Preeclampsia

Decreased Level of Cord Blood Circulating Endothelial Colony–Forming Cells in Preeclampsia

Rocio Muñoz-Hernandez, Maria L. Miranda, Pablo Stiefel, Ruei-Zeng Lin, Juan M. Praena-Fernández, Maria J. Dominguez-Simeon, Jose Villar, Rafael Moreno-Luna, Juan M. Melero-Martin

See Editorial Commentary, pp 23–25

Abstract—Preeclampsia is a pregnancy-related disorder associated with increased cardiovascular risk for the offspring. Endothelial colony–forming cells (ECFCs) are a subset of circulating endothelial progenitor cells that participate in the formation of vasculature during development. However, the effect of preeclampsia on fetal levels of ECFCs is largely unknown. In this study, we sought to determine whether cord blood ECFC abundance and function are altered in preeclampsia. We conducted a prospective cohort study that included women with normal (n=35) and preeclamptic (n=15) pregnancies. We measured ECFC levels in the umbilical cord blood of neonates and characterized ECFC phenotype, cloning-forming ability, proliferation, and migration toward vascular endothelial growth factor-A and fibroblast growth factor-2, in vitro formation of capillary-like structures, and in vivo vasculogenic ability in immunodeficient mice. We found that the level of cord blood ECFCs was statistically lower in preeclampsia than in control pregnancies (P=0.04), a reduction that was independent of other obstetric factors. In addition, cord blood ECFCs from preeclamptic pregnancies required more time to emerge in culture than control ECFCs. However, once derived in culture, ECFC function was deemed normal and highly similar between preeclampsia and control, including the ability to form vascular networks in vivo. This study demonstrates that preeclampsia affects ECFC abundance in neonates. A reduced level of ECFCs during preeclamptic pregnancies may contribute to an increased risk of developing future cardiovascular events. (Hypertension. 2014;64:165-171.) • Online Data Supplement

Key Words: fetal blood • preeclampsia • pregnancy

Preeclampsia is a multisystem syndrome affecting 2% to 8% of pregnancies, and it is a major cause of maternal and fetal morbidity and mortality.1,2 Offspring of preeclamptic pregnancies have an increased risk of developing postnatal cardiovascular events, including hypertension and stroke.3,4 Epidemiological studies have shown that several cardiovascular diseases have origins during development.5 However, the effects of preeclampsia on the fetal cardiovascular system remain poorly understood.

Endothelial colony–forming cells (ECFCs) are circulating progenitor cells that give rise to highly vasculogenic endothelial cells.6,7 ECFC levels in fetal blood are elevated during the third trimester of pregnancy.6–10 and these cells are postulated to contribute to the rapid formation of fetal vasculature and to the maintenance of vascular integrity.11,12 Recent studies have shown that cord blood ECFC level and function are impaired in several pregnancy-related disorders associated with long-term cardiovascular risks, including gestational diabetes mellitus, fetal bronchopulmonary dysplasia, and intrauterine growth restriction.13–16 However, it remains unclear whether cord blood levels of ECFCs are also altered during preeclampsia.

Here, we conducted a prospective cohort study to determine the umbilical cord blood levels of ECFCs in preeclampsia and analyzed the results in light of potential confounding obstetric factors. We also compared the functional properties of ECFCs derived from preeclamptic and normal pregnancies.

Methods

Study Subjects

Fifteen (preeclampsia) and 35 (control) white mother–offspring pairs were included in this study. Preeclampsia was defined as high blood...
pressure (>140/90 mm Hg) and excess protein in the urine (>0.3 g in 24 hours) after 20 weeks of pregnancy. Pre-existing chronic hypertension was not an exclusion criterion for preeclampsia. All preeclamptic mothers were treated with α-methyldopa; in addition, 5 patients also received labetalol. Intrauterine growth restriction was defined as a fetus with an individualized weight percentile <10% and with asymmetry in several ultrasound measurements, including a significant decrease in abdominal perimeter compared with long bone length and biparietal diameter. Exclusion criteria included multiple gestation, maternal infections, respiratory disease, and women who carried fetuses with chromosomal abnormalities or congenital malformations. In the control group, women with hypertensive disorders were excluded. The local ethics committee at the Hospital Universitario Virgen del Rocío approved this research, and all the parents gave written informed consent for extraction of data from their obstetric records and for the use of umbilical cord blood in accordance with the Declaration of Helsinki. Methods on obstetric factors are described in Materials and Methods in the online-only Data Supplement.

Enumeration and Characterization of ECFCs

Umbilical cord blood samples (20–50 mL) were collected ex utero using heparinized tubes and processed within 2 hours. Enumeration and characterization of ECFCs were performed following previously described methods17–19; details can be found in expanded Materials and Methods in the online-only Data Supplement.

Statistical Analysis

Data from preeclampsia and control subjects were compared and analyzed with IBM SPSS v. 19.0 software (IBM Corp, Armonk, NY). Categorical variables were expressed by absolute frequencies and percentages (n, %). Noncategorical variables were expressed by mean±SD or median and 25th to 75th interquartile range. Categorical variables were analyzed with Fisher exact tests except for tobacco use and offspring sex, which were analyzed with Pearson χ² tests. Noncategorical variables were analyzed with 2-tailed unpaired Student t tests, with the exception of maternal age and gestational age, which were not normally distributed and therefore analyzed with Mann–Whitney U tests. Shapiro–Wilk tests were used to determine normality. Univariate correlations were performed with the use of Spearman correlation coefficient. Data from experiments performed in vitro and in mice were analyzed using GraphPad Prism v. 5 software (GraphPad Software, La Jolla, CA). These data were expressed as mean±SE and mean values were compared using unpaired Student t tests. For all analyses, P<0.05 was considered significant.

Results

Patient Demographics

We studied 15 (preeclampsia) and 35 (control) mother–offspring pairs (Table). Based on the severity of the pathology, the preeclampsia group included subjects with mild (n=6), severe (n=7), and hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome (n=2). In addition, 2 subjects in the preeclampsia group had pre-existing chronic hypertension. The prevalence of cesarean deliveries in the preeclampsia group was statistically higher than in control (P=0.001). Offspring born from mothers with preeclampsia had lower gestational age, birth weight, and birth weight percentile than those in the control group (P=0.001, P=0.004, and P=0.006, respectively). Preeclamptic mothers had higher pregestational diastolic blood pressure (P=0.003) than control. There were no statistical differences in the remainder of the obstetric characteristics analyzed (P>0.05).

| Table. Obstetric Characteristics of Preeclampsia and Control Groups |
|----------------------------------|------------------|------------------|
|                                  | Control (n=35)   | Preeclampsia (n=15) | P Value |
| Maternal                         |                  |                  |
| Age, y                           | 30.9±5.7         | 30.4±6.3         | 0.68    |
| Primipara, n (%)                 | 23 (65.7)        | 13 (86.7)        | 0.18    |
| In vitro fertilization, n (%)    | 3 (8.6)          | 2 (13.3)         | 0.63    |
| Cesarean delivery, n (%)         | 4 (11.4)         | 9 (60.0)         | 0.001   |
| Tobacco use, n (%)               | 16 (45.7)        | 5 (33.3)         | 0.42    |
| Gestational diabetes mellitus, n (%) | 0 (0.0)          | 1 (6.7)          | 0.30    |
| Prepregestational BMI, kg/m²     | 26.1±5.1         | 26.4±5.6         | 0.85*   |
| <25, n (%)                       | 15 (42.8)        | 5 (33.3)         | …       |
| 25–30, n (%)                     | 12 (34.3)        | 6 (40.0)         | …       |
| >30, n (%)                       | 8 (22.9)         | 4 (26.7)         | …       |
| Gestational weight gain, kg      | 12.2±6.6         | 12.5±5.3         | 0.88*   |
| Pregestational blood pressure, t mm Hg |                  |                  |
| Diastolic                         | 63.1±7.7         | 73.1±11.1        | 0.003   |
| Systolic                          | 106.7±11.2       | 112.4±16.6       | 0.20*   |
| Blood pressure at onset of PE, mm Hg |                  |                  |
| Diastolic                         | …                | 97.1±4.6         | …       |
| Systolic                          | …                | 158.5±9.8        | …       |
| Neonatal                         |                  |                  |
| Male, n (%)                      | 23 (65.7)        | 10 (66.7)        | 0.95    |
| Gestation age, wk                | 39.1±2.1         | 36.6±2.6         | 0.001   |
| Intrauterine growth restriction, n (%) | 0 (0.0)          | 2 (13.3)         | 0.08    |
| Preterm birth, n (%)             | 5 (14.3)         | 5 (33.3)         | 0.14    |
| Birth weight, kg                 | 3.3±0.6          | 2.6±0.8          | 0.004*  |
| Birth weight percentile, %       | 53.4±34.8        | 25.9±34.1        | 0.006   |
| Cord blood MNC level, ‡ millions per 10 mL blood | 36.7±23.3        | 28.1±16.8        | 0.28    |

Categorical variables are represented by absolute frequencies and percentages (n, %). Noncategorical variables are represented by mean±SD. Categorical variables were analyzed with Fisher exact tests except for tobacco use and offspring sex that were analyzed with Pearson χ² tests. P values are from comparison of control and preeclampsia groups. BMI indicates body mass index; MNC, mononuclear cell; and PE, preeclampsia.

*Noncategorical variables that were not normally distributed were analyzed with Student t tests. Noncategorical variables that were not normally distributed were analyzed with Mann–Whitney U tests.

‡Values for prematurity blood pressure are from n=27 control subjects.

†Values for MNC level are from n=29 control subjects.

Cord Blood Levels of ECFCs in Preeclampsia

We quantified the number of ECFCs in the umbilical cord blood of neonates at the time of delivery. ECFCs were identified in culture as outgrown colonies containing ≥50 endothelial cells. The endothelial nature of the colonies was corroborated by the cobblestone-like morphology of the cells (Figure 1A) and by binding of fluorescently labeled Ulex europaeus agglutinin type 1 lectin (Figure 1B). Colonies in the control group emerged in culture as early as 1 week (7% of the colonies), and most of the colonies emerged between 2 weeks (60%) and 3 weeks (31%; Figure 1C), which is consistent with previous reports.18 In contrast, the time needed
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P = 0.27 and control, respectively; Table S2), mode of delivery (P = 0.77 in preeclampsia and control groups demonstrated that the level of ECFCs was independent of both the severity of the pathology (mild/severe/hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome; P = 0.06), the time of onset of preeclampsia (early/late; P = 0.42), diastolic and systolic pregestational blood pressure (P = 0.51 and P = 0.94; Table S2), and diastolic and systolic blood pressure at the time of onset of preeclampsia (P = 0.52 and P = 0.27; Table S2).

**Variation of Cord Blood ECFC Levels With Maternal Body Mass Index and Gestational Age**

Previously, we demonstrated that maternal body mass index (BMI) is a potential confounding factor for cord blood levels of ECFCs. To address whether the difference in ECFC abundance between preeclampsia and control was confounded by maternal weight, we categorized the study into prepregnancy maternal BMI <25 kg/m² (normal weight; n = 15/5 control/preeclampsia), 25 to 30 kg/m² (overweight; n = 12/6), and >30 kg/m² (obese; n = 8/4; Figure 2A). ECFC levels in control subjects increased from normal prepregnancy maternal weight (mean of 4 colonies) to overweight (11 colonies) and obese (7 colonies) subjects, with statistically significant differences between these subgroups (Figure 2A). In contrast, the level of ECFCs in preeclampsia was consistently low, irrespective of the value of maternal BMI, with mean ECFC abundances of 2, 3, and 3 colonies in cord blood samples from normal weight, overweight, and obese mothers, respectively (Figure 2A). In addition, the difference in ECFC levels between control and preeclampsia for maternal BMI 25 to 30 kg/m² was statistically significant (P < 0.05). Taken together, these results confirmed that maternal BMI is a confounding factor for ECFC level and demonstrated that the reduction in ECFC abundance observed in preeclampsia was more prominent among subjects in the overweight (BMI = 25–30 kg/m²) group.

To address whether the difference in ECFC abundance between preeclampsia and control was influenced by gestational age, we categorized the study into premature (<37 gestational weeks; n = 5/5 control/preeclampsia) or term (≥37 weeks; n = 30/10) deliveries (Figure 2B). Our study did not include extremely premature infants, and the lowest gestational age for both groups was 31 weeks. We observed that ECFC abundance in the control group was increased in prematurity (Figure 2B), which is consistent with previous reports. However, the level of ECFCs in preeclampsia did not change with gestational age (P > 0.05), and it remained

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**Figure 1.** Cord blood levels of endothelial colony–forming cells (ECFCs) in preeclampsia. A, Phase contrast micrograph of a representative ECFC colony from a preeclamptic (PE) pregnancy. Arrowheads delimitate the border of the colony (scale bar, 200 μm). B, Binding of fluorescently labeled *Ulex europeaus* agglutinin type 1 lectin (UEA-1) to a colony of ECFCs (scale bar, 200 μm). C, Weekly appearance of ECFC colonies in culture. Bars represent means±SE levels of ECFCs in 10 mL of cord blood. Percentages represent the proportion of total ECFCs appeared each week. D, Total number of ECFC colonies in 10 mL of cord blood from normal (n = 35) and PE (n = 15) pregnancies. Lines represent mean ECFC abundance. n values are denoted on top of each group. *P < 0.05. CTR indicates control.

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**Figure 2.** Variation of cord blood endothelial colony–forming cell (ECFC) levels with maternal body mass index (BMI) and gestational age. A, ECFC abundance in cord blood from subjects categorized by prepregnancy maternal BMI. B, Cord blood level of ECFCs from deliveries categorized by gestational age as preterm (<37 weeks) and term (≥37 weeks). Lines represent mean ECFC abundance in 10 mL of cord blood. n values are denoted on top of each group. *P < 0.05 between control (CTR) and preeclamptic (PE) groups for maternal BMI 25 to 30 kg/m² and gestational age <37 weeks.
significantly lower than the control for both preterm (4±1 colonies in preeclampsia and 14±2 colonies in control; \( P=0.02 \)) and term deliveries (2±1 colonies in preeclampsia and 6±2 colonies in control; \( P=0.06 \); Figure 2B). In addition, the difference in ECFC levels between control and preeclampsia for gestational age <37 weeks was statistically significant (\( P<0.05 \)). These results confirmed that gestational age is a confounding factor for ECFC level (increased in prematurity) and demonstrated that the overall reduction in ECFC abundance observed in preeclampsia was more prominent among premature deliveries.

Phenotypic and Functional Characteristics of Cord Blood ECFCs in Preeclampsia

We then examined whether there were functional differences among ECFCs from the control and preeclampsia groups. To this aim, ECFCs were first expanded in culture and purified by virtue of CD31 expression (Figure 3A). The endothelial phenotype of CD31-selected cells was verified via expression of CD31 and vascular endothelial-cadherin at the cell–cell borders, and the expression of von Willebrand factor in a punctuate pattern in the cytoplasm (Figure 3B). Quantitative reverse transcription polymerase chain reaction analyses demonstrated similar levels of expression of endothelial cell markers (CD31, von Willebrand factor, vascular endothelial-cadherin, and endothelial nitric oxide synthase) and absence of mesenchymal cell markers (CD90 and platelet-derived growth factor receptor-\( \beta \)) in ECFCs from both preeclampsia and control (\( P>0.05 \); Figure 3C). We also observed that ECFCs from both groups expressed high levels of growth factor receptors vascular endothelial growth factor (VEGF) receptor-1, VEGF receptor-2, and fibroblast growth factor (FGF) receptor-1 and low levels of VEGF receptor-3, FGF receptor-2, and FGF receptor-3 (Figure 3D), which is consistent with a vascular endothelial phenotype.\(^\text{20}\)

To assess ECFC function, we randomly selected 6 ECFC cultures from each group and performed several in vitro functional assays (Figure 4). In the preeclampsia group, one of the ECFC cultures selected corresponded to a subject with intrauterine growth restriction. We observed no statistical difference between preeclampsia and control in ECFC cloning-forming ability (Figure 4A; \( P=0.48 \) and Figure 4B; \( P=0.60 \)) and in the capacity of ECFCs to assemble into capillary-like structures on Matrigel (Figure 4C and 4D; \( P=0.95 \) and Figure 4E; \( P=0.81 \)). We observed a moderate decrease in the mitogenic and migratory response to VEGF-A (Figure 4F; \( P=0.52 \) and Figure 4H; \( P=0.20 \)) and FGF-2 (Figure 4F; \( P=0.15 \) and Figure 4H; \( P=0.29 \)) in cord blood ECFCs from preeclampsia, although these differences were not statistically significant for \( n=6 \). We also examined the in vivo vasculogenic ability of ECFCs after transplantation into immunodeficient mice (Figure 5). In both preeclampsia and control groups, transplanted ECFCs formed extensive networks of perfused microvessels by day 7, as revealed by hematoxylin and eosin-stained sections of the explants (Figure 5A) and confirmed by immunohistochemical staining of human-specific CD31 (Figure 5B). Microvessels also stained positively for \textit{Ulex europaeus} agglutinin type 1 lectin, a lectin that specifically binds to human (but not murine) endothelial cells (Figure 5C). In addition, ECFC-lined microvessels had extensive perivascular coverage at day 7, as revealed by positive \( \alpha \)-smooth muscle actin expression (Figure 5C), which indicated vascular stability. Importantly, quantitative histological evaluation of human-specific microvessel density demonstrated no statistical difference between ECFCs from preeclampsia and control (Figure 5D; \( P=0.71 \)).

Discussion

The mechanisms that govern the abundance of ECFCs in health and disease are insufficiently known. The maternal vascular pathophysiologic features of preeclampsia are well characterized and involve widespread endothelial dysfunction.\(^\text{21}\)

However, the effects of preeclampsia on fetal levels of circulating progenitor cells have not been examined systematically. A previous study by Hwang et al\(^\text{22}\) demonstrated a decrease of cord blood AC133+/KDR+/CD34+ endothelial progenitor cells and their progeny in pregnancies complicated by preeclampsia. However, there is increasing consensus on the distinction between cells that originate from AC133+/KDR+/CD34+ endothelial progenitor cells and those that are defined as ECFCs.\(^\text{23–25}\)

Indeed, Yoder et al\(^\text{23}\) demonstrated that early endothelial

![Figure 3. Phenotype of cord blood endothelial colony-forming cells (ECFCs) in preeclampsia.](http://hyper.ahajournals.org/)

A. Phase contrast micrograph of CD31-selected culture-expanded ECFCs from preeclampsia (PE) cord blood (scale bar, 200 \( \mu m \)). B. ECFC expression of CD31, vascular endothelial (VE)-cadherin, and von Willebrand factor (vWF) demonstrated by indirect immunofluorescence. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; scale bar, 50 \( \mu m \)). C. Quantitative reverse transcription polymerase chain reaction analyses of ECFCs for endothelial (CD31, vWF, VE-cadherin, and endothelial nitric oxide synthase [eNOS]) and mesenchymal (platelet-derived growth factor receptor [PDGFR]-\( \beta \), CD90) cell markers and for FGF-2 (Figure 3D), which is consistent with a vascular endothelial phenotype.\(^\text{20}\)

![Figure 4.](http://hyper.ahajournals.org/)
progenitor cells that generate endothelial cell colony-forming units are hematopoietic in origin, fail to form perfused vessels in vivo, and are clonally distinct from ECFCs. Thus, in addition to variations in the number of AC133+/KDR+/CD34+ endothelial progenitor cells, it remains unclear whether preeclampsia alters baseline levels of cord blood ECFCs. Here, we unambiguously identified ECFCs based on well-known endothelial cell markers and functional properties and demonstrated that the level of cord blood circulating ECFCs is decreased in preeclampsia. This reduction was statistically significant, independent of common obstetric factors, and was not associated with changes in cell phenotype or function.

Recent studies have emphasized the importance of several confounding factors on circulating levels of ECFCs, including maternal BMI and gestational age.8,18 Previously, we demonstrated a positive correlation between maternal BMI and ECFC abundance in umbilical cord blood of neonates born from nonobese healthy mothers with nonpathological pregnancies.14 This association suggested a potential physiological adaptation that occurs in the rapidly growing fetus in response to intrauterine conditions imposed by maternal weight. In this study, we examined the influence of maternal prepregnancy weight and found that ECFCs levels were consistently lower in preeclampsia than in control pregnancies, irrespective of maternal BMI. Gestational age has also been recognized as a source of variation for ECFC levels. Previous studies have shown that levels of circulating ECFCs are more elevated in premature deliveries (gestational age, 28–35 weeks) than at term,8 although extremely premature infants (<28 weeks) have been associated with fewer ECFCs.14 We examined the influence of gestational age in an equal number of premature infants (<37 gestational weeks) and observed that independent of gestational age, ECFCs levels were consistently lower in preeclampsia than in control pregnancies, irrespective of maternal BMI. Gestational age has also been recognized as a source of variation for ECFC levels. Previous studies have shown that levels of circulating ECFCs are more elevated in premature deliveries (gestational age, 28–35 weeks) than at term,8 although extremely premature infants (<28 weeks) have been associated with fewer ECFCs.14 We examined the influence of gestational age in an equal number of premature infants (<37 gestational weeks) and observed that independent of gestational age, ECFCs levels were consistently low in the pathological group. This implicated that the difference in ECFC abundance between preeclampsia and control was more significant in premature deliveries than at term. Taken together, our data suggest an impaired mobilization of ECFCs in preeclampsia that is more evident in preterm deliveries and not associated with changes in cell phenotype or function.

Figure 4. In vitro functional properties of cord blood endothelial colony-forming cells (ECFCs) in preeclampsia. A, Clonogenic properties of ECFCs expressed as (A) percentage of cells with cloning-forming ability and (B) mean number of cells per colony after 10 days in culture. Cell nuclei were identified by 4',6-diamidino-2-phenylindole (DAPI) staining (inset). C, Representative phase contrast micrographs of capillary-like networks formed by ECFCs on Matrigel (scale bar, 300 μm). The ability to form capillary-like networks was quantified and expressed as (D) total number of capillaries per field and (E) total capillary length per field. F, Cell proliferation in response to vascular endothelial growth factor (VEGF)-A (10 ng/mL) and fibroblast growth factor (FGF)-2 (1 ng/mL) expressed as fold increase in cell number. G, Representative phase contrast micrographs depicting the closure of a gap created in an ECFC monolayer (scale bar, 200 μm). Gap closure was monitored in response to VEGF-A (10 ng/mL) and FGF-2 (1 ng/mL). H, Migratory capacity of ECFC in response to VEGF-A and FGF-2 expressed as percentage of gap closure after 15 hours. Bars represent means±SE (n=6). CTR indicates control; and PE, preeclamptic.
and reduced proliferative and vasculogenic properties, including a decrease in the ability to form chimeric vessels after transplantation into immunodeficient mice. Similarly, ECFCs derived from pregnancies complicated by intrauterine growth restriction exhibit altered vasculogenic potential. In this study, we observed a considerable delay in the average time of colony appearance in preeclampsia, with a significant proportion of ECFC colonies emerging during the fourth week of culture. However, with the exception of the delayed endothelial colony formation, ECFCs from preeclamptic pregnancies were otherwise deemed functionally normal. The ability to grow to clonal density and the capacity to form capillary-like networks were highly similar between ECFCs from the preeclamptic group and their nonpathological counterparts. The proliferative and migratory responses to angiogenic factors VEGF-A and FGF-2 were reduced in ECFCs from the preeclamptic group, although the differences with control ECFCs were not statistically significant. More importantly, ECFCs from the preeclamptic group displayed full vasculogenic capacity after transplantation into immunodeficient mice, forming extensive networks of perfused blood vessels with complete perivascular coverage. Taken together, ECFC function was deemed similar between preeclampsia and control. Nevertheless, whether a larger sample size may reveal small functional differences not appreciated in our study remains a possibility.

Perspectives
In this study, we demonstrated a decreased level of umbilical cord blood circulating ECFCs in preeclampsia. Cord blood ECFCs from preeclamptic pregnancies required more time to emerge in culture as endothelial colonies than control ECFCs, but they displayed otherwise normal vascular activity in vitro and in vivo. Epidemiological studies have indicated that several cardiovascular diseases originate during development, and thus there is increasing interest in understanding the relation between the activity of fetal progenitor cells and the appearance of cardiovascular pathologies in the offspring. To date, the pathophysiological implications of having reduced levels of circulating ECFCs during pregnancy are not well understood. Further studies should examine whether the reduced level of cord blood ECFCs observed in preeclampsia correlates with elevated risk of developing subsequent cardiovascular events, such as stroke and hypertension.

Acknowledgments
We thank Dan Li and Dr Shou-Ching Jaminet (Center for Vascular Biology, Department of Pathology, Beth Israel Deaconess Medical Center) for quantitative reverse transcription polymerase chain reaction analyses and the study midwives (Unidad Clinica de Obstetricia y Urgencias del Hospital de la Mujer, Hospital Universitario Virgen del Rocio) for assistance in the collection of umbilical cord blood samples.

Sources of Funding
This work was supported by the National Institutes of Health (R00EB009906 to Dr Melero-Martin); Sistema Andaluz de Salud, Consejería de Salud (SAS1111241 to Dr Moreno-Luna) and Consejería de Economía, Innovación y Ciencia (P08-CV1-4352 to Dr Villar) of the Junta de Andalucía, and Instituto de Salud Carlos III (PH10/02473 to Dr Stiefel).

Disclosures
None.

References


What Is New?

To our knowledge, this is the first prospective cohort study that examines cord blood endothelial colony–forming cell (ECFC) level and function in preeclampsia.

What Is Relevant?

Preeclampsia is a pregnancy-related disorder associated with increased cardiovascular risk for the offspring. ECFCs participate in the formation of new vasculature and the maintenance of vascular integrity; thus, an impaired ECFC level during pregnancy may contribute to an increased risk of developing postnatal cardiovascular events.

Novelty and Significance

Summary

Cord blood ECFC function is normal and highly similar between preeclampsia and control. However, ECFC level is significantly decreased in preeclampsia. This reduction in ECFC abundance is independent of other obstetric characteristics, including gestational age and maternal body mass index. Further studies should examine whether a reduced level of cord blood ECFCs correlates with elevated risk of developing subsequent cardiovascular events, such as stroke and hypertension.
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_Hypertension._ 2014;64:165-171; originally published online April 21, 2014;
doi: 10.1161/HYPERTENSIONAHA.113.03058

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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Running title: Neonatal endothelial progenitors in preeclampsia
EXPANDED MATERIAL AND METHODS

Obstetric factors
The following maternal and neonatal data were obtained from the obstetric records: severity of preeclampsia (mild/severe/HELLP syndrome); time of onset of preeclampsia (early/late); maternal age; mode of delivery (cesarean/vaginal delivery); mode of conception (natural/in vitro fertilization); parity (primipara/multipara); offspring sex; offspring birth weight; maternal height; pre-gestational (6-8 weeks gestation) maternal weight; end-of-pregnancy (right before delivery) maternal weight; pre-gestational blood pressure; and blood pressure at the onset of preeclampsia. Brachial systolic and diastolic blood pressures were measured on the right arm with a handheld analog device operated by trained nursing staff. Patients were lying in supine position with a bed slope of 45° (semi-sitting) for 10 min. Three blood pressure readings were taken at 5 min intervals and the mean was used for data analysis. Gestational age was recorded according to the obstetricians’ best estimate of gestation. Maternal BMI was calculated as the weight in kilograms divided by the square of the height in meters (kg/m²). Gestational weight gain was calculated as the difference between the weight at the end of pregnancy and the weight at first consultation. Newborn birth weight percentiles were calculated according to World Health Organization (WHO) standards. Maternal pre-pregnancy tobacco use was dichotomized into never- and ever-users.

Enumeration of endothelial colony-forming cells
Umbilical cord blood samples (20–50 mL) were collected ex utero using heparinized tubes and processed within 2 hours. Mononuclear cells (MNC) were obtained and cryopreserved as previously described 1,2. Cryopreserved mononuclear cells were thawed, thoroughly washed, and cultured on fibronectin-coated 6-well tissue culture plates (BD Bioscience, San Jose, CA, USA) using endothelial cell-medium (EGM-2 without hydrocortisone, Lonza; 20% FBS; 1X glutamine-penicillin-streptomycin) 3. Unbound cells were removed at 48 hours and the bound fraction maintained in endothelial cell-medium, with media being replenished every 2-3 days. Endothelial colonies were identified as well-circumscribed monolayers of ≥ 50 cells with cobblestone morphology. Colonies were enumerated on days 7, 14, 21, and 28 by visual inspection using an inverted microscope.

Characterization of endothelial colony-forming cells
Colonies were incubated for 20 min with fluorescently labeled Ulex Europaeus Agglutinin type 1 lectin (UEA-1; 1:200) to corroborate their endothelial nature. ECFCs were expanded in culture and purified by expression of CD31, as we have previously shown 3. Endothelial cell phenotype was characterized in vitro by testing: (1) expression of endothelial cell markers, (2) cloning-forming ability, (3) proliferation and migration towards VEGF and FGF-2, and (4) formation of capillary -like structures, using methods previously described by our laboratory 2,4. The vasculogenic ability of ECFCs was evaluated in vivo using a previously developed xenograft model of human endothelial cell transplantation into immunodeficient mice 3,5. Animal experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee at Boston Children’s Hospital.

References


**SUPPLEMENTAL TABLES**

**Table S1.** Cord blood ECFC levels in control and preeclampsia groups: analysis of categorical obstetric variables

<table>
<thead>
<tr>
<th>Obstetric variable</th>
<th>Control (n=35)</th>
<th></th>
<th>P Value</th>
<th>Preeclampsia (n=15)</th>
<th></th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YES</td>
<td>NO</td>
<td></td>
<td>YES</td>
<td>NO</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.2±8.4</td>
<td>5.3±5.8</td>
<td>.32</td>
<td>2.1±2.8</td>
<td>5.5±6.4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(n=23)</td>
<td>(n=12)</td>
<td></td>
<td>(n=13)</td>
<td>(n=2)</td>
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<tr>
<td>Primipara</td>
<td>7.0±6.6</td>
<td>7.2±7.8</td>
<td>.83</td>
<td>2.5±3.2</td>
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<tr>
<td></td>
<td>(n=4)</td>
<td>(n=31)</td>
<td></td>
<td>(n=9)</td>
<td>(n=6)</td>
<td></td>
</tr>
<tr>
<td>Cesarean delivery</td>
<td>6.3±8.5</td>
<td>7.3±7.7</td>
<td>.86</td>
<td>1.5±2.1</td>
<td>2.6±3.5</td>
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<tr>
<td></td>
<td>(n=3)</td>
<td>(n=32)</td>
<td></td>
<td>(n=2)</td>
<td>(n=13)</td>
<td></td>
</tr>
<tr>
<td>In vitro fertilization</td>
<td>7.6±7.4</td>
<td>6.4±8.2</td>
<td>.64</td>
<td>1.8±2.6</td>
<td>4.0±4.4</td>
<td>.40</td>
</tr>
<tr>
<td></td>
<td>(n=23)</td>
<td>(n=12)</td>
<td></td>
<td>(n=10)</td>
<td>(n=5)</td>
<td></td>
</tr>
<tr>
<td>Male neonate</td>
<td>13.6±5.3</td>
<td>6.1±7.5</td>
<td>.02</td>
<td>3.6±2.5</td>
<td>2.0±3.6</td>
<td>.16</td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td>(n=30)</td>
<td></td>
<td>(n=5)</td>
<td>(n=10)</td>
<td></td>
</tr>
<tr>
<td>Preterm birth</td>
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<td>7.2±7.6</td>
<td>-</td>
<td>0</td>
<td>2.7±3.3</td>
<td>-</td>
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<td></td>
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<td>(n=35)</td>
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<td>(n=1)</td>
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<td>Gestational diabetes</td>
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<td>5.5±3.5</td>
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<tr>
<td>Intrauterine growth restriction</td>
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<td>(n=35)</td>
<td></td>
<td>(n=2)</td>
<td>(n=13)</td>
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</tbody>
</table>

ECFC levels are represented by mean ± SD. P values are from comparison of YES and NO subgroups within control and preeclampsia groups (Mann–Whitney U). ECFC = endothelial colony-forming cell.
### Table S2. Cord blood ECFC levels in control and preeclampsia groups: analysis of quantitative obstetric variables

<table>
<thead>
<tr>
<th>Obstetric variable</th>
<th>Control</th>
<th>Preeclampsia</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>r</td>
<td>P Value</td>
<td>n</td>
</tr>
<tr>
<td><strong>Maternal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>35</td>
<td>-.05</td>
<td>.77</td>
<td>15</td>
</tr>
<tr>
<td>Pre-gestational BMI</td>
<td>35</td>
<td>.16</td>
<td>.35</td>
<td>15</td>
</tr>
<tr>
<td>Gestational weight gain</td>
<td>35</td>
<td>.30</td>
<td>.08</td>
<td>15</td>
</tr>
<tr>
<td><strong>Pre-gestational blood pressure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic</td>
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<td>-.10</td>
<td>.61</td>
<td>15</td>
</tr>
<tr>
<td>Systolic</td>
<td>27</td>
<td>-.40</td>
<td>.04</td>
<td>15</td>
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<td><strong>Blood pressure at onset of PE</strong></td>
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<tr>
<td>Diastolic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td>Systolic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>15</td>
</tr>
<tr>
<td><strong>Neonate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestation age</td>
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<td>-.21</td>
<td>.24</td>
<td>15</td>
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<tr>
<td>Birth weight</td>
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<td>.87</td>
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</tr>
<tr>
<td>Birth weight percentile</td>
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<td>-.07</td>
<td>.71</td>
<td>15</td>
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<td>Cord blood MNC level</td>
<td>29</td>
<td>.01</td>
<td>.97</td>
<td>15</td>
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</tbody>
</table>

Univariate correlations between each obstetric variable and ECFC levels were examined with Spearman rho test. n = number of subjects analyzed for each obstetric variable. r = Spearman correlation co-efficient. P = statistical significance for each correlation. ECFC = endothelial colony-forming cell; BMI = body-mass index; PE = preeclampsia; MNC = mononuclear cell.