Estrogens and estrogen receptor agonists have been shown to increase reactive hyperemia, the NO-mediated increase in flow following vascular occlusion [23].

Prostacyclin, a potent vasodilator produced through the cyclooxygenase pathway, has also shown enhanced production with estrogen [6]. In a systematic review, pretreatment of endothelial cells with tamoxifen blocked the increased production of prostacylin induced by estrogen application. In another study, prostacylin production induced by raloxifene was not impeded by an estrogen receptor antagonist [24]. Carlsson et al. found that in humans ibuprofen decreased post-occlusive reactive hyperemia of the forearm, suggesting that prostacyclin may contribute acutely to vascular reactivity [25]. Prostacyclin also has important anti-atherosclerotic effects by inhibiting the formation of foam cells and also cholesterol deposits via its effects on vascular

smooth muscle cells. Additionally, it works synergistically with NO to inhibit the aggregation of platelets [6].

Furthermore, estrogen has been demonstrated to interfere with endothelin, a powerful vasoconstrictor produced by endothelial cells. Through specific receptors, endothelin stimulates vascular smooth muscle contraction and the proliferation of smooth muscle cells and fibroblasts. It is also proinflammatory, activating macrophages and promoting adherence of monocytes to the vessel wall [23]. Estrogen suppresses the endothelial production of endothelin and interferes with its activity at receptors. Endothelin counteracts the effects of NO, and the balance of these two molecules is believed to create the basal tone of vascular smooth muscle [26]. Thus, in the estrogen-poor state of menopause, the level of potentially harmful endothelin would be disproportionately high compared to the protective NO.

With these effects that estrogen has been shown to have, it may be an important contributor to the maintenance of overall endothelial health and protection against cardiovascular disease. By contributing to the preservation of the balance between NO and endothelin, estrogen may help to prevent endothelial dysfunction [7]. This dysfunction is a pathological process central to the progression of vessel disease. In menopause without estrogen to support NO production, its inhibition of smooth muscle proliferation and platelet aggregation would diminish [27]. Endothelin would further enhance the atherogenic process. Furthermore, activation of the cyclooxenase pathways during estrogen deficiency may further damage the endothelium through the production of oxygen radical species [7]. In the background of a damaged endothelium, autonomic

release of ACh, which would result in vasodilation with normal physiology, would instead cause smooth muscle constriction in the absence of relaxing factors

Laser Doppler Flowmetry

Several research techniques have been used to evaluate vascular blood flow and the mechanisms by which estrogen modulates it. Laser Doppler flowmetry (LDF) is gaining prominence as a noninvasive measure of blood flow. Monochromatic light is delivered to the tissue under study, where it is frequency-shifted by moving red blood cells in proportion to the concentration of moving RBCs and their velocities. The resulting signal (in volts) provides a measure of RBC "flux" in 1 mm^3 of tissue (the approximate volume monitored) per unit time [28]. The laser Doppler provides spatial and temporal resolution previously unattainable through other measures of blood flow, such as halide clearance or thermistor anemometry methods [29].

Utility of LDF in research has been verified in numerous applications. Studies comparing it to established techniques have found estimation of flow to be similar. In the small bowel of anesthetized dogs, Lynch et al. demonstrated 85% sensitivity in laser Doppler flow velocity and 94% sensitivity in the laser Doppler index, comparable to Doppler ultrasound and perfusion fluorometry [30]. Another study comparing LDF to fluorometry in canine island flaps found similar results in the two methods [31]. Measurements of endometrial perfusion by LDF, xenon clearance and hydrogen clearance techniques yielded similar results [32]. In the forearm of humans, Braverman et al. correlated degree of superficial vascularity of cutaneous biopsy to flow rates found by LDF [33]. The application of these various techniques may ultimately depend on the desired level of flow estimation. For instance, clearance techniques typically measure

entire organs or tissue bodies, whereas Doppler ultrasound measures flow within a specific vessel. LDF has the benefit of temporal and spatial resolution, as well as its non-invasive application without need of dye injections.

Another useful of application of LDF is the measure of reactive hyperemia (RH), or the increase in blood flow that occurs in response to certain stimuli such as heat or flow obstruction. Post-occlusive reactive RH is created by the endothelial response [11], which is mediated by different factors depending on the method used, the tissue anatomy, and duration of flow deprivation. Flow occlusion using a blood pressure cuff will elicit a response in the larger arteries and arterioles as well as local tissue microvasculature, whereas local compression of skin will isolate the response to the cutaneous microvasculature. The duration of vascular occlusion determines the proportion of response mediated by the myogenic and metabolic components. A brief occlusion of flow causes a dilation of the resistance vessels, which would result in a myogenic increase in flow with release of the obstruction. A longer suspension of flow (1-3 minutes) would result in a local increase in vasoactive metabolites, further enhancing the hyperemia. [25] The contribution of particular metabolic components has been demonstrated to vary by the targeted vessels. Having found a significantly augmented response with statin pre-treatment, Binggeli et al. reasoned prostaglandins to play a significant role in skin post-occlusion hyperemia [34]. Alternatively, flow-dependent dilation in large peripheral conduit arteries is primarily mediated by nitric oxide [35]. Local anatomy likely also has an impact upon the response, as well as the specific innervation of the vasculature.

LDF has demonstrated potential in a wide variety of clinical situations. Primary care physicians may find it useful assessing peripheral vascular disease and treatment

response in hypertensive and diabetic patients. In one investigation, LDF evaluation of lower limb blood flow was able to differentiate between healthy subjects and patients with known atherosclerosis [36]. Schonberger et al. found with LDF a diminished response to vasoactive substances in diabetic subjects compared to healthy subjects, likely a reflection of decreased compliance due to peripheral vascular disease [37]. It may also be used by OB/GYN and reproductive specialists to monitor responses to hormone treatments in estrogen deficient women and those undergoing fertility treatments. Through intra-uterine Doppler, Gannon et al. identified significantly elevated mean endometrial perfusion in the early follicular and early secretory phases of the menstrual cycle [32]. Clinical LDF evaluation of the latter phase may improve the probability of successful embryo implantation or *in vitro* fertilization [38,39]. Surgeons of various specialties may find the utility in LDF measurement of perfusion. In selective devascularization of pig bowel, LDF intraoperative detection of low flow rates was predictive of subsequent ischemic necrosis [40]. In graft surgeries, such as flap or coronary bypass, LDF may be able to confirm effective perfusion and predict outcomes.

Despite this promise, LDF measure of cutaneous flow as a reflection of systemic hemodymic parameters may be confounded by multiple variables. The cutaneous vasculature is highly responsive to various factors including temperature, blood volume, activity, and mental stress. The specific innervation and neurotransmitter receptor distribution is an important determinant of this responsiveness. For instance, despite an insignificant change in blood pressure, volunteers subjected to the mental stress of arithmetic demonstrated a 37% decrease in blood flow in the finger's superficial vasculature, which has a high density of ά-adrenergic receptors [41]. In a similar study

using the cold pressor test, with the immersion of the hand in ice water, the blood flow of the finger of the contralateral hand decreased by 48% compared to the 2% decrease in flow of the ear [42]. Considering these findings, the regional placement of LDF probes is of essential importance. The forehead recently has become a site for pulse oximetry measurement, as well as the experimental measure of blood flow using LDF [37].

Another ongoing concern is the reproducibility of data found with LDF. Using endoscopic LDF as a measure of human gastric blood flow, Kvernebo et al. reported temporal and spatial variability to be within acceptable limits [29]. In unpublished data, Gannon et al. analyzed LDF variance and found that although intrasubject variation was high, variability could be reduced within treatment groups by increasing the number of sampling sites[32]. However, considering the high reactivity of the cutaneous vasculature and difficulty in reducing the numerous influential variables, LDF measurement of the superficial blood flow of the skin may not correlate well to the perfusion of visceral tissues. To overcome this, further evaluation of intra-subject variability must be conducted. This includes both spatial variability, the difference between probe measures on the same subject in a similar region, and temporal variability, the difference in one subject from one period to the next.

Purpose:

In our study design, we attempted to address both the nature and mechanism of estrogenergic effects upon blood flow, and to evaluate the reproducibility of LDF data. We chose to examine the impact of estrogen upon nitroglycerin-induced vasodilatation and reactive hyperemia in women compared to male controls and to further evaluate the utility of laser Doppler as a clinical non-invasive measurement of blood flow. We

compared flow rates of the forehead cutaneous microvasculature in women during both high and low estrogen states of their menstrual cycle, and compared this to the flow rates in male subjects. To evaluate differences in vascular reactivity, we subjected the microvasculature to two challenges: the cutaneous application of nitroglycerin to the site of the probe; and transient interruption of local flow to evince a hyperemic response. Furthermore, to investigate the reproducibility of laser Doppler data, we tested both temporal and spatial variability, and used each subject as his/her own control. In evaluating vascular responsiveness, our primary endpoints were the response to topical nitroglycerin and the degree of reactive hyperemia following occlusion of blood flow. Secondary endpoints were baseline flow rates and biological zero values, and time to peak hyperemic response and to recovery of pre-occlusion flow rate. We hypothesized women to have an increased responsiveness of flow parameters during high estrogen state compared to low estrogen state.

Methods

The protocol of this investigation was submitted by Dr. David Silverman and approved through the Human Investigations Committee of Yale University School of Medicine. Prior to their participation, informed consent was obtained by the author from all subjects in writing.

Subjects

Participants were recruited by the author through an advertisement at the Yale-New Haven Medical Center to compose two subject groups: 8 healthy females with regular menstrual cycles and 6 healthy males. Volunteers with a history of cardiovascular disease, diabetes, migraines, or fainting were excluded.

Doppler flowmetry readings

Each subject participated in two 30 minute recording sessions on two different days. Female participants were monitored once during the low estrogen period in their menstrual cycle (days 1-6) and once during the high estrogen period (days 21-23, or while on monocyclic oral contraception pills). The readings were scheduled based upon the upcoming phase of their cycle so that the high-estrogen reading of 3 female subjects preceded the low-estrogen reading. Male controls were monitored on two non-specified days. All scheduling and recording sessions were performed by the author.

All data recording sessions took place in a temperature-regulated room (68 ± 1 º F). Subjects refrained from smoking tobacco or ingesting caffeinated beverages for 4 hours prior to their participation. The forehead was prepared by lightly swabbing with alcohol, followed by wet and dry gauze. Four laser Doppler probes were applied to the forehead of the subjects, avoiding the large vessels of the temple and medial forehead. Each of the three experimental probes was applied with a standardized 6 mm diameter hole-punched section of a translucent 0.6 mg/hr 20 cm^2 Minitran^{TM} homogenous nitroglycerin patch (3M, Minnesota). This portion of the patch delivered drug at a rate of approximately 0.008 mg/hr, calculated from the specified rate of 0.03 mg/hour per 100 mm^2 area of the patch. The patch section was adhered to the laser Doppler probe with a double stick disk. The fourth probe was a control, applied to the forehead with a double stick disk and no drug. Each lead was adhered to the forehead using Double-Stick Discs (#M Health Care, Neuss, Germany) to allow undisturbed monitoring. Laser Doppler fluxmetry was performed continuously at each site for 30 to 32 minutes. Between 28 and 32 minutes of the trial, uniform pressure was applied to the control lead to occlude blood

flow to the site for 5 seconds. Occlusion of flow was confirmed by observing flattening of the lead tracing. Release of the pressure created a characteristic and transient increase in flow rate, known as reactive hyperemia (Figure 1).

Chart for Windows (ADInstruments, Colorado Springs, CO) was utilized for the collection of data at a rate of 1000Hz. All laser Doppler sensors were calibrated using motility standards (Perimed, Sweden).

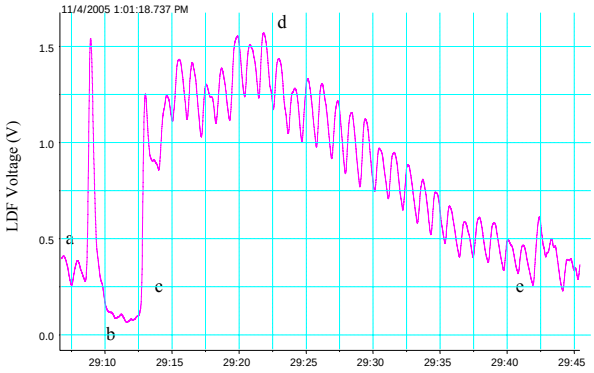

Figure 1 **Reactive hyperemia curve.** This curve is typical of occlusive reactive hyperemia. It includes the pre-occlusive baseline (a), the occlusive period from which the biological zero is taken (b), the release point and sudden resumption of flow (c), the peak hyperemic response (d), and the gradual return to the pre-occlusive baseline (e). This image was taken from the Chart for Windows recording of one of our male subjects and modified with labels in Microsoft Word.

Data analysis

Each data tracing was analyzed by blinded researchers, including the author and another member of the lab. For each of the four monitoring probes, baseline and peak flow rates were calculated. Mean flow rates were calculated for the baseline and peak flow values during the two minutes after application of the probe for the visibly lowest 10 second period (baseline) and for the visibly highest 10 second period within 26-28 minutes following application of the probe (peak flow). To assess reactive hyperemia following a five second compression of local skin, the peak response value was recorded along with the calculated time to peak and time to the return to pre-occlusive baseline.

Motion artifact was avoided when choosing baseline or peak periods. Fluctuations from the trending flow rate visualized in more than two leads were suspected to be systemic reflexes rather than a local response and were also avoided.

In analysis of the reactive hyperemia, a pre-occlusive flow rate average was calculated. The lowest point of the Doppler wave was recorded as the biological zero. The peak response was recorded at the point of greatest flow rate during the transient post-occlusive hyperemic response. Time to peak response was measured from immediately prior to the release of the occlusive pressure to the point of greatest flow. The former point also began the measure of time to return to pre-occlusive flow rate. The end point of this measure was the point at which the Doppler wave first equaled the calculated pre-occlusive flow rate average.

Statistical analysis

Statistical analysis was performed by another member of the lab using SPSS for Macintosh (SPSS, Inc. Chicago, IL) software. The Wilcoxon signed ranks test was used to make comparisons within each subject group and between recording sessions. Student t-tests were used to compare between males and females.

During the course of the study, the high-hormone recording session was held prior to the low hormone for three women, while the opposite was true of the remaining 5 women. To correct for the 3 women whose high-hormone reading occurred first, the order of the readings of 2 randomly chosen male subjects were reversed for many of the calculations. This reversal was made when it was important to compare high hormone to low hormone states. To assess variability, the recordings were organized by the order in which they occurred.

Results

LDF Variability

To assess the reliability and reproducibility of the laser Doppler as an estimation of flow rate, we examined both spatial variability and temporal variability. To assess spatial variability, the range of baseline values for the four probes applied to a single subject was calculated for the male subjects. The maximum flow rate was between 2 to 6.4 times greater than the lowest within a single subject [Figure 2]. Temporal variability was evaluated by comparing the baseline values from the first session to the second within each subject. Because of the high spatial variability found, the median of the three nitroglycerin probes was used in the calculations. Use of the median value was effective in decreasing the relative variability (Figure 3 and 4). The ratio of median baseline values from the second to the first reading ranged from 0.32 to 1.4 in the males and 0.47 to 1.8 in the females. In 2 of the men and 6 of the women, the median baseline of the second reading was decreased compared to the first. The overall change in baseline was increased in the males (-0.1) and decreased in the females (0.06). However, none of these differences were found to be significant.

Baseline values

In the comparisons of baseline flow rates, the median value recorded within a session was used as above. In women, there was no difference of baseline seen between high or low hormone states (Figure 5). Likewise, no significant difference was seen in men between sessions, when correcting for the altered order of the women's readings (Figure 6). When the baseline rates of the males were compared from the first recording session to the subsequent, again no difference was seen.

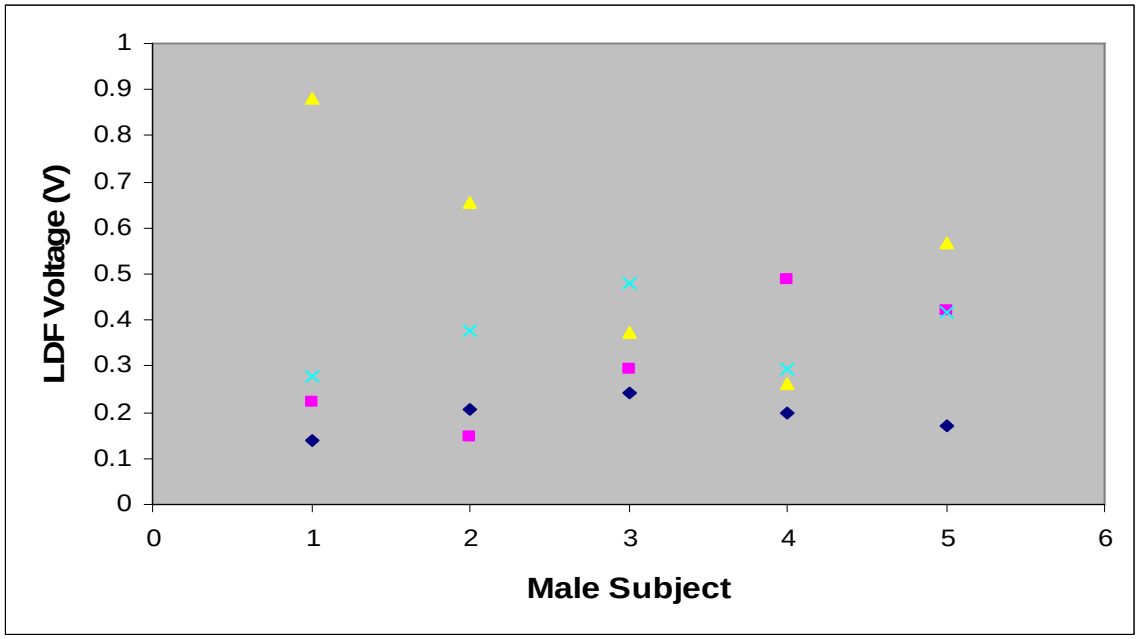

Figure 2 **Range of baseline flow rates in males.** Range of the flow rate values found for each of the 4 laser Doppler probes applied to each male subject during their first data recording session. The (x) is the control lead.

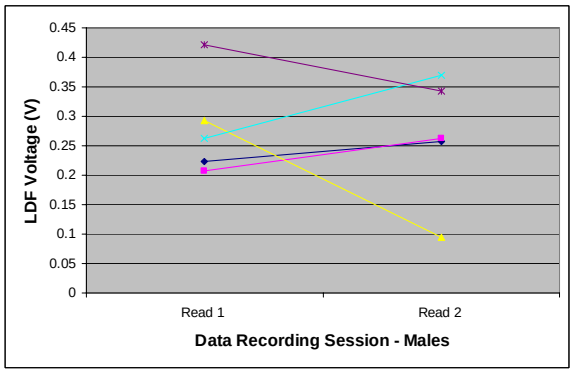

Figure 3 **Median baseline values in males.** A comparison of the median baseline flow values between the first data recording session and the subsequent session.

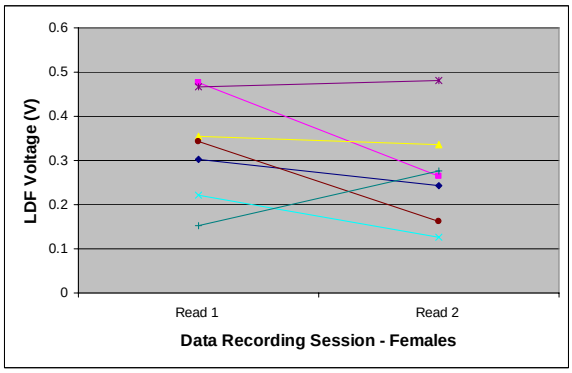

Figure 4 **Median baseline values in females.** A comparison of the median baseline flow values between the first data recording session and the subsequent session

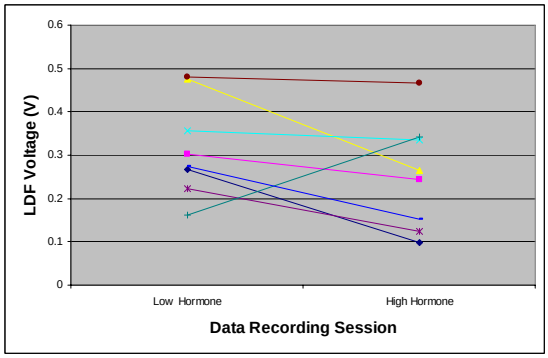

Figure 5a

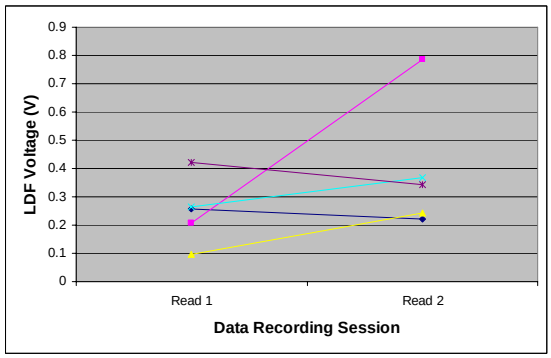

Figure 5b

Figures 5a and b **Median baseline flow rates** in a) females and b) males. Males were corrected for the order reversal in females (The order of the recordings of subjects chosen randomly, represented by the yellow triangle and dark blue circle, were reversed.)

Response to nitroglycerin

To assess whether hormonal state had an impact upon endothelial-independent vasodilation, we compared the relative rise in flow after local cutaneous application of nitroglycerin at the two during hormone states. No significant was found in the mean or median relative rise of three leads with applied topical nitroglycerin (Figure 7; average rise not shown). The median relative rise increased during the high hormone state of 6 females and during the second reading of 1 male. Little difference in the absolute rise in response to nitroglycerin was seen in females between their low and high hormone states (Figure 8).

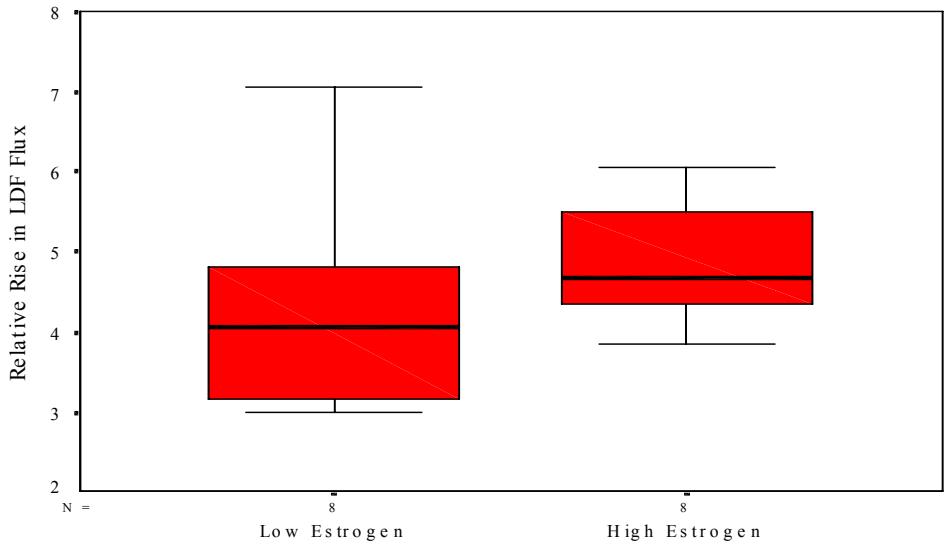

Figure 7a

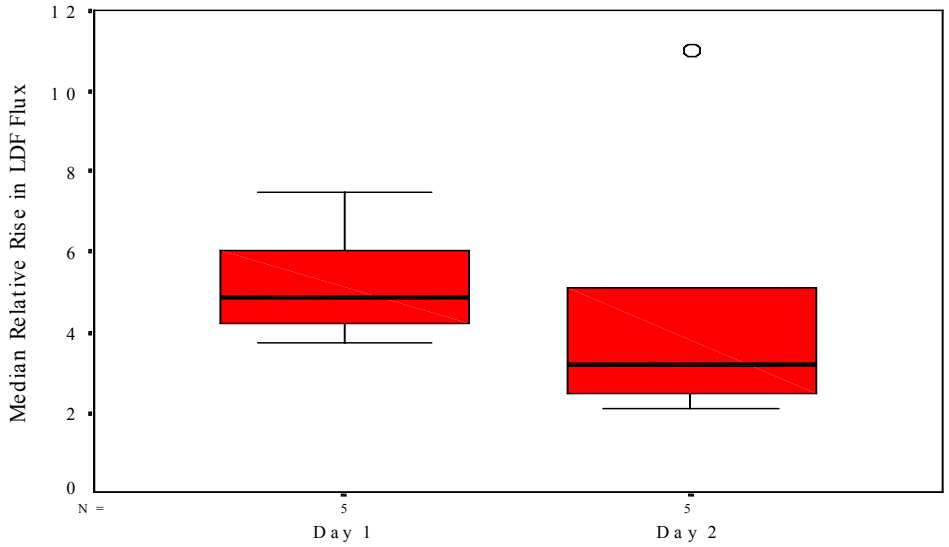

Figure 7b

Figure 7a and 7b. **Relative rise of flow rates in response to nitroglycerin.** In both graphs, the median value of the three experimental LDF leads were used for the comparison. **7a)** Response in females is compared by hormonal status. **7b)** Response in males by recording session, including correction for the order of female subjects readings.

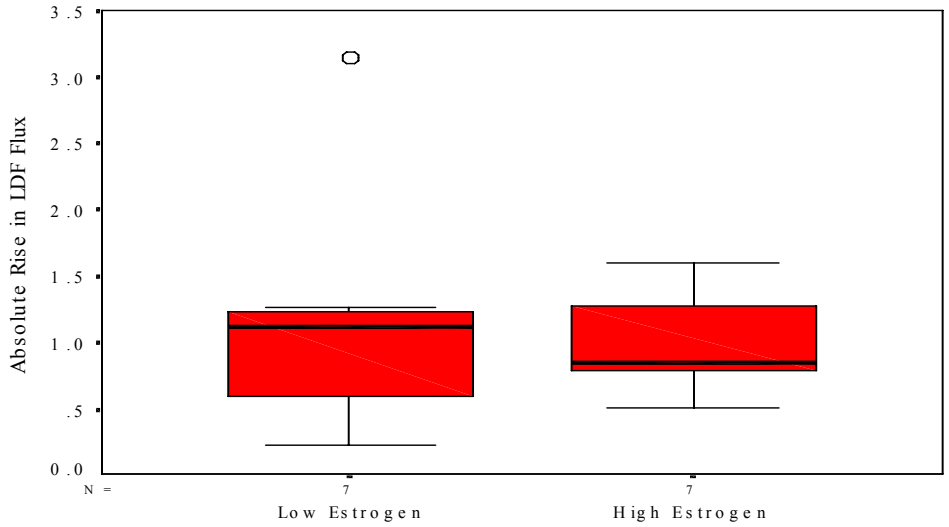

Figure 8 **Absolute rise of flow rates in response to nitroglycerin by hormonal status in females.**

Reactive hyperemia

To further evaluate the impact of hormonal state upon vascular reactivity, the reactive hyperemia response was assessed using peak response value and the relative rise

(peak/pre-occlusive baseline), as well as calculated time to peak and the time to return to pre-occlusive flow rate. The lowest absolute flow rate during occlusion was measured as the biological zero. There was no significant difference of biological zero from one reading to the next in either group (Figure 9). Four females demonstrated an increase in biological zero during the high hormone state, compared to three males during their second reading.

The difference in peak response also was not significant (Figure 10). Three men decreased during the second reading and 6 women decreased in their high hormone state. The relative rise of the peak response compared to pre-occlusive baseline also showed no significant differences (Figure 11). During the high hormone state 4 women showed an increase; and during the second session, 2 males had a relative increase. Similarly, there was no difference in the duration of the response, or the time to return to the pre-occlusive baseline (Figures 12 and 13). Two males and three females took longer to return to the pre-occlusive baseline flow rate during the second reading and high hormone state respectively.

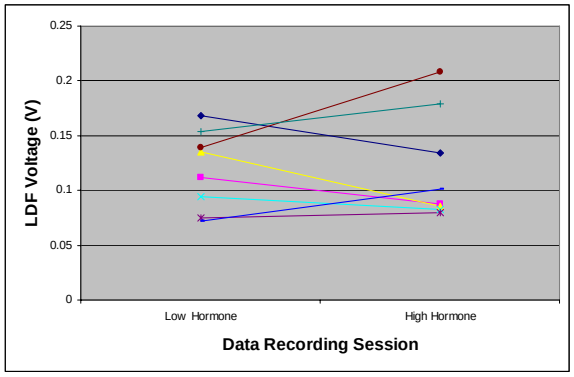

Figure 9a **Comparison of biological zero in females.** Biological zero attained during occlusion pressure was compared between low and high hormone states.

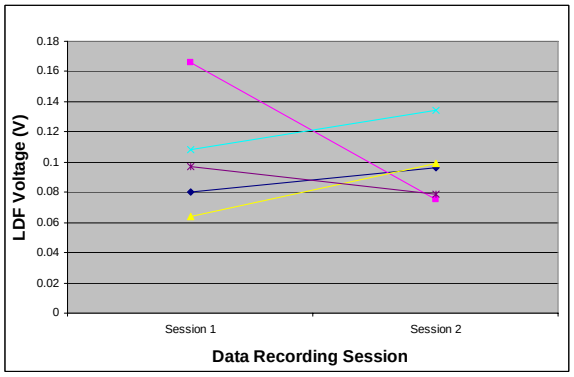

Figure 9b **Comparison of biological zero in males.**

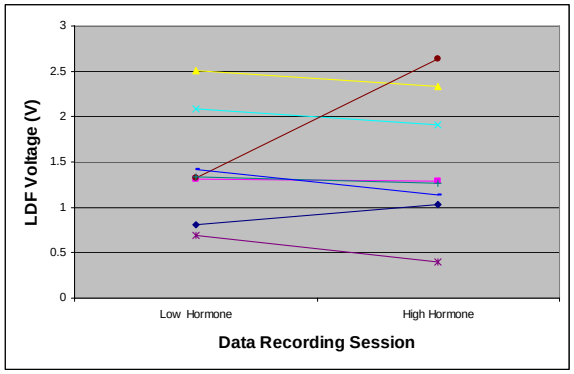

Figure 10a **Peak hyperemic response in females.**

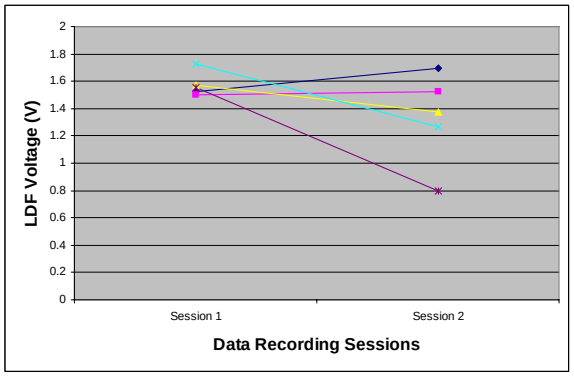

Figure 10b **Peak hyperemic response in males.**

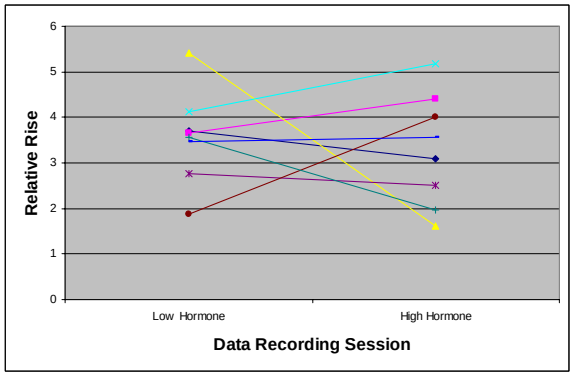

Figure 11a **Relative rise of hyperemic response in females.** A comparison of the relative rise of the hyperemic response over the pre-occlusive baseline flow in females in both low hormone and high hormone states.

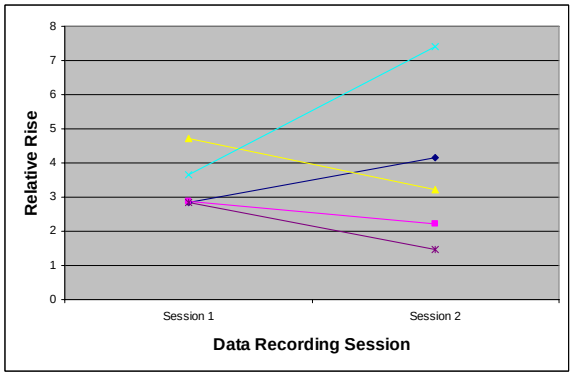

Figure 11b **Relative rise of hyperemic response in males.**

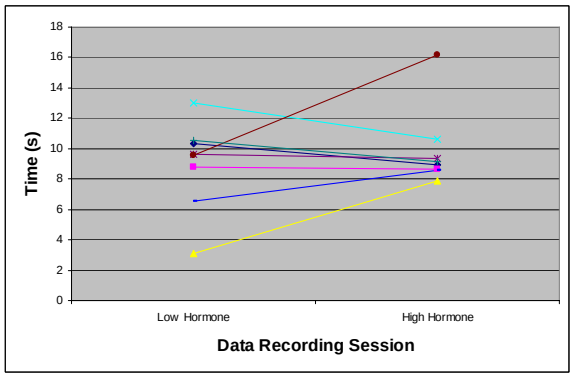

Figure 12a **Time to peak hyperemic response in females.**

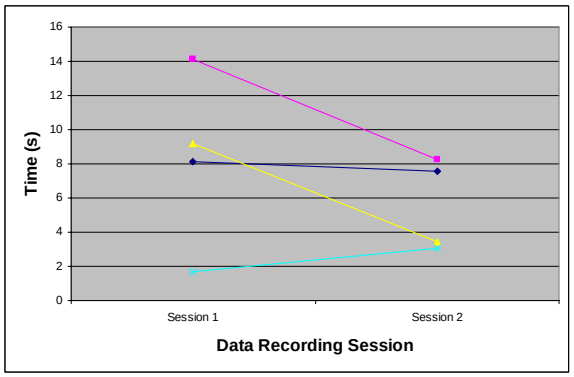

Figure 12b **Time to peak hyperemic response in males.**

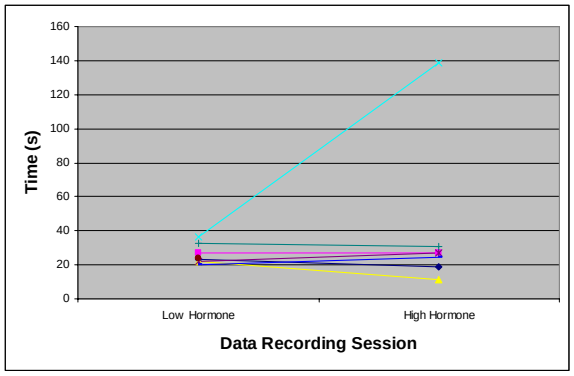

Figure 13a **Duration of hyperemic response in females.**

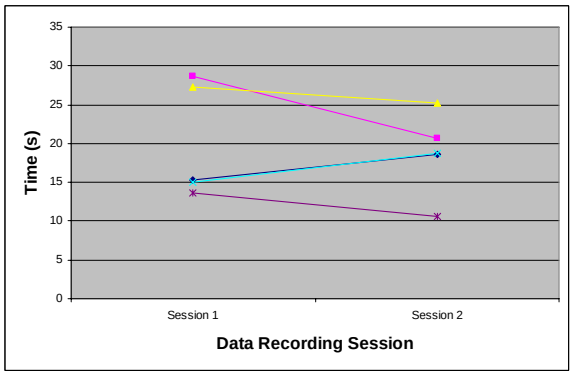

Figure 13b **Duration of hyperemic response in males.**

Discussion

The clinical implications of estrogen or estrogen receptor agonist therapy could well extend beyond reproductive medicine to contribute to the fields of cardiovascular medicine, neurology, and surgery. Considering the protective effects it has upon vascular function, it may once again become a mainstay in the health management of post-menopausal women, particularly for those having hypertension, atherosclerosis, or other significant cardiac risk factors [7]. Estrogen may also gain prominence as a preventative measure for menstrual migraines [15]. For those at risk for stroke or suffering from other ischemic brain diseases, it may play a role in improving blood flow and protecting cortex. In ovarectomized rats, estrogen replacement was protective against cortical damage during middle cerebral artery occlusion [43]. Battaglia et al. found improvement in pulsatility and peak systolic flow of the carotids and ophthalmic artery during HRT in menopausal women [44]. Many surgical procedures have an inherent risk of postoperative ischemia that potentially might be predicted by intra-operative LDF analysis and alleviated by temporary estrogen therapy. In addition to improving flow parameters, it may actually promote neovascularization, as was found in a rabbit model

of ischemic limb injury [45]. With this potential, estrogen may be a beneficial adjunct to abdominal surgeries, flap and transplant operations, as well as cardiovascular procedures.

Although previous research investigations have overwhelmingly found a positive effect of estrogen upon blood flow [2,7,13,16,18,23,32], we did not find any significant differences in women between high hormone and low hormone states. This may either confirm the results of those studies that found no effect [3,46], or the negative results in this case may simply be due to differences in methods used and/or subjects studied. Another important possibility to consider is that the current manner in which the laser Doppler is applied is subject to too many variables.

As discussed previously, laser Doppler evaluation of cutaneous microvasculature flow is subject to many physiologic and anatomic variables. In this investigation, we attempted to evaluate these by analyzing both spatial and temporal variability. For the former, we compared four sites within each subject for both baseline flow and vasodilatory responsiveness. For the latter, we compared the same parameter in subjects on two different days. We found a wide range in baseline flow rates in both females and males without a significant day or hormone effect. Although the impact of variability may be minimized by increasing the number of subjects and sites evaluated as Kvernebo et al. suggested [29], a greater benefit may be obtained by actually reducing intersite variability at the microvascular level. Using skin biopsy, Braverman et al. demonstrated the relationship between blood flow values found by LDF and the underlying arrangement of cutaneous microvasculature [33]. Using a similar method of mapping, it may be possible to develop a non-invasive technique to apply LDF probes, perhaps through site sampling. Regional occlusion of flow might also be used on limbs to visually

demonstrate sites of high and low vessel concentration through the hyperemic flush produced upon reperfusion [33]. Using this reactive hyperemic flush, however, might not be reliable in patients with vascular disease including those who might benefit most from clinical monitoring with LDF, such as patients with diabetes and atherosclerosis. Another method that might prove more valuable may be the use of the integrating LDF probe, which averages the values gained through continuous LDF monitoring over several neighboring sites. Members of our lab are currently evaluating the reproducibility of this probe. We are furthermore attempting to develop a method of LDF site selection and probe application that would ease the production of reliable data in both clinical and research situations.

For the purpose of evaluating the effects of estrogen upon microvasculature flow in this study, the timing of the LDF readings may have also significantly impacted our results. For the high hormone state, we chose to perform the reading between the 21-23 days of the women's cycles. Previous studies have used a similar time period or have attempted to approximate the day of the pre-ovulatory peak [11,14,39]. The benefit to the latter period is the ability to isolate estrogenergic effects from those of elevated progesterone. Progesterone has on occasion been demonstrated to strongly counteract the effects of estrogen upon vascular function, although this seems to be an effect of only certain progesterones [6,12,15]. On other occasions, progesterone has been found to have effects similar to estrogen [6,12,15]. Due to this possible interaction, day 13 may be optimal to evaluate the vascular effects of estrogen. However, this pre-ovulatory estrogen surge is short lived, and may be easily missed due to cyclic differences in females. Because our protocol and HIC approval did not include IV blood draws (to confirm the

pre-ovulatory estrogen surge) and our subject population included women on OCP's (combined estrogen and progesterone), we felt day 21-23 to be a better time period for our investigation.

Another impact upon outcome might be due to differences in autonomic and auto-regulatory factors. As discussed earlier, the skin microvasculature is dramatically responsive to many factors including temperature and mental stress. Furthermore, certain regions are less responsiveness than others. But an important influence may be in the regulatory differences in the cutaneous and subcutaneous vasculature to that of the internal organs. This difference may interfere with non-invasive LDF evaluation of cutaneous vessel function as a representation of the health of internal organ vasculature.

Weaknesses in study design that may have further contributed to the lack of positive findings include subject selection bias and low number of subjects. Our subjects were all young and healthy selected through advertisement at Yale-New Haven Medical Center. Considering that the studies referenced utilized many different methods in many different subject groups, it would be useful to evaluate the impact of age and health upon LDF measurement of blood flow and the impact of gonadal hormones.

Regarding the potential of estrogenergic effects upon vascular function, it is likely that the lack of positive findings in this study is due to the variables as discussed, most particularly the variability of flow between probe sites. Despite the lack of positive findings in this case, through this study we have identified an area of further research that may significantly progress the use of LDF in both research and clinical applications.

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