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Pulmonary gas transfer related to markers of angiogenesis during the menstrual cycle

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Farha S, Asosingh K, Laskowski D, Licina L, Sekigushi H, Losordo DW, Dweik RA, Wiedemann HP, Erzurum SC. Pulmonary gas transfer related to markers of angiogenesis during the menstrual cycle. *J Appl Physiol* 103: 1789–1795, 2007. First published August 23, 2007; doi:10.1152/jappphysiol.00614.2007.—Gas transfer in the female lung varies over the menstrual cycle in parallel with the cyclic angiogenesis that occurs in the uterine endometrium. Given that vessels form and regress in the uterus under the control of hormones, angiogenic factors, and proangiogenic circulating bone marrow-derived progenitor cells, we tested the possibility that variation in pulmonary gas transfer over the menstrual cycle is related to a systemic cyclic proangiogenic state that influences lung vascularity. Women were evaluated over the menstrual cycle with weekly measures of lung diffusing capacity and its components, the pulmonary vascular capillary bed and membrane diffusing capacity, and their relation to circulating CD34⁺CD133⁺ progenitor cells, hemoglobin, factors affecting hemoglobin binding affinity, and proangiogenic factors. Lung diffusing capacity varied over the menstrual cycle, reaching a nadir during the follicular phase following menses. The decline in lung diffusing capacity was accounted for by ~25% decrease in pulmonary capillary blood volume. In parallel, circulating CD34⁺CD133⁺ progenitor cells decreased by ~24% and were directly related to angiogenic factors and to lung diffusing capacity and pulmonary capillary blood volume. The finding of a greater number of lung microvessels in ovariectomized female mice receiving estrogen compared with placebo verified that pulmonary vascularity is influenced by hormonal changes. These findings suggest that angiogenesis in the lungs may participate in the cyclic changes in gas transfer that occur over the menstrual cycle.

endothelial progenitor cell

THE FIRST BREATH OF THE NEWBORN marks the transition from fluid to air respiration. From that time on, the primary essential function of the respiratory system is gas exchange. Oxygen diffuses along a pressure gradient from the inhaled air across the surfaces of the terminal bronchioles, alveolar ducts, and alveoli into the rich vascular network of capillaries, and on entering the bloodstream it readily crosses the erythrocyte membrane and binds to the ferrous heme of hemoglobin (10). Thus gas transfer in the lung is modeled as a series of resistances: the molecular diffusion of the gas across the terminal bronchiolo-alveolar-capillary membranes, the diffusion within the blood, and the chemical reaction of the gas with hemoglobin. The Roughton and Forster (31) equation summarizes these concepts: $1/DL = 1/Dm + 1/\theta Vc$. DL represents the total lung diffusing capacity, Dm the diffusing capacity of the

membrane that separates the air from the blood in the terminal ventilatory unit, Vc the pulmonary capillary bed available for gas transfer, and θ the rate of reaction of the gas with the red blood cell (31). Dm is a function of the diffusion coefficient of the gas in the alveolar membrane, the thickness and the area of the membrane, the solubility of the gas in the lung tissue, and its molecular weight and is independent of lung blood volume in this model. The second element on the right-hand side of the equation represents the diffusion of the gas within the blood and its reaction with hemoglobin; however, it can be simplified to θVc for gases such as carbon monoxide, because the rate at which the gas is removed from the plasma by combining to the hemoglobin molecule is faster relative to that at which it is removed by circulating blood. As such, when measuring carbon monoxide lung diffusing capacity, the effect of cardiac output can be neglected.

During postnatal development, gas transfer capacity increases as body mass increases. The increase in gas transfer is attributed to an increase in alveoli number and size allowing the respiratory system to increase overall lung diffusing capacity to meet the rising metabolic demands of larger and more active tissues (38). Metabolic demands also increase as a normal physiological process in women during reproduction (8), and gas transfer has been noted to vary cyclically in women during their reproductive years (33, 35). The mechanism of menses-related changes in gas transfer, which are typified by a maximal diffusing capacity for carbon monoxide (DL_{CO}) just before menses that rapidly drops and reaches a nadir on the third day of menstruation, is not clear (33). Here we hypothesized that the increase in gas transfer over the course of the menstrual cycle may be related to increase of the surface area of pulmonary capillaries available for gas exchange, Vc, through the process of new growth of vessels that subsequently regress over the time of menstruation.

Angiogenesis is routine during the reproductive cycle, with vessels forming and regressing in the endometrium and ovaries during the menstrual cycle under the control of hormones and angiogenic factors, such as vascular endothelial growth factor (VEGF) (1, 24, 29). VEGF levels vary over the menstrual cycle with two peaks, around ovulation and during the luteal phase (1, 24). Although tissue-resident endothelial cells participate in the new vessel formation, bone marrow-derived endothelial progenitor cells (EPC) also actively promote the formation of new blood vessels and the maintenance of vascular homeostasis (16, 17, 28). Mobilized EPC in the circulation are defined

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Table 1. Population of men and women

Variable	Women (n = 10)	Men (n = 4)	P Value
Age, yr	31±1	45±2	0.002
Weight, kg	65±1	87±3	<0.0001
O ₂ saturation, %	98.7±0.1	97.6±0.3	0.0006
Respiratory rate, breaths/min	11.7±0.2	12.9±0.4	0.008
DL _{CO} , ml·min ⁻¹ ·mmHg ⁻¹	24.0±0.4	30.4±0.8	<0.0001
VA, liter	5.5±0.1	6.6±0.1	<0.0001
Dm, ml·min ⁻¹ ·mmHg ⁻¹	62±6	58±4	0.6
Vc, ml	55±3	71±6	0.01
FVC, liters	3.97±0.06	4.58±0.08	<0.0001
FEV ₁ , liters	3.28±0.05	3.75±0.06	<0.0001
NO, ppb	14.7±1.1	17.0±1.9	0.3
Hgb, g/dl	13.5±0.2	15.7±0.3	<0.0001

Values are means ± SE. DL_{CO}, diffusing capacity for carbon monoxide; VA, alveolar volume; Dm, membrane diffusing capacity; Vc, pulmonary capillary bed; FVC, forced vital capacity; FEV₁, forced expiratory volume at 1 s; NO, nitric oxide; Hgb, hemoglobin.

by expression of CD34, a common cell surface marker for hematopoietic stem cells and endothelial cells, and the co-expression of the stem cell marker CD133 (4, 30). The strong association of increased circulating EPC numbers to neovascularization has led to the measure of circulating CD34⁺CD133⁺ EPC as a sensitive and specific marker of new vessel formation (4, 20, 30). The contribution of EPC to vascular pathological processes is increasingly recognized as a central component in pathogenesis; hence strategies aimed at modifying EPC are effective in regulating angiogenesis and therapies in acute myocardial infarction (6, 34) and cancer (12). The cyclic physiological neovascularization in the reproductive organs of women, i.e., corpus luteum and endometrium, has also recently been shown to be dependent on bone marrow-derived EPC (3). In this study, we investigated the changes in DL_{CO} over the menstrual cycle by weekly measurements of the subdivisions Vc and Dm together with factors that might alter θ , especially hemoglobin. To further investigate angiogenesis as a possible cause of the changes in Vc, proangiogenic factors and circulating proangiogenic EPC were determined. To evaluate the concept of hormonal effects on lung microvessel density, murine lung vascularity was quantitated in ovariectomized mice treated with estrogen at levels comparable to those present in women during the luteal phase or with placebo.

MATERIALS AND METHODS

Healthy, nonsmoking women with regular menstrual cycles were enrolled in the study. Volunteers were seen weekly for physiological tests, including pulse, oxygen saturation, and lung diffusion capacity, and for blood sampling. Healthy, nonsmoking men were enrolled as control subjects. This study was approved by the Institutional Review Board at the Cleveland Clinic, and all subjects gave written informed consent.

Lung function and diffusing capacity for carbon monoxide. The forced expiratory volume at 1 s (FEV₁) and forced vital capacity (FVC) were measured with an Eagle spirometer (InSpire Health, Louisville, CO) (27). The single-breath carbon monoxide diffusing capacity measurement was performed weekly with Eagle equipment (InSpire Health) with volunteers rested and in a seated position (23). On each visit, DL_{CO} was measured at two different oxygen concentrations (21% and 42%). DL_{CO} measurements were performed at the same time of the day on each visit to minimize the effect of diurnal variations. The single-breath DL_{CO} procedure was performed in du-

plicate, ~3 min apart, with a washout volume of 750 ml and an alveolar volume (VA) of 750 ml. The breath-hold time was ~10 s. DL_{CO} was adjusted for hemoglobin (Hgb) according to the American Thoracic Society (ATS) guidelines (23). Exhaled O₂ was obtained from the alveolar gas at each measurement of the diffusing capacity. Dm and Vc were calculated from DL_{CO} measurements at inspired O₂ concentrations of 21% and 42% as described by Roughton and Forster (31); θ was calculated from the formula described by Cotes (10, 32): $1/\theta = [0.34 + (0.0061 \times \text{PcO}_2)] \times (14.6/\text{Hgb})$. PcO₂ represents the capillary partial pressure of oxygen and was determined to be nearly equivalent to the alveolar partial pressure of oxygen (PAO₂) measured at end expiration (32): $\text{PcO}_2 = \text{PAO}_2 - 5$.

End-expiration PAO₂ was determined from the direct measure of end-expiration oxygen concentration and the daily barometric pressure. Since measurements of Dm and Vc are best made when Hgb is fully saturated with oxygen, DL_{CO} was measured over a range of inspired O₂ concentrations, 21%, 42%, 60% and 80%, to confirm that 1/DL_{CO} relative to inspired O₂ fraction (FI_{O2}) was linear ($R^2 = 0.97$). This validated the use of inspired oxygen concentrations of 21% and 42% to determine Dm and Vc in the study.

Before DL_{CO} determinations, single-breath online measurement of fractional nitric oxide (NO) concentration in expired breath (FENO) was also measured at each visit with the NIOX (Aerocrine, NY) (2).

Hemoglobin, carboxyhemoglobin, and 2,3-diphosphoglycerate. The NPT7 series (Radiometer, Copenhagen, Denmark) was used to measure Hgb and carboxyhemoglobin (COHgb) levels from whole blood samples collected in a heparin tube with spectroscopy. 2,3-Diphosphoglycerate (2,3-DPG) levels were measured in whole blood samples collected in heparin tubes with a spectrophotometric assay of NADPH oxidation (Roche Diagnostics, Mannheim, Germany). The concentration of 2,3-DPG was expressed in micromoles per gram of hemoglobin.

Estrogen, VEGF, and stem cell factor. Estrogen was measured in serum samples by radioimmunoassay (Specialty Lab). VEGF and stem cell factor (SCF) levels were measured in serum samples with Quantikine ELISA kits (R&D Systems).

Flow cytometry evaluation of EPC numbers and colony forming assay. Mononuclear cells (1×10^6) isolated from the peripheral blood were labeled with anti-human CD34-FITC (Becton Dickinson) and anti-human CD133-PE (Miltenyi Biotec, Auburn, CA) monoclonal

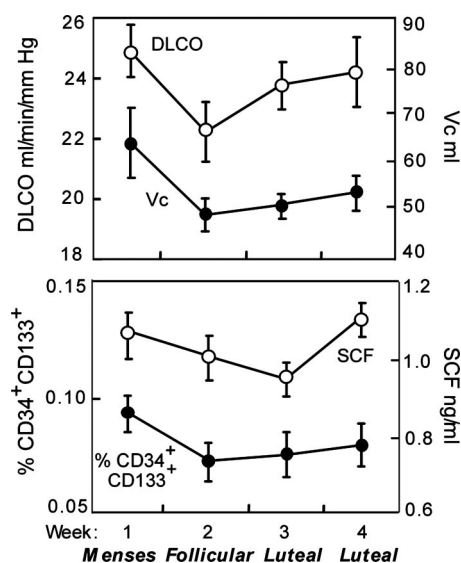


Fig. 1. Coordinate changes in gas transfer [diffusing capacity for carbon monoxide (DL_{CO})], pulmonary vascular capillary bed (Vc), endothelial progenitor cells (EPC) (CD34⁺CD133⁺), and stem cell factor (SCF) over the menstrual cycle. No changes occur in men over the same time period (not shown).

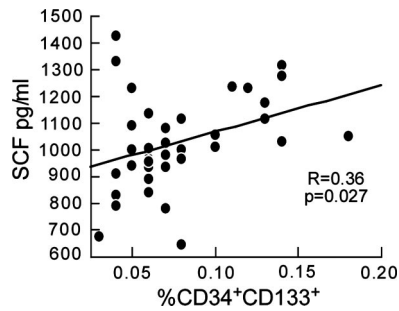


Fig. 2. Association of SCF with circulating CD34⁺CD133⁺ EPC. The points represent all measurements obtained for women over the study period.

antibodies to quantify the CD34⁺CD133⁺ progenitor cells. To control for nonspecific antibody binding, isotype-matched irrelevant antibodies were used. After incubation, cell suspensions were washed with PBS-1% BSA-0.02% sodium azide and suspended in FACSflow (Becton Dickinson). The FACScan flow cytometer (Becton Dickinson) was used to count 0.5×10^6 events. Data were analyzed with Cell-Quest 3.3 Software (Becton Dickinson). The endothelial nature of EPC was confirmed by colony formation as described by Hill et al. (15).

Animal studies. Female FVB/N wild-type mice (4 wk old) (Jackson Laboratory, Bar Harbor, ME) were anesthetized by intraperitoneal injection of 150 mg ketamine/kg body wt and ovariectomized, followed by subcutaneous implantation of 1.7 mg 17 β -estradiol pellets (Innovative Research of America) into the back. Control mice received placebo pellets. Circulating 17 β -estradiol levels were measured at days 7 and 14 with a commercially available enzyme-linked immunoassay (estradiol ELISA, Cayman Chemical). After 2 wk, the animals were euthanized. The lungs were insufflated by injecting 10 ml of saline via the trachea, and then 5 ml of 2% paraformaldehyde was injected via the trachea to rapidly fix the lungs. The pulmonary arteries and veins were banded, and the lungs were harvested and placed in 2% paraformaldehyde. Subsequently, the lungs were placed into 10% buffered formalin for 48 h. All animal procedures were performed under protocols reviewed and approved by the St. Elizabeth's Medical Center (Boston, MA) Animal Care and Use Committee.

To analyze the lung microvessel density, 5- μ m sections of paraffin-embedded tissues were stained with polyclonal rabbit

anti-human von Willebrand factor, which cross-reacts with the mouse antigen (Dako Cytomation, Glostrup, Denmark). Samples were pretreated with proteinase K for 4 min, and staining was visualized with diaminobenzidine and hydrogen peroxidase. All samples were stained with an automated biotin-avidin peroxidase system (Ventana-320-ES; Ventana Medical Systems, Tucson AZ). Secondary antibody alone was used as negative control. Sections were counterstained with hematoxylin. To quantify the microvessel density, pictures were taken from five random fields at a final magnification of $\times 400$ with a Leica DMR light microscope equipped with a Retiga EX digital camera and with Q imaging software. The number of blood vessels was counted on each picture, and the sum of five fields was calculated for each mouse. Quantification of the lung blood vessel density was performed in blinded fashion, and the results were confirmed in a second blinded analysis. Alveolus areas were calculated with Image-Pro Plus v. 5.1 software in each image. The software calibrated each image for the specific microscope and objective used. The alveolar radius was derived from the area with the formula: radius (r) = $\sqrt{(\text{area}/\pi)}$, and the volume of the alveolus was calculated with the formula: volume (μm^3) = $(4/3)\pi \times r^3$.

Statistical analysis. The study data consisted of multiple observations for each subject, and it was assumed that observations of a variable within a subject might be positively correlated. Therefore, a method accounting for this correlation was used to provide estimates of mean values for study variables, and to be the basis for measures of association within subjects and for comparisons of weeks or phases. Linear mixed models with random subject intercepts satisfied these requirements. Associations between subjects were studied by first obtaining averages of variables over each patient's set of observations, resulting in only a single value of each variable per patient. Spearman correlations were then used to estimate the correlations of the average patient values. Spearman correlations were also estimated based on the entire set of observations in the data, although the associations estimated with the data in this manner represent a combination of between- and within-subject relationships. The linear mixed model analyses and computation of associated estimates and P values were performed with SAS PROC MIXED version 9.1 (SAS Institute, Cary, NC). Spearman correlations and associated P values were calculated with R version 1.9 (www.r-project.org). All data are presented as means \pm SE.

For the mouse data, the Wilcoxon test was used to calculate the P values with JMP 5.0.1 (SAS Institute).

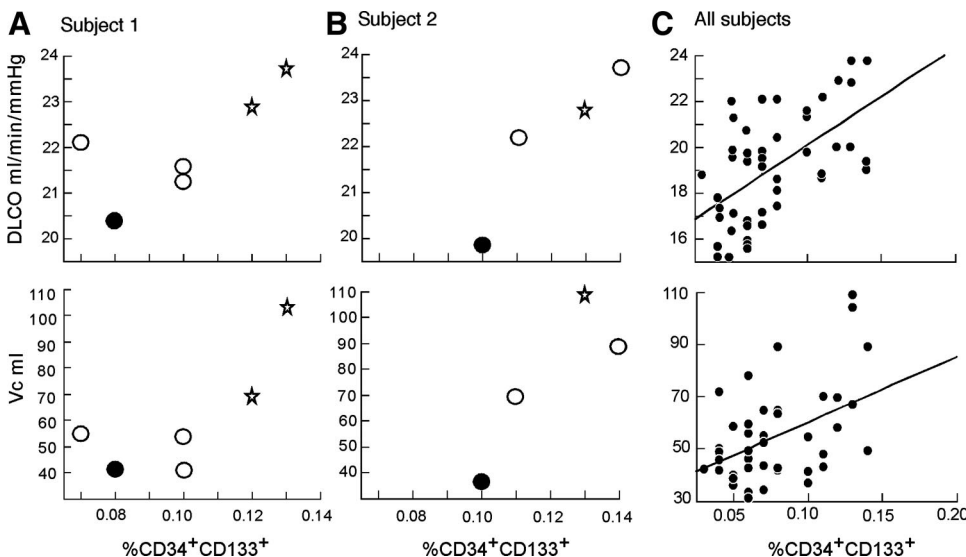


Fig. 3. Relation of DLCO measured at 42% O₂ and Vc to circulating CD34⁺CD133⁺ EPC. Values from 2 representative women over the study period are shown [studied over 6 (subject 1; A) and 4 (subject 2; B) wk]. Each point was measured at a different week of the menstrual cycle, with the 1st week starting at the 1st day of menses. ☆, Menses; ●, follicular phase; ○, luteal phase. C: relation of DLCO measured at 42% and Vc to CD34⁺CD133⁺ EPC for all women over the menstrual cycle.

RESULTS

Ten healthy, nonsmoking women (age 31 ± 1 yr) were enrolled in the study. They were on no medications except that 4 of the 10 were on hormonal contraceptives. Hormonal contraceptives were permitted because previous studies have shown that changes in DL_{CO} were detectable in menstruating women whether on hormonal contraceptives or not (33). Four healthy, nonsmoking men (age 45 ± 2 yr) with no medical history and on no medications were also enrolled as gender-discordant controls. All volunteers were longitudinally assessed over 4 wk with one visit each week. Four women were followed for 7 wk with duplicate measurements. The menstrual cycle was divided into weeks. *Week 1* was defined by the first day of menstruation. Menstrual cycles varied from 28 to 31 days in length.

Lung function and physiology in men and women. DL_{CO} , V_A , and Hgb were lower in women than in men (Table 1). However, O_2 saturation was higher in women compared with men ($P = 0.0006$). Table 1 summarizes the mean values for men and women.

Lung diffusing capacity over the menstrual cycle. DL_{CO} in women decreased by 10% between *week 1* and *week 2* of the menstrual cycle ($P = 0.013$). The values subsequently increased over *weeks 3–4* (Fig. 1). Men had no significant variation in DL_{CO} among consecutive weeks ($P = 0.72$). For control of reproducibility of measures, the within-day variability was determined in subjects. As per ATS recommendations, DL_{CO} measurements were made during the same time of day to eliminate diurnal variation. The same-day variability per subject was $<5\%$ in all individuals. Repetitive measurements were performed over 6 different days in one volunteer. The maximal within-day variability over 6 days was 5.4%. Duplicate measures performed in four women over 7 wk also provided a measure of variability within week of the menstrual cycle. The maximal mean variability of the duplicate measurements of the four women followed over 7 wk was 4.3% [% variability of DL_{CO} : *week 1*, 0.5 ± 0.4 ; *week 2*, 4.3 ± 1.5 ; *week 3*, 0.5 ± 0.3 ; *week 4*, 2.3 ± 2.2].

Hgb concentration varied slightly over the menstrual period, increasing from *week 1* to *week 4* by 2.5% ($P = 0.032$) [Hgb

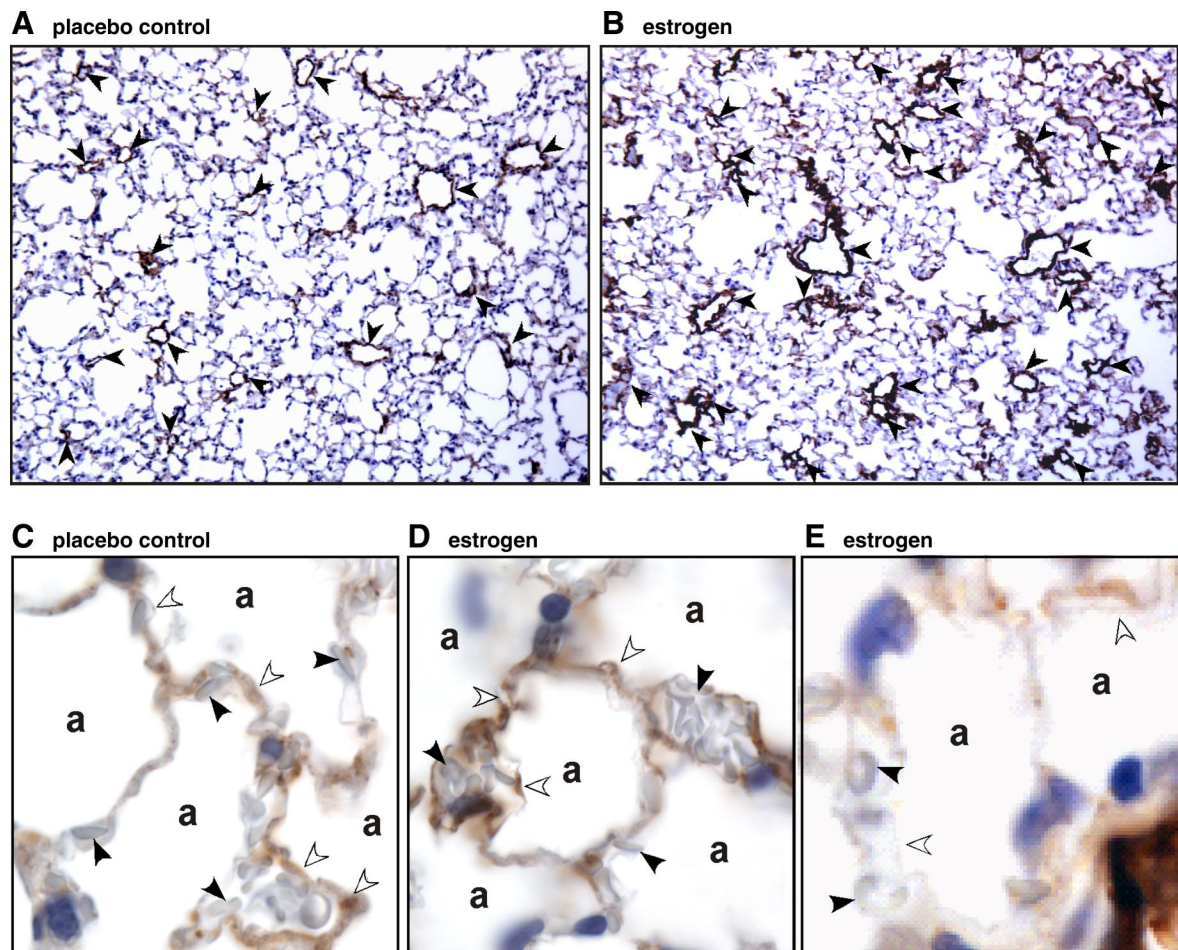


Fig. 4. Lung tissues from ovariectomized female mice exposed to estrogen (B, D, E) or placebo (A, C) for 2 wk. A and B: lungs of estrogen-exposed mice have greater numbers of vessels as identified by endothelial cells that are positive (brown staining) for von Willebrand factor but smaller alveoli than control ovariectomized female mice. Arrowheads identify microvessels. Magnification $\times 400$. C and D: alveolar capillary unit from the lungs of ovariectomized mice exposed to estrogen compared with placebo. Immunopositivity for von Willebrand factor identifies endothelial cells. Magnification $\times 1,000$. E: capillaries containing erythrocytes are identified as they traverse through alveolar walls. Magnification $\times 1,500$. a, Alveolar spaces; open arrowheads, capillary lining; black arrowheads, red blood cells.

(g/dl): follicular phase 13.3 ± 0.2 , luteal phase 13.5 ± 0.2 ($P = 0.03$); *week 1* 13.2 ± 0.3 , *week 2* 13.4 ± 0.3 , *week 3* 13.6 ± 0.3 , *week 4*, 13.6 ± 0.3 ($P = 0.18$). Change in DL_{CO} was not associated with the change in Hgb, e.g., there was no change in Hgb from *week 1* to *week 2* ($P = 0.8$), the time period when DL_{CO} was decreasing. Similarly, COHgb, which could affect DL_{CO} measurement, did not change significantly between *week 1* and *week 2* ($P = 0.37$). In addition, V_A was unchanged over the menstrual cycle in women. The affinity of carbon monoxide toward Hgb is high, and its plasma solubility is low; hence cardiac output effect on DL_{CO} is trivial and not taken into consideration as a significant determinant (10). Nevertheless, pulse rate was measured as an indicator of cardiac effects and, as in previous studies (9, 22), showed no significant change over the menstrual cycle ($P = 0.88$).

In parallel to the change in DL_{CO} , V_c decreased by 25% between *week 1* and *week 2* ($P = 0.046$) (Fig. 1). In contrast, D_m did not change over the menstrual cycle ($P = 0.87$). Similarly, θ did not change overall ($P = 0.79$). There was no change in 2,3-DPG (mean $16 \pm 1 \mu\text{mol/g Hgb}$; $P = 0.25$) or exhaled NO over the menstrual cycle ($P = 0.62$). To evaluate a potential effect of contraceptives, the subgroup of women on oral contraceptives were compared with those not on contraceptives. Estrogen varied as expected over the menstrual cycle, with the lowest value at *week 1* (200 pg/ml), and was lower overall in the follicular phase compared with the luteal phase [estrogen (pg/ml): follicular phase 266 ± 32 , luteal phase 361 ± 33 ; $P = 0.037$] Estrogen was lower in women on birth control during *week 4* [estrogen (pg/ml): 185 ± 170 vs. 455 ± 129 ; $P = 0.006$]. Nevertheless, DL_{CO} of women on birth control behaved similarly to that of those not on birth control (all comparisons $P > 0.5$), while V_c in *week 2* of those women not on birth control tended to be lower than that of those on birth control (44 ± 4 vs. 59 ± 5 ; $P = 0.04$).

Circulating EPC over the menstrual cycle. The $CD34^+CD133^+$ EPC in the circulation varied over the menstrual cycle, also decreasing by $\sim 24\%$ over the time of menses from *week 1* to *week 2* ($P = 0.021$) in parallel with the drop in V_c and DL_{CO} (Fig. 1). Unlike previous studies (1, 24), the changes in VEGF in this study did not achieve significance at the times studied over the menstrual cycle; however, SCF showed variability ($P = 0.041$), with the lowest values in *weeks 2* and *3* (Fig. 1). The fact that VEGF did not reach statistical significance for variation during the menstrual cycle in this study may be secondary to the timing and definition of the weeks of the cycle. In previous studies (1, 24), the phases were defined by the luteinizing hormone surge at midcycle and by serial vaginal ultrasounds to evaluate changes in vascularity and track dominant follicle growth. Consistent with a coordinate role in regulating endothelial precursors, SCF correlated with number of $CD34^+CD133^+$ cells, with greater numbers of $CD34^+CD133^+$ cells associated with higher levels of SCF within volunteers ($P = 0.038$) and across all volunteers ($R = 0.36$, $P = 0.026$) (Fig. 2).

Correlation of lung gas transfer and EPC. DL_{CO} at 21% O_2 or at 42% O_2 was related to number of $CD34^+CD133^+$ cells in peripheral blood when all measurements were taken into account ($R = 0.47$, $P = 0.001$ and $R = 0.56$, $P < 0.001$, respectively) (Fig. 3). This correlation held within volunteers for DL_{CO} measured at 42% O_2 ($P = 0.023$; Fig. 3) but did not reach statistical significance for DL_{CO} at 21% O_2 ($P = 0.24$).

The membrane diffusing capacity D_m did not correlate with number of $CD34^+CD133^+$ cells ($P = 0.91$); however, V_c was directly related to $CD34^+CD133^+$ cell numbers for all measurements ($R = 0.36$, $P = 0.01$) and within volunteers ($P = 0.013$) (Fig. 3).

Vasculogenesis in the lungs of mice with inductive ovulation. To directly investigate changes in lung vasculature under the influence of estrogen *in vivo*, ovariectomized mice were implanted with either estrogen or placebo pellets. The estradiol pellets ensured continuous release of estrogen at a dose comparable to the upper physiological range found in nonpregnant premenopausal women during the midcycle to the luteal phase of the normal menstrual cycle, but levels that are approximately threefold higher than those in the nonpregnant mouse (19, 41). The number of microvessels per lung field was greater in the estrogen-treated mice than in control ovariectomized mice after 2 wk of estrogen [number of microvessels/lung field (0.18 mm^2): estrogen-treated 18 ± 1 , placebo group 15 ± 1 ; $P = 0.037$]. Interestingly, alveolar size and volume were smaller in the estrogen-treated mice compared with the placebo control [average area of alveolus (μm^2): estrogen-treated group 394 ± 32 , placebo group 554 ± 45 ($P = 0.015$); average volume of alveolus (μm^3): estrogen-treated group $6,000 \pm 748$, placebo group $9,990 \pm 1,244$ ($P = 0.015$)] (Figs. 4 and 5). Thus mice receiving estrogen had greater numbers of vessels and smaller alveoli, suggesting a potential better matching of ventilation to perfusion (26). Furthermore, the smaller size of alveoli with associated rich networks of capillaries (Fig. 4B) predicts that surface area available for gas transfer is greater in estrogen-exposed mice. In fact, the ratio of number of microvessels to number of

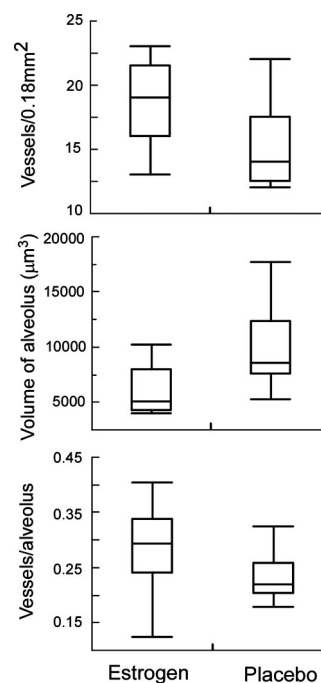


Fig. 5. Lung histology quantification of vessels and alveoli in mice by treatment group. Estrogen-exposed mice have greater numbers of microvessels and smaller alveoli than mice exposed to placebo. There is a trend toward more vessels per alveolus in estrogen-treated mice. Box plots represent 25th and 75th quartiles and the middle line of the box the median sample value. Lines extend to the outermost data points from the quartiles.

alveoli per high-power field trended to be greater in the estrogen-exposed mice compared with the control mice (no. of vessels/alveolus: estrogen-treated group 0.28 ± 0.08 , placebo group 0.23 ± 0.04 ; $P = 0.047$) (Fig. 5).

DISCUSSION

This study details the unique characteristics of gas transfer in women and provides evidence that gas transfer varies over the menstrual cycle because of cyclic changes in the pulmonary vascular capillary bed that are linked to circulating proangiogenic EPC.

Here the changes in gas transfer over the menstrual cycle are similar to previous findings (33). Analysis of the components of gas transfer reveals that the change in gas transfer is due to change in the pulmonary capillary vascular bed, and not to alterations in membrane diffusion or hemoglobin affinity. Seaton (35) also identified an increase in gas transfer in the luteal phase, which was attributed to changes in the pulmonary vascular bed, but not blood volume. An increase in blood and/or plasma volume is also unlikely to have caused the changes observed in the present study, given minimal change in hemoglobin. Furthermore, estrogen causes plasma volume expansion during the preovulatory phase of the menstrual cycle (37). Thus the pulmonary vascular bed would have been expected to increase from *week 1* to *week 2*, rather than decrease. Another possible explanation for the change in the vascular bed could be pulmonary vasodilation under the influence of NO. However, unlike previous findings (21), exhaled NO did not vary over the menstrual cycle in this study. Even so, the changes in exhaled NO observed in a previous study predict NO-related vasodilation at *week 2* compared with *week 1*, which is in opposition to our findings.

The observed change in the physiological pulmonary vascular capillary bed in women over the menstrual cycle may be related to new vessel formation in the lungs, based on two major findings. First, a greater number of pulmonary microvessels were present in the lungs of mice after 2 wk of estrogen at levels comparable to those present during the luteal phase of menstruating women. This provides support for the hypothesis that estrogen can modify vessel density in the lung and that vascular changes can occur over a period of time that is relevant to the female human menstrual cycle. In fact, a recent study showed that EPC migration and proliferation are regulated by estrogen through estrogen receptors and phosphatidylinositol 3-kinase pathways (41). Unexpectedly, a smaller size of alveoli was also found in the estrogen-treated mice. This situation predicts a more efficient matching of ventilation to blood perfusion (26, 39), with a greater total surface area available for gas transfer per unit alveolar volume in the mice exposed to estrogen. Second, changes in circulating EPC numbers and angiogenic factor SCF, and their strong association to gas transfer and the physiological pulmonary capillary vascular bed capacity, provide support for angiogenesis in the lung over the menstrual cycle. Current concepts suggest that neovascularization in the adult involves bone marrow-derived stem cells and local pulmonary stem cells. Whether or not bone marrow-derived endothelial progenitors directly incorporate into vessels or whether they provide factors to support expansion of tissue-resident endothelial cells for angiogenesis is still not

resolved. Furthermore, while endothelial cells and neovascularization may be derived from resident endothelial stem cells, a recent study suggests that epithelial-to-mesenchymal cell transformation may also give rise to endothelial cells for new vessel formation (36). Regardless of the source of the stem cells giving rise to the new vessels, circulating EPC are clearly related to new blood vessel formation in tissues (3, 4, 6, 12, 20, 30, 34). Hence, the direct correlation of circulating EPC to the physiological pulmonary vascular capillary bed capacity is also supportive of cyclic neovascularization in the female lung, perhaps through release of proangiogenic factors by EPC (18, 40).

Abundant evidence supports that new pulmonary vessels can form and regress rapidly, and well within the time frame of the menstrual cycle changes observed in this study, under the direction of proangiogenic factors such as VEGF and SCF and that EPC are associated with the process (11, 25). For example, a model for the formation and regression of vessels in the airways over 28 days has been studied by Baluk et al. (7) in lungs of mice. New blood vessels in mouse trachea form by sprouting angiogenesis under the influence of VEGF expression in the airway. Endothelial sprouts are seen as soon as 1 day after VEGF expression, and maximum vessel density is reached within 7 days and maintained as long as VEGF is expressed. On VEGF withdrawal, vessels regress within 3 days. In a recent study, expression of new capillary vessels in the lung microvasculature occurred within 12–24 h of mobilization of EPC from the bone marrow into the blood, which predicts that new capillary vessels in the lung, and hence V_c and DL_{CO} , should be temporally closely associated to EPC numbers in the blood (5). In multiple model systems, microscopic imaging techniques reveal that basement membrane sleeves and pericytes are left behind after endothelial cell degeneration and provide the scaffold for microvascular re-growth that occurs as rapidly as 1 wk after VEGF is again made available (11, 25). Empty basement membrane sleeves remain for as long as 21 days after vessel regression (25), and so would be available to facilitate vascular restoration over the time period of the menstrual cycle within which changes in estrogen and proangiogenic factors occur. The plasticity of tumor vasculature by these processes is evident (13, 14), and a similar process may subservise a physiological role in the lung for the preparation of women for the greater metabolic needs of childbearing. It is important to note that although a significant association among DL_{CO} , V_c , estrogen, and $CD34^+CD133^+$ progenitor cells was found in this study, this does not necessarily imply causality. In particular, other possible mechanisms that may account for the changes in gas transfer, such as recruitment, vasodilation, and/or vasoconstriction of existing pulmonary vessels, were not excluded. Further study of the physiological processes that allow cyclic peaks of gas transfer in women is warranted, given the potential for application to regenerative strategies to augment oxygen uptake in patients with advanced lung diseases.

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