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Endothelial nitric oxide synthase haplotypes are associated with preeclampsia in Maya mestizo women

Lizbeth Díaz-Olguín[a], Ramón Mauricio Coral-Vázquez[b], Thelma Canto-Cetina[c], Samuel Canizales-Quinteros[d], Belem Ramírez Regalado[a], Genny Fernández[e] and Patricia Canto[a,*]
[a]*División de Investigación Biomédica, Subdirección de Enseñanza e Investigación, Centro Médico Nacional 20 de Noviembre, Instituto de Seguridad y Servicios Sociales de los Trabajadores del Estado, México, D.F., México*
[b]*Sección de Posgrado, Escuela Superior de Medicina, Instituto Politécnico Nacional, México, D.F., & División de Medicina Genómica, Centro Médico Nacional "20 de Noviembre", Instituto de Seguridad y Servicios Sociales de los Trabajadores del Estado, México, D.F., México*
[c]*Laboratorio de Biología de la Reproducción, Departamento de Salud Reproductiva y Genética, Centro de Investigaciones Regionales "Dr. Hideyo Noguchi", Mérida Yucatán, México*
[d]*Facultad de Química, Universidad Nacional Autónoma de México, México, D.F., México, Unidad de Biología Molecular y Medicina Genómica, Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán", México, D.F., Mexico*
[e]*Servicio Prenatal del Hospital Materno Infantil, S.S., Mérida, Yucatán, México*

Abstract. Preeclampsia is a specific disease of pregnancy and believed to have a genetic component. The aim of this study was to investigate if three polymorphisms in *eNOS* or their haplotypes are associated with preeclampsia in Maya mestizo women.
A case-control study was performed where 127 preeclamptic patients and 263 controls were included. Genotyped and haplotypes for the -768T→C, intron 4 variants, Glu298Asp of *eNOS* were determined by PCR and real-time PCR allelic discrimination. Logistic regression analysis with adjustment for age and body mass index (BMI) was used to test for associations between genotype and preeclampsia under recessive, codominant and dominant models. Pairwise linkage disequilibrium between single nucleotide polymorphisms was calculated by direct correlation r^2, and haplotype analysis was conducted.
Women homozygous for the Asp298 allele showed an association of preeclampsia. In addition, analysis of the haplotype frequencies revealed that the -786C-4b-Asp298 haplotype was significantly more frequent in preeclamptic patients than in controls (0.143 vs. 0.041, respectively; OR = 3.01; 95% CI = 1.74–5.23; $P = 2.9 \times 10^{-4}$).
Despite the Asp298 genotype in a recessive model associated with the presence of preeclampsia in Maya mestizo women, we believe that in this population the -786C-4b-Asp298 haplotype is a better genetic marker.

Keywords: Preeclampsia, maya-mestizo women, polymorphisms of *eNOS*, *eNOS* haplotypes.

1. Introduction

Preeclampsia is a specific disease of pregnancy [1]. This syndrome occurs in 5–7% of pregnant women, although in some populations this may be increased up to three times because of various social, economic, racial, geographic and/or social factors [2]. Despite being one of the leading causes of maternal death and a major contributor of maternal and perinatal morbidity, the precise cause for this syndrome has not been completely elucidated [3].

The mechanisms causing preeclampsia are still unclear; however, endothelial activation/dysfunction is a central pathogenic feature in women with this syn-

*Corresponding author: Patricia Canto, M.D., Ph.D., División de Investigación Biomédica C.M.N. 20 de Noviembre, ISSSTE, San Lorenzo No. 502, 2nd piso, Col. del Valle, Delegación Benito Juárez, C.P. 03100, México, D.F., México. Tel.: +52 55 52003513; Fax: +52 55 55754879. E-mail: ipcanto@yahoo.com.mx.

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drome [4,5]. Nitric oxide (NO) induces vasodilatation, inhibits platelet aggregation, and prevents platelet adhesion to endothelial cells [6–9]. An increment of NO production has been described in normal pregnancy [10,11]; on the other hand, reduced NO formation has been implicated in the pathogenesis of preeclampsia [12,13]. The endothelial NO synthase (eNOS) is the enzyme that generates NO in blood vessels and regulates vascular function, as well as playing a protective role to endothelial cells [14,15].

Susceptibility to preeclampsia is believed to have a genetic component [16–18]. Several candidate genes are under investigation in relation with the development of preeclampsia, and these include the *eNOS*. Three polymorphisms in the *eNOS* gene have been associated with preeclampsia: a single-nucleotide polymorphism (SNP) in the promoter region, the –786T→C (rs2070744), a variable number of tandem repeats in intron 4 (a 27 bp-repeat), and a SNP in exon 7 (Glu298Asp, rs1799983) [19–22]. However, the results of studies seeking associations of these polymorphisms with preeclampsia have not always been consistent in different population analyses [20,23,24]. As proposed by Sandrim et al. [21], these results are probably due to only analyzing the genotypes of one polymorphism instead of assessing the association of *eNOS* haplotypes with preeclampsia.

Because the mestizo and amerindian populations of Mexico are genetically heterogeneous [25] and considering the putative role of polymorphisms in genetic susceptibility to preeclampsia, the principal aim of this study was to analyze the possible association between three polymorphisms in *eNOS* gene (as well their haplotypes) and preeclampsia in maya mestizo women.

2. Subjects and methods

The study was approved by the Institute's Human Research Committee of the Centro de Investigaciones Regionales "Dr. Hideyo Noguchi". Informed consent was obtained from all patients and controls before participation in the study. One hundred twenty seven preeclamptic women (without history of preeclampsia) and 263 pregnant non-preeclamptic women (controls) were analyzed. All patients were of Maya mestizo ethnic origin, resulting from the admixture between Maya and European (Spanish) population with at least one Maya surname. All subjects lived in the state of Yucatán.

A case-control study was performed to investigate the possible association between three *eNOS* polymorphisms and preeclampsia. The study was conducted at the Materno-Infantil Hospital of the Secretaria de Salud from August 2002 to September 2003. This Institution is responsible for providing maternity services to low-income women residing in Mérida, Yucatán, Mexico. All women admitted with a diagnosis of preeclampsia who agreed to participate in the study were recruited and selected consecutively according to their regular visits to this hospital. Upon each visit, blood pressure was measured in a seated position by physicians or obstetrical nurses via the auscultatory method using a mercury sphygmomanometer. Korotkoff phase V was generally used for defining diastolic blood pressure.

Clinical findings of the patients and controls were described previously [26]. Preeclampsia was defined as the development of hypertension and proteinuria ($>$ 300 mg urinary protein in 24 h) in women with no baseline proteinuria. Hypertension was defined as systolic blood pressure $\geqslant$ 140 mmHg and/or diastolic blood pressure $\geqslant$ 90 mmHg measured on two consecutive occasions at least 24 h apart [27]. Women with previously diagnosed hypertension were excluded from the study. The control group was comprised of women with uncomplicated pregnancy admitted for natural childbirth or caesarean section, with normal length pregnancy, blood pressure $\leqslant$ 120/80 mmHg and without proteinuria.

2.1. Genotyping

Peripheral blood samples were obtained from all individuals, and genomic DNA was purified as described by Kempter and Grossbadern [28]. Three *eNOS* gene polymorphisms were studied: the -786 T→C polymorphism in the 5'-flanking region of gene (rs2070744), the variable number of tandem repeat (27 bp-repeat) polymorphisms in intron 4 and the G-894T→Glu298Asp polymorphism in exon 7 (rs1799983).

Genotypes for intron-4 polymorphism were determined by PCR. DNA was amplified by the polymerase chain reaction (PCR) in 25 μl of reaction mixture containing 200 ng of genomic DNA, 0.2 mM dNTPs, 2.0 U of *Platinum Taq* DNA polymerase (Invitrogen, Life Technologies Corporation, Brazil), and 0.2 μM of each specific set of intron 4 of the *eNOS* primers (the sequence of the primers have been described previously) [29]. Thirty cycles of PCR amplifications were performed in a Thermal Cycler (Multigene II, Labnet International Inc., USA). Except for the last, all cycles

were 1 min at 96°C, 1 min at 59°C, and 1 min at 72°C. In the last cycle the annealing temperature was at 72°C for 5 min. After amplification, PCR products were electrophoresed on 1.2% agarose gels and stained with ethidium bromide to verify the correct size of the expected fragments; the negative control in the PCR having all reagents except DNA. Two alleles were obtained when this region was amplified: "*eNOS*a," which was 234 base pair (bp) long and consisted of one 27-bp repeating unit and "e*NOS*b," which was 261-bp long and consisted of two 27-bp repeating units.

On the other hand, real-time PCR allelic discrimination TaqMan assay (AB) was used for genotyping Glu298Asp and -786T→C polymorphisms. All PCR reactions contained 10 ng of DNA, 5.0 μl TaqMan Universal Master Mix (AB) (2X), 0.25 μl primers and probes (10X) and water for a final volume of 10 μl, including the appropriate negative controls in all assays.

In case of the Glu298Asp polymorphism, the assay used probes and primers designed by Applied Biosystem assay-on-demand services (assay ID: C_3219460_20), whereas for the -786T→C polymorphism the probe and primers were designed by us. Primers sequences were as follows: forward 5'-ACC AGGGCATCAAGCTCTTC-3' and reverse 3'- GCAG GTCAGCAGAGAGACTAG-3'. The probe for each allele is as follows: wild-type FAM 5'-AGGGTCAGC C**A**GCCAG-3' and mutant VIC 5'-AGGGTCAGCC**G** GCCAG-3'. Real-time PCR was performed on an ABI Prism 7500 Fast (Applied Biosystems). Conditions for Glu298Asp were 50°C for 2 min, 95°C for 10 min, and 50 cycles of amplification (92°C for 15 sec and 60°C for 1.30 min). For the -786T→C polymorphism, conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of amplification (92°C for 15 sec and 60°C for 1 min). For each cycle, the software determined the fluorescent signal from the VIC or FAM-labeled probe (Applied Biosystems). Allelic discrimination was performed using specific primers and probes for each allele.

2.2. *Statistical analysis*

Data from the overall patient population in the study were summarized as mean ± standard deviation in the case of quantitative variables. Deviations from Hardy-Weinberg equilibrium were tested using the χ^2 test. Power calculation of 80% assuming a 10% difference in genotype percentages was estimated using the epidemiological data obtained by Duran and Couoh [30], and the mathematical calculation was performed according to Pértegas and Fernández [31].

Logistic regression analysis with adjustment for age and body mass index (BMI) was used to test for associations between genotype and preeclampsia under recessive, codominant and dominant models (SPSS v. 10.0, Chicago, IL). Pairwise linkage disequilibrium between SNPs was calculated by direct correlation r^2 (Haploview v. 3.2), and haplotype analysis was conducted using PHASE 2.1 software.

Table 1
Clinical characteristics of preeclamptic women and controls

	Preeclampsia	Controls	P
N	127	263	
Age (years)	22.0 ± 5.6	21.59 ± 4.5	0.349
BMI (kg/m^2)	33.5 ± 5.4	22.5 ± 3.2	< 0.0001
SBP (mmHg)	149.6 ± 11.4	112.8 ± 7.2	< 0.0001
DBP (mmHg)	101.3 ± 8.4	76.7 ± 5.1	< 0.0001
Birth weight (g)	2318.6 ± 408.81	3085.8 ± 404.1	< 0.0001

Data are means ± SD.
BMI = Body Mass Index.
SBP = Systolic Blood Pressure.
DBP = Diastolic Blood Pressure.

3. Results

The mean age of women with preeclampsia and control group was 22 (± 5.6) and 21.59 (± 4.5) years, respectively. No cases with HELLP (hemolysis, elevated liver enzymes and low platelet count) were diagnosed. As expected, women with preeclampsia showed significantly higher systolic/diastolic blood pressures and BMI compared to control women, and these differences were significant. Likewise, the birth weight was significantly different between the preeclamptic group and the control group (Table 1).

Hardy-Weinberg equilibrium test was performed for the polymorphisms under study and showed that the distribution of the observed genotypes did not differ from the expected one, in either the patient or control group ($P > 0.05$). The statistical power of the study was 89% at $P < 0.05$ to detect previous associations observed in women with preeclampsia.

-768T→C, variable number of tandem repeat polymorphisms in intron 4, Glu298Asp of *eNOS* genotype and allele frequencies are presented in Table 2. Low pairwise linkage disequilibrium was present among the polymorphisms ($r^2 < 0.228$). Genotype and allele distributions showed no significant differences under dominant and codominant models (Table 2). However,

Table 2
Genotype and allele frequencies of the -768T→C, intron 4 variants, Glu298Asp of *eNOS* polymorphisms in Maya mestizo women with preeclampsia

Polymorphism	Cases ($n = 127$)	Controls ($n = 263$)	*P value
-768T→C			
Genotype frequencies			
T/T	94 (74.0)	195 (74.1)	0.668
T/C	28 (22.0)	61 (23.2)	
C/C	5 (3.9)	7 (2.7)	
Allele frequency			
T	216 (85.0)	451 (85.7)	
C	38 (14.9)	75 (14.3)	
Intron-4			
Genotype frequencies			
b/b	110 (86.6)	233 (88.6)	0.534
b/a	14 (11.0)	26 (9.9)	
b/c	1 (0.8)	1 (0.4)	
a/a	2 (1.6)	3 (1.1)	
Allele frequency			
b	235 (92.5)	493 (93.7)	
a	18 (7.1)	32 (6.1)	
c	1 (0.4)	1 (0.2)	
Glu298Asp			
Genotype frequencies			
Glu/Glu	94 (74.0)	206 (78.3)	0.405
Glu/Asp	28 (22.0)	55 (20.9)	
Asp/Asp	5 (3.9)	2 (0.8)	
Allele frequency			
Glu	216 (85.0)	416 (88.8)	
Asp	38 (14.9)	59 (11.2)	

Note: For the number of individuals (n), values in parentheses indicate percentage.
$*P$ value adjusted for maternal age and body mass index (under dominant model).

women homozygous for the Asp298 allele showed an association of preeclampsia (recessive model) as compared to those carrying the Glu allele (Asp298Glu and Glu298Glu genotypes), with an odds ratio (OR) = 5.4 and 95% confidence interval (CI) = 1.02–27.9; $P = 0.047$, adjusted for age and BMI. This significant association was after adjusting for age and BMI.

Furthermore, five haplotypes with a frequency higher than 5% were identified (Table 3). Interestingly, the haplotype frequencies showed significant differences in cases and controls ($P < 0.0001$). The analysis revealed that the -786C-4b-Asp298 haplotype was significantly more frequent in patients with preeclampsia than in controls (0.143 vs. 0.041, respectively; OR 3.01; 95% CI = 1.74–5.23; $P = 2.9 \times 10^{-4}$). Moreover, 786T-4a-Glu298 was the second most common haplotype but showed no significant group differences; whereas the -786T-4b-Asp298 and -786C-4b-Glu298 haplotypes were significantly less frequent or absent in patients as compared to controls (0.004 and 0.007 vs. 0.072 and 0.060, respectively, $P < 0.0001$).

4. Discussion

Preeclampsia is a complex pregnancy-specific condition involving endothelial dysfunction and activation [2,4,5] and has been suggested as a potential role of NO deficiency in the pathogenesis of preeclampsia. Furthermore, several studies found a significant association among clinically important polymorphisms as well as in *eNOS* gene haplotypes and this syndrome [19–22], suggesting that genetic variations in this gene may predispose to this hypertensive condition of pregnancy.

In the present study we analyzed the possible association of the polymorphisms -786T→C, the variable number of tandem repeat polymorphisms in intron 4 and Glu298Asp, as well as the haplotypes of *eNOS* gene and preeclampsia in Maya mestizo women, finding that Glu298Asp polymorphism and three haplotypes were associated with this syndrome. Interestingly, as reported [20], we found that the pairwise linkage disequilibrium among the allelic association of the three polymorphisms was low.

Table 3
Estimated haplotype frequency distribution of the -768T→C, intron 4 variants, Glu298Asp of *eNOS* polymorphisms in Maya mestizo women with preeclampsia

Haplotypes			Cases	Controls	*P*
-768T→C	Intron-4	Glu298Asp			
T	b	Glu	0.776	0.767	–
C	b	Asp	0.143	0.041	2.9×10^{-4}
T	a	Glu	0.068	0.019	NS
T	b	Asp	0.007	0.072	< 0.0001
C	b	Glu	0.004	0.060	< 0.0001

P considered significant when < 0.008 (0.05/haplotype number). Only haplotypes with a frequency higher than 5% in cases or controls were showed. NS, non-significant.

Serrano et al. [20] reported the association among the same three polymorphisms and haplotypes of *eNOS* and presence of preeclampsia in women from Colombia. In accordance with our results, the main finding of their study was that women homozygous for the Asp298 allele, in addition to the presence of a high-risk –786C-4b-Asp298 haplotype, were associated with preeclampsia. Noteworthy, in our population the differences of this haplotype between preeclamptic and control women was significantly higher ($P = 2.9 \times 10^{-4}$). Additionally, frequency of the same haplotype in our population was higher compared with the Colombian women with preeclampsia (14.3% vs. 9.3%, respectively).

Likewise, Sandrim et al. [21] studied the relationship between the same polymorphisms of *eNOS* and Brazilian women with preeclampsia; nevertheless, there were no significant differences in genotype distribution. Albeit, they found significant differences in the distribution of the haplotype frequencies when women with preeclampsia were compared with the control group, especially the -786C-4a-Glu298 haplotype. However, this haplotype is not common in our population ($< 1\%$). In addition, the authors found that the -786T-4a-Glu298 haplotype apparently protects against preeclampsia. In contrast, we found an inverse association where this haplotype was more frequent in cases (6.8%) than in controls (1.9%), although this difference was not significant.

Inconsistencies of the results in different populations regarding the association of the -786T→C, variable number of tandem repeat polymorphisms in intron 4 and Glu298Asp polymorphisms of the *eNOS* and preeclampsia may be caused by differences in ethnic groups [19,20,32,33]. We have already observed this in other studies related to preeclampsia in Maya mestizo women [26,34]. For that reason, differences in the frequency and type of haplotypes described by Serrano et al. [20], Sandrim et al. [21] and in this study may be due to genetic background.

Several studies have suggested that the Glu298Asp polymorphism of *eNOS* has functional consequences. Savvidou et al. [35] examined the vascular responses in healthy pregnancy, demonstrating that healthy pregnant women homozygous for the Asp298 polymorphism had a lower flow-mediated dilatation of the brachial artery (21% lower) in comparison with the women who carried the Glu298 allele in a homozygous state. Because the flow-mediated dilation is an NO-dependent response, the authors proposed that women with the Glu298Asp polymorphism may reduce the threshold for endothelial dysfunction and thus be more likely to develop preeclampsia. On the other hand, in vitro analysis of the *eNOS* Glu298Asp polymorphism has provided insight that in a recessive model Asp298 may affect the function of the protein because the eNOS Asp298 undergoes selective proteolytic cleavage in endothelial cells and vascular tissue, thereby reducing vascular NO generation [36,37]. However, Fairchild et al. [38] and McDonald et al. [39] reported contradicting results about the function of this polymorphism, suggesting that this observation may be due to an artifact.

Moreover, in vitro assay of -786T→C polymorphism of *eNOS* has shown that only the C allele reduces promoter activity of the *eNOS* gene [40,41]. In addition, subjects with the C allele presented a decreased serum concentration of nitrite/nitrate [41]. Jeerooburkhan et al. [42] observed that this polymorphism does not significantly influence the plasma levels of NO. On the other hand, studies of the variable number of tandem repeats in intron 4 polymorphism of *eNOS* indicate that carriers of this variant have lower nitric oxide plasma levels and decreased protein expression [43,44], whereas other studies have shown no evidence for this findings [42,45].

Recently, Sandrim et al. [22] evaluated, for the first time, the association among plasma nitrite concentrations and three eNOS polymorphisms in Brazilian women with normal pregnancy as well as in women with preeclampsia, finding that women with normal pregnancy and the common haplotype (-786T-4b-Glu298) showed a high nitrite concentration in comparison with the preeclampsia group ($P < 0.004$). These authors suggested that perhaps the last haplotype protects against the development of preeclampsia due to increased nitric oxide formation. In contrast, in our population this haplotype was similar between controls and cases (76.7% vs. 77.6%, respectively), suggesting that these differences may be due to the ethnic diversity.

It has been suggested that the haplotypes of the *eNOS* gene may affect response to antihypertensive treatment in women with preeclampsia [46]; nevertheless, in the population of Brazilian women the most common haplotypes that affect treatment response were -786C-4a-Glu298 and -786T-4a-Asp298, which are very different from the major haplotype found in our study (-786C-4b-Asp298). These findings all underline the importance of determining, in different populations, the *eNOS* gene haplotypes in women with preeclampsia.

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