Endothelial function across an oral contraceptive cycle in women using levonorgestrel and ethinyl estradiol

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Abstract

Endothelial function across an oral contraceptive cycle in women using combination ethinyl estradiol/levonorgestrel OCPs has been shown to be antagonized by some (16, 49), but not all, progestin types (45). Thus any effect of estrogen on arterial vasculature may be preserved or antagonized depending on the progesterin, and this must be considered when assessing the impact of OCPs on vascular health in women.

Recently, Rickenlund et al. (44) demonstrated that treatment with combination ethinyl estradiol/levonorgestrel OCPs improves endothelium-dependent vasodilation in amenorrheic and sedentary women but not in young healthy athletes (44). However, endothelial function was not assessed between the hormone phases of the oral contraceptive cycle in these subjects. As of yet, no study on OCPs and endothelial function has considered cyclic fluctuations (26, 44, 48).

Therefore, our goal was to evaluate endothelial function during the active hormone phase and compare it with the placebo phase of an oral contraceptive cycle in healthy young women using low-dose monophasic ethinyl estradiol and levonorgestrel OCPs. This description of endothelial function in oral contraceptive users is important for several reasons: 1) it may provide clinicians with greater insight into the arterial effects of the current lower estradiol dosing, 2) it may establish whether cyclic fluctuations in endothelial function occur across OCP cycles, and 3) it may provide insight into the role of the commonly prescribed progesterin, levonorgestrel, on the arterial vasculature. We hypothesized that endothelium-dependent vasodilation would be higher during the active phase when ethinyl estradiol and levonorgestrel are administered and lower during the placebo phase. Additionally, we investigated two doses of monophasic ethinyl estradiol plus levonorgestrel OCPs since we further hypothesized that a greater change in endothelium-dependent vasodilation between the active phase and placebo phase of an OCP cycle.
methods

The participants in this study were 15 healthy premenopausal women taking combined monophasic ethinyl estradiol and levonorgestrel progestin OCPs as prescribed by their health care provider. The subjects were young (18–26 yr), normally active women not taking any other medications. All subjects had used their oral contraceptive method for ≥3 mo. All subjects were required to take a pregnancy test and show negative results before the start of each study. Approval of this investigation was granted by the Institutional Review Board of the University of Oregon. Each subject provided written and oral consent for the protocol before participation. Exclusion criteria were smoking, cardiovascular disease, hypertension, hypercholesterolemia, metabolic disorders, personal or family history of blood clots, personal history of menstrual disorders, or any contraindications against the use of OCPs.

Participants in this study were recruited from one of two groups: group VLD or group LD, based on the dose of exogenous hormones in their OCPs. To see a 1.5–2.0% change in endothelium-dependent vasodilation with a power of 80% between hormone phases, 6–8 subjects are required per group using the custom analysis DICOM system used in the present study (51). Group VLD (n = 8) oral contraceptive users took hormone pills containing 100 μg of levonorgestrel and 20 μg concentrations of the estrogenic compound ethinyl estradiol across weeks 1, 2, and 3 (Alesse, Wyeth, Madison, NJ; or Levite, Berlex, Montville, NJ). The group LD (n = 7) oral contraceptive users took pills containing 150 μg of levonorgestrel and 30 μg concentrations of the estrogenic compound ethinyl estradiol in weeks 1, 2, and 3 (Nordette, Wyeth; or Levlen, Berlex). In both groups, week 4 pills serve as placebo pills and did not contain any exogenous hormone. All subjects participated in the experimental protocol on 2 study days, once during days 5–7 of week 3 and once during days 5–7 of week 4. Subjects gave verbal confirmation that they had taken their pills according to the prescription. Subjects were instructed to abstain from exercise and vitamins or supplements for 24 h, and alcohol, caffeine, or food for 12 h before the study. All studies were conducted in a temperature-controlled room (22° to 24°C) between the hours of 7:00–11:00 AM. Subjects were brought in to the laboratory at the same time of day for each of their two participatory study days. The order of experimental study days was counterbalanced.

measurement techniques

heart rate and blood pressure. Heart rate was monitored continuously using a five-lead electrocardiogram (CardioCap, Datex-Ohmeda; Tewksbury, MA) dually interfaced with our Doppler ultrasound system and data acquisition computer. Arterial blood pressure was continuously monitored noninvasively using a portable finger blood pressure cuff (Portapres Model-2, TNO-TPD Biomedical, Amsterdam, The Netherlands).

blood samples. To verify the suppression of endogenous estradiol and progesterone by the oral contraceptives, venous blood samples were collected on each study day from an antecubital vein after subjects had 20 min of supine rest. Samples were collected in 7.5-ml serum separation blood collection tubes (BD Vacutainer; Franklin, NJ) and were separated within 30 min of collection by centrifuging at 1,300 g relative centrifugal force for 15 min at 4°C. Serum samples were stored frozen at −70°C until transport to Oregon Medical Laboratories (Eugene, OR) for analysis of estradiol and progesterone, which were measured using standard radioimmunoassay technique. A serum value corresponding to <20 pg/ml was beyond the detectable limit for estradiol, and a serum value <0.2 ng/ml was beyond the detectable limit for progesterone. Because of this, serum hormone values are reported as the median value.

brachial artery measurements. All subjects were supine with their right arm supported at an ~80–90° angle from their torso at heart level. A blood pressure cuff was placed on the subject’s forearm just below the antecubital fossa. With the use of a high-resolution Doppler ultrasound machine (Acuson 128XP), a high-frequency 7.0-MHz linear array probe was placed on the brachial artery 3–10 cm proximal to the antecubital fossa for longitudinal imaging and blood velocity tracing. The transducer probe was held in place by a clamp to maintain the same position over the brachial artery for the entire study. Ultrasonic parameters were set to optimize longitudinal B-mode images of the lumen and arterial wall interface while insonating the lumen of the artery at an angle of 60° to determine blood velocity. Endothelium-dependent and -independent vasodilatory responses were tested using the standard 5-min distal occlusion flow-mediated dilatation (FMD) technique (11, 12) followed by sublingual nitroglycerin (NTG) administration, respectively. Subjects repeated these tests on each study day.

protocol

Following the venous blood sample collection, subjects rested for 20 min during instrumentation and before endothelial function testing. Brachial artery diameters were measured before, during, and after distal reactive hyperemia, followed by measurements before and after 0.4 mg sublingual NTG administration. The operating parameters remained constant throughout each study period, and the same settings were used during the second study period.

endothelium-dependent vasodilation. After the initial resting period, baseline scans assessing vessel diameter were obtained for 1 min. Following the baseline scan, the blood pressure cuff on the forearm was rapidly inflated to 300 mmHg, held for 5 min, and then deflated rapidly. The increase in arterial blood flow immediately following cuff deflation, called reactive hyperemia, results in an increase in shear stress across the vessel endothelium resulting in FMD of the artery. Images were recorded for 1.5 min before cuff deflation and continuously for 2 min post-cuff deflation.

endothelium-independent vasodilation. After 20 min of rest following the endothelium-dependent testing, new baseline images were recorded for 1 min followed by a sublingual administration of 0.4 mg NTG. The brachial artery was continuously imaged for 10 min following NTG administration. Subjects remained supine for an additional 15 min before leaving the laboratory area.

FMD stimulus quantification. To quantify the reactive hyperemia stimulus, we chose to use an estimate of shear rate rather than blood flow. It has been previously shown by Pyke et al. (41) that smaller blood vessels experience a larger shear rate and smaller flow than larger vessels during the FMD reactive hyperemia test. Furthermore, Pyke et al. (41) demonstrated that, in young healthy subjects, there is no relationship between the peak blood flow (ml/min) and the peak shear rate (blood velocity/vessel diameter) during the FMD reactive hyperemia test. Additionally, because the peak vasodilation may not be achieved until 60 s of post-cuff release (3, 39), we calculated the area under the curve of the shear rate over 90-s post-cuff release (42). Shear stress = (ηV)/D where η is blood viscosity, V is blood velocity, and D is the diameter of the blood vessel (22). Shear stress was estimated in the present study using the following equation: shear rate = η (in cm/s)/D (in mm), which does not account for subject differences or changes in blood viscosity during the study period, but it is a satisfactory estimate of the stimulus (4, 13, 41, 53).

data analysis. Heart rate and blood pressure data were recorded on a computer at 20 Hz and saved for later analysis (WinDaq; Dataq Instrument; Akron, OH). Brachial artery images and blood velocity were recorded from an Acuson 128XP ultrasound Doppler System to a data acquisition computer that is interfaced with custom analysis software (DICOM; Perth, Australia) to capture real-time video images, encode, and store the images at 30 frames/s (51). This system allows for off-line review and automated edge-detection and wall-
tracking analysis of vessel diameter and synchronous measurement of blood velocity. The system also allows for analysis of vessel diameter in synchrony with end diastole, providing greater reproducibility of measurement than with previous methods, reduces observer error significantly, and allows the detection of significant changes with substantially fewer subjects (51).

Reproducibility. The same observer undertook the brachial artery imaging and data recording and analyzed all data. The intraobserver variability for measuring brachial artery diameter was assessed by comparing separate baseline diameter measurements in each of the subjects across the 2 study days. The coefficient of variation (SD/mean × 100) for baseline diameter measurements was 1.7% for this study.

Statistical analysis. The within-group data were analyzed by using paired t-tests to compare differences between the placebo pill phase and the active pill phase for each variable. Baseline subject characteristic data were compared between groups using two-way ANOVAs. Between-group comparisons were not made for vascular responsiveness. Statistical significance was defined as P < 0.05. All data are expressed as means ± SE.

Follow-up Protocol

To confirm that a very low dose of oral ethinyl estradiol could increase endothelium-dependent vasodilation when administered unopposed by levonorgestrel, we measured endothelium-dependent vasodilation in two additional women who were using OCPs. We measured endothelium-dependent vasodilation on 2 study days, once during the placebo phase of the oral contraceptive cycle (no ethinyl estradiol or progestin administered) and once during the same interval of the following placebo phase after administering 10 μg/day of oral ethinyl estradiol for 5 consecutive days. Thus we compared endothelium-dependent vasodilation in a hormone-free placebo phase to an unopposed ethinyl estradiol phase.

RESULTS

Subject Characteristics

Baseline characteristics for the study groups are shown in Table 1. Subjects did not differ in height, weight, body mass index, or in the duration of time that they had been using their OCPs. Baseline characteristics between the active and placebo phase of the oral contraceptive cycle for the study groups are shown in Table 2. There were no significant differences in heart rate, systolic blood pressure, or mean arterial pressure between the oral contraceptive phases in each group. However, diastolic blood pressure was higher at baseline during the active phase compared with the placebo phase in group LD (P = 0.04), and this was not observed in group VLD. Heart rate and blood pressure did not vary significantly during the study protocol.

Group VLD had larger baseline diameters than group LD (Table 3; P = 0.001). As a result, group VLD also experienced a lower shear stress during FMD testing than group LD (Table 3; P = 0.002). Because of this group difference, no statistical comparisons were made between flow-mediated, endothelium-dependent vasodilation or endothelium-independent vasodilation of group VLD compared with group LD.

Endogenous estradiol and progesterone levels were suppressed across the cycle in all subjects. In group LD, serum estradiol levels were <20 pg/ml in the active phase and <20 pg/ml during the placebo phase. Group LD serum progesterone levels were 0.4 ng/ml in the active phase and 0.3 ng/ml during the placebo phase. Group VLD estradiol levels were <20 pg/ml during the active phase and <20 pg/ml during the placebo phase. Group VLD progesterone levels were 0.3 ng/ml during the active phase and 0.4 ng/ml during the placebo phase.

Endothelium-Dependent Vasodilation of the Brachial Artery

There was no difference in baseline diameters or shear rate between the active phase and placebo phase in either group (Table 3). FMD was lower in group VLD during the active phase compared with the placebo phase of the cycle (P = 0.024). This difference in group VLD is displayed by showing the relationship of endothelium-dependent vasodilation of the brachial artery to the cycle phase (Fig. 1A). In contrast, there were no differences in the FMD response in group LD between the active phase and placebo phase of the oral contraceptive cycle (P = 0.647; Fig. 1B).

Endothelium-Independent Vasodilation of the Brachial Artery

There were no significant differences in endothelium-independent vasodilation between the active phase or the placebo phase in group VLD or group LD (P > 0.05; Table 3).

Table 1. Baseline characteristics in group VLD and group LD

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group VLD</th>
<th>Group LD</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>21.0±2.0</td>
<td>20.0±1.0</td>
<td>0.21</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>59.7±6.8</td>
<td>57.9±5.7</td>
<td>0.58</td>
</tr>
<tr>
<td>Height, cm</td>
<td>163.2±4.1</td>
<td>163.7±5.2</td>
<td>0.85</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>22.4±1.9</td>
<td>22.0±1.4</td>
<td>0.37</td>
</tr>
<tr>
<td>Time on oral contraceptives, months</td>
<td>9.0±6.0</td>
<td>16.0±12.0</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Values are in means ± SE; n = 8 very low-dose group (VLD) oral contraception and n = 7 low-dose group (LD) oral contraception.

Table 2. Baseline characteristics in group VLD and group LD during active and placebo phases of the oral contraceptive cycle

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group VLD</th>
<th>Group LD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic</td>
<td>120±6</td>
<td>120±7</td>
<td>0.93</td>
</tr>
<tr>
<td>Diastolic</td>
<td>60±7</td>
<td>63±7</td>
<td>0.23</td>
</tr>
<tr>
<td>MAP</td>
<td>80±5</td>
<td>82±6</td>
<td>0.35</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>63±10</td>
<td>64±10</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 group VLD oral contraception and n = 7 group LD oral contraception. MAP, mean arterial pressure.
Table 3. Endothelial function values across an oral contraceptive cycle

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group VLD</th>
<th>Group LD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Active phase</td>
<td>Placebo phase</td>
</tr>
<tr>
<td>Baseline diameter, mm</td>
<td>3.49±0.10</td>
<td>3.49±0.12</td>
</tr>
<tr>
<td>FMD diameter, mm</td>
<td>3.68±0.11</td>
<td>3.74±0.14</td>
</tr>
<tr>
<td>Baseline pre-NTG, mm</td>
<td>3.48±0.11</td>
<td>3.55±0.11</td>
</tr>
<tr>
<td>NTG, diameter, mm</td>
<td>4.05±0.11</td>
<td>4.09±0.12</td>
</tr>
<tr>
<td>NTG, %change</td>
<td>16.59±1.11</td>
<td>15.43±1.35</td>
</tr>
<tr>
<td>Shear rate 90s AUC, Velocity/diameter</td>
<td>4,810±762</td>
<td>4,362±657</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 group VLD oral contraception and n = 7 group LD oral contraception. FMD, flow-mediated dilation; NTG, nitroglycerin; AUC, area under the curve.

Follow-Up Protocol

In both subjects tested, endothelium-dependent vasodilation was higher when 10 μg of oral ethinyl estradiol were administered compared with the hormone-free placebo phase as displayed in Fig. 2.

DISCUSSION

In contrast to our hypothesis, we found that endothelium-dependent vasodilation was not higher during the active phase compared with the placebo phase of the cycle in women using low-dose ethinyl estradiol/levonorgestrel OCPs. This finding was surprising based on the evidence that ethinyl estradiol has been shown to alter many factors that promote endothelium-dependent vasodilation including increased expression of endothelial nitric oxide synthase in human endothelial cells (32), decreased endothelin-1 levels in healthy young women (35), and increased circulating nitric oxide levels in postmenopausal women (10). Although the doses we studied are categorized as low-dose and very low-dose formulations, these doses are physiologically higher than naturally circulating sex hormones. In fact, our follow-up data show that doses of unopposed ethinyl estradiol as low as 10 μg can increase endothelium-dependent vasodilation compared with a placebo phase in young women using OCPs. This supports our speculation that the activity of the progestin levonorgestrel antagonized ethinyl estradiol induced-vasodilation in the present study.

We observed no changes in baseline diameter, shear rate, and cardiovascular responses between phases in either group. Thus the decrease in endothelium-dependent vasodilation in group VLD during the active phase of the cycle does not appear to be the result of changing sympathetic stimulation or overall vascular tone. This indicates that for the same shear stimulus, the brachial artery dilated more during the placebo phase in group VLD.

Since we observed variation in vasodilation between two groups of women using different doses of hormones, we believe a discussion on these contraception formulations is warranted to aid in the interpretation of our results. Although both formulations in this study administer the same ethinyl estradiol-to-progestin ratio of 1:5 during each day of the active phase, there are pharmacokinetic differences in the in vivo ratio of ethinyl estradiol and levonorgestrel between the low-dose and the very low-dose formulations. For instance, the mean area under the curve (0–24 h) hormone concentrations during the active phase corresponds to in vivo ethinyl estradiol-to-levonorgestrel ratios of 0.008 for group VLD and 0.013 for group LD (2, 14, 33). In addition, the maximum serum hormone concentrations (C_max) during the active phase corresponds to in vivo ethinyl estradiol-to-levonorgestrel ratios of 0.013 for group VLD and 0.018 for group LD (2, 14, 33). This indicates that despite the 33% reduction in hormone dose, there is a higher concentration of levonorgestrel to ethinyl estradiol during the active phase in group VLD. Although this is difficult to relate directly to the present data, the higher levonorgestrel to ethinyl estradiol balance may contribute to the decrease in vasodilation during the active phase in group VLD.

The progestin in this study, levonorgestrel, is a second generation androgenic progestin that has been shown to impact vasculature in several ways. First, levonorgestrel can produce hypertension and increase the vasoconstrictor sensitivity to α₁- and α₂-receptor agonists in rat arterial vasculature when administered alone (6, 7). Second, the known side effects of the levonorgestrel intrauterine systems of decreased menstrual blood loss and amenorrhea are partially explained by local actions of levonorgestrel on the endometrial vasculature including significant decreases in subendometrial blood flow (54), the downregulation of estrogen receptors (28), and increases in blood flow resistance of uterine arteries during the luteal phase (25). Finally, levonorgestrel can antagonize estrogen-induced improvements on both carotid artery blood flow (27) and the plasma lipid profile (9, 27). Our data are the first to demonstrate that levonorgestrel may antagonize the vasodilatory effect of ethinyl estradiol on the brachial artery in young women using oral contraception. Our findings are similar to those described for the progestin medroxyprogesterone acetate, which has been shown to antagonize estrogen-induced vasodilation in the brachial artery, forearm, and aorta (21, 30, 46, 49). In comparison, the progestin norethisterone has not been found to antagonize estradiol in producing vasoprotective substances in vitro (38) but does decrease estrogen-induced vasodilation in the brachial artery in women with hypercholesterolemia (16).

These data highlight the idea that progestin bioactivity is an important component for assessing vascular physiology in women using exogenous hormones.

There are limitations in the present study that should be taken into consideration. Foremost, we studied women at only two time points across the cycle. Our choice of study days at the end of week 3 of the active phase and at the end of week 4 of the placebo phase provided reliable time points to show whether or not exogenous hormones administered in the oral contraceptives had an effect on endothelial function compared with a placebo phase when no exogenous hormones are administered and when endogenous estradiol and progesterone levels are low. In addition, we recognize that we have a...
understand how variations in dose, progestin type, and formulation can impact vascular health in women. The issue of estradiol dosing is important, since estradiol has the potential to not only increase nitric oxide bioavailability and vasodilation but also increase other vasodilatory and anticoagulant substances that promote healthy vasculature (29, 36). Certain lower-dose ethinyl estradiol/progestin formulations may provide contraceptive benefits to young women but may not provide the vascular benefits attributed to estrogens if estrogen activity is antagonized by the progestin.

The question of why cyclic changes in vascular function are potentially important may be related to the cascade of events that begin with natural female sex hormones impacting the release of vasoactive substances and the subsequent effects that these substances have on vascular smooth muscle, atherosclerotic processes, and overall vascular tone. Endogenous female sex hormones have been shown to cause cyclic fluctuations in cardiovascular responses across the menstrual cycle including

Conclusions and Perspectives

From the fact that growing numbers of women are using hormonal contraception, there is an increasing importance to understand how variations in dose, progestin type, and formulation can impact vascular health in women. The issue of estradiol dosing is important, since estradiol has the potential to not only increase nitric oxide bioavailability and vasodilation but also increase other vasodilatory and anticoagulant substances that promote healthy vasculature (29, 36). Certain lower-dose ethinyl estradiol/progestin formulations may provide contraceptive benefits to young women but may not provide the vascular benefits attributed to estrogens if estrogen activity is antagonized by the progestin.

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**Fig. 1.** A: very low-dose group (VLD; $n = 8$). B: low-dose group (LD; $n = 7$). Flow-mediated, endothelium-dependent vasodilation of the brachial artery during the active phase and placebo phase of an oral contraception cycle is shown. Values are means ± SE. *$P = 0.024$ vs. placebo phase.

**Fig. 2.** Follow-up protocol. Flow-mediated, endothelium-dependent vasodilation of the brachial artery during a ethinyl estradiol only vs. a hormone-free, placebo phase in subjects 1 (top) and 2 (bottom).
endothelium-dependent vasodilation of the brachial artery (15, 23, 31, 50), sympathetic outflow (37), α-adrenergic vasoconstriction (8), arterial distensibility (20), and circulating levels of some endothelium-derived vasoactive factors (35). If cyclic fluctuations occur due to hormone-dependent modifications in vasoactivity that decrease the atherogenic processes, then this process may be an important adaptation that helps to regulate the response to vascular injury. For instance, during the menstrual cycle, the endometrial vasculature goes through a cycle of ischemia and reperfusion vascular injury, platelet aggregation and thrombotic processes, and estradiol-induced promotion of new and healthy vasculature (17, 52). It may be that vascular health is maintained by balancing vascular insults with periods of vascular modifications that negate pathological processes.

In summary, endothelium-dependent vasodilation was not higher during the active phase of an oral contraceptive cycle in women using ethinyl estradiol/levonorgestrel OCPs. In fact, we found endothelium-dependent vasodilation was lower during the active phase in women using very low-dose ethinyl estradiol/levonorgestrel OCPs. In contrast to a natural menstrual cycle, which promotes increased endothelium-dependent vasodilation when hormones are elevated, these findings indicate that combination ethinyl estradiol/levonorgestrel hormone use during an OCP cycle does not promote endothelium-dependent vasodilation in young healthy women.

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GRANTS

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