ORIGINAL ARTICLE

Evaluation of Endothelial Nitric Oxide Synthase and Nitric Oxide Levels in Preeclamptic and Normotensive Pregnant Women

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Abstract:

Background: Preeclampsia is a pregnancy specific disease, causes maternal, fetal morbidity and mortality. The exact cause of preeclampsia is not clearly known. However, endothelial dysfunction contributes significantly to the preeclampsia pathophysiology. *Aim and Objectives:* To measure maternal serum endothelial Nitric Oxide Synthase (eNOS) and Nitric Oxide (NO) concentrations in preeclamptic and normotensive pregnant women and their correlation with blood pressure. *Materials and Methods:* Prospective case-control study conducted in the Department of Biochemistry in collaboration with Department of Obstetrics and Gynecology of RL Jalappa Hospital and Research Centre, Sri Devaraj Urs Medical College, Karnataka, India. Normotensive (n=120) and preeclamptic (n=120) women were included in this study. Based on preeclampsia severity, subjects were divided into mild (n=44) and severe preeclampsia (n=76). Blood samples collected from the study subjects allowed to obtain clear serum, stored at -80°C and processed. EDTA blood used to measure hemoglobin and platelet count. Routine parameters such as Random Blood Sugar (RBS), urea, creatinine, uric acid, Asparatate Transaminase (AST), Alamine Transaminase (ALT) and NO, eNOS concentrations were estimated. Corresponding urine samples tested for protein by dipstick. *Results:* Low gestational age (36.89 ± 3.27 weeks) and increased Body Mass Index (BMI) (27.28 ± 3.85 kg/m^2) were recorded. Elevated blood pressure [systolic (157.3 ± 15.06 mmHg), diastolic (101.31 ± 10.67 mmHg), mean arterial pressure (120.08 ± 11.18 mmHg)], pulse rate (88.00 ± 5.65 bpm), serum uric acid (5.83 ± 1.86 mg/dL), AST (25.04 ± 11.61 IU/L), ALT (18.92 ± 10.16 IU/L) levels were observed in preeclampsia than healthy pregnant women. eNOS (4.89 ± 2.18 ng/mL) and NO (5.93 ± 2.25 µmoles/L) levels were decreased significantly in preeclampsia than healthy pregnant women. eNOS levels were significantly different between mild (5.80 ± 2.15 ng/mL) and severe preeclampsia (4.33 ± 2.03 ng/mL). eNOS has negative correlation with blood pressure [systolic (r=-0.229), diastolic (r=-0.178) and mean arterial pressure (r=-0.197)]. NO levels were negatively correlated with systolic (r=-0.250), diastolic (r=-0.208) and mean arterial pressure (r =-0.229). *Conclusion:* Study concludes that reduced eNOS and nitric oxide, negative correlation with blood pressure in preeclampsia indicated altered endothelial function.

Keywords: Endothelial Dysfunction, Normotensive Pregnant Women, Preeclampsia, Vasoconstriction

Introduction:

Preeclampsia (PE) is a human pregnancy disease contributes to maternal, fetal morbidity and mortality and potentially life-threatening with a complication of 3-8% of pregnancies, commonly seen after 20 weeks of gestation [1-2].

This complication accounts for 16% of maternal deaths in developed countries whereas higher range in developing countries [3]. It is a multi-organ disease involves brain, liver, kidney and

endothelium. Placenta an interface between fetus and mother is a key component in preeclampsia onset. Despite of the extensive research the exact mechanism of the disease is unclear. However, poor and shallow placentation with impairment of spiral artery remodeling with endothelial dysfunction is one of the contributing factors in pathogenesis of preeclampsia comprising oxidative stress, platelet and thrombin activation, inflammation and imbalance between angiogenic and anti-angiogenic molecules [4-5].

The endothelium plays a crucial role in vascularization in pregnancy, vasoactive function like vasodilation depends on release of vasodilator molecules namely nitric oxide or endothelium derived relaxant factor, produced from endothelial cells of blood vessels [6].

During pregnancy, adequate uteroplacental blood is required and is dependent on vasodilation. Therefore, the production of vasodilator molecules from the endothelium is very essential to maintain a healthy pregnancy through the regulation of blood pressure. The compromised endothelial function reported in preeclampsia linked to symptoms such hypertension, edema, proteinuria [7].

Nitric oxide exerts its actions via activation of cyclic Guanosine Monophosphate [cGMP], which promotes Ca^{2+} efflux and causes smooth muscle relaxation [8]. Nitric Oxide (NO) known to have an important role in the regulation of feto-placental vascular permeability and resistance, cellular respiration, proliferation, apoptosis, trophoblast invasion, placental development, placental vascular dilation and thus placental blood flow, prevents platelet aggregation in placenta and prevents adhesion of platelets to endothelial cells and acts as an anti-coagulant [9-10].

Endothelial Nitric Oxide Synthase (eNOS) catalyzes the conversion of L-arginine into nitric oxidein endothelial cells [9].The activity of this enzyme depends on phosphorylation and elevated calcium concentration in endothelial cells [7].The expression of eNOS and its action in syncytiotrophoblast, villous endotheliumis important [11]. Dearranged eNOS/NO implicated in pathophysiology of pregnancy in utero-placental region [8].

Although, several endothelial dysfunction markers like endothelin-1 (ET-1), Thromboxane A2 (TXA2) and ADMA were reported [7-8].As per the literature search, information on maternal serum eNOS and NO concentrations in preeclampsia are limited. Moreover, few studies have reported conflicting results about eNOS and NO levels in preeclampsia [12-15]. Even though, various factors influence pregnancy hypertension and its complications, yet there is a scope to study eNOS and NO in relation to blood pressure and other routine parameters in normal pregnancy and preeclampsia. Hence, the aim of the present study is to measure maternal serum eNOS and nitric oxide concentrations in preeclamptic and normotensive pregnant women and their correlation with blood pressure.

Material and Methods:

The prospective case-control study design was carried out in Department of Biochemistry in collaboration with Department of Obstetrics and Gynecology of RL Jalappa Hospital and Research Centre, a teaching hospital of Sri Devaraj Urs Medical College, a constituent of Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka, India, after obtaining Institutional Ethical Clearance.

Sample size was calculated by using the formula $n=2Sp^2[Z_1-\alpha/2 + Z_1-\beta]^2/\mu d^2$, $Sp^2 = S_1^2 + S_2^2/2$with 90% power and 95% confidence interval [8]. A total of 240 pregnant women were included in this study after obtaining the written informed consent, among them 120 normotensives and 120 preeclamptic cases. Further, depending on the severity, 120 preeclampsia cases were sub grouped into mild (n=44) and severe preeclampsia (n=76).

Exclusion criteria consisted of pregnant with history of renal disease, liver disease, thyroid disorder, chronic systemic hypertension, gestational diabetes, hypertensive encephalopathy, cardiovascular diseases, pregnancy with fetal anomaly, multiple pregnancies, patients with history of smoking or alcohol use and malignancy conditions.

Diagnosis of Preeclampsia:

Preeclampsia was diagnosed according to the American College of Obstetrics and Gynecologists Criteria (ACOG practice bulletin 2013),with blood pressure of systolic ⩾ 140 and diastolic ⩾ 90 mmHg noted for the first time during pregnancy on two occasions at least four hours apart, after 20 weeks of gestation with proteinuria of ⩾ 300 mg/ 24 hours or 1+ protein in a single voided sample or in the absence of proteinuria, at least one organ involvement is considered in addition to hypertension and the following findings were considered as systemic involvement; thrombocytopenia (a platelet count <100 000/μL), renal insufficiency (serum creatinine >1.1 mg/dL or doubling of serum creatinine levels in the absence of any renal disease), hepatic dysfunction (elevated liver enzymes in the blood levels to two times of the normal), symptoms associated with cerebral or ophthalmic and edema. However, mild preeclampsia was defined with blood pressure of ⩾ 140/90 mmHg on 2 occasions at least 4 hours apart after 20 weeks of gestation and with proteinuria (dipstick reading of +1) whereas severe preeclampsia was defined as the presence of any of the following criteria: Blood pressure of ⩾ 160/110 mmHg on 2 occasions at least 6 hours apart, elevated serum creatinine levels >1.1 mg/dL or doubling of the serum creatinine levels in the absence of other renal diseases, elevated liver transaminases to twice normal concentration, platelet count less than 100,000/microliter, headache, visual impairment, epigastric pain or pain in the right upper quadrant [16].

Collection of Blood Sample:

Five milliliters of venous blood sample was collected from each study participant and aliquoted into plain tube (3 mL) and EDTA (2mL) tubes during their antenatal checkup. Blood samples were allowed to clot for 30 minutes, centrifuged at 3000 rpm for 10 minutes at 4° C to obtain the clear serum. Thus, obtained clear serum was stored at -80°C until testing. Complete blood count was done by using EDTA blood. Serum was used for the estimation of Random Blood Sugar (RBS) (GOD-POD), urea (urease), creatinine (sarcosine oxidase), uric acid (uricase), Asparatate Transaminase (AST) (IFCC), Alamine Transaminase (ALT) (IFCC) by using Vitros 5.1 FS Dry chemistry analyzer. Maternal serum nitric oxide (Griess reaction) by spectrophotometer and eNOS was done by ELISA method. Five milliliters of corresponding urine sample was collected for urinary protein analysis by dipstick method. Height and weight were recorded and Body Mass Index (BMI) was calculated.

Determination of Maternal Serum eNOS:

Human eNOS concentration in serum was measured by enzyme-linked immunosorbent assay (ELISA) technique as per the procedure supplied by Sincere Biotech Co. Ltd, Beijing, China (Human eNOS kit catalogue No : E13650645).

This assay was based on the principle of quantitative sandwich technique. Purified human eNOS antibody was pre-coated onto the microtiter plate wells; make solid phase antibody, then added standards and samples to the wells. eNOS present in the serum was bound to the human eNOS antibody which with Horse Radish Peroxidase (HRP) labeled, become antibody-antigen-enzyme-antibody complex. After removing any unbound substances by washing procedure, added 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution. TMB substrate solution becomes blue color at HRP enzyme-catalyzed. The reaction was terminated by adding stop solution (2 mol/L sulfuric acid) and then color changes to yellow, the absorbance of the color was measured at 450 nm. The concentration of human eNOS in the samples was then determined by comparing with standard curve and represented as ng/mL.

Determination of Maternal Serum Nitric Oxide:

Nitrite and nitrate concentration can be determined by using Griess reagent in which NO reacts with 3% sulphanilamine and 10% Ethylenediamine dihydrochloride forming chromophore which can read at 543 nm in spectrophotometer (Perkin Elmer lamda 1.2) [17].

Statistical Analysis:

The results were expressed in mean ± SD. Mann-Whitney *U* test was used for continuous non-normally distributed variables. Categorical variables were expressed as percentage.

Spearman's correlation applied to find the association of variables. The level of significance considered for p value <0.05. Analysis was performed using SPSS software, licensed version 22.0.

Results:

Table 1 describes the demographic variables obtained indicated low gestational age (36.89 ± 3.27 weeks) and increased BMI(27.28 ± 3.85 kg/m^2), systolic (157.3 ± 15.06 mmHg), diastolic (101.31 ± 10.67 mmHg), mean arterial pressure (120.08 ± 11.18 mmHg) and pulse rate (88.00 ± 5.65 bpm) were observed in preeclampsia cases compared to healthy pregnant women.

Table 2 presents the routine parameters like serum uric acid (5.83 ± 1.86 mg/dL), AST (25.04 ± 11.61 IU/L), ALT (18.92 ± 10.16 IU/L) levels were increased and eNOS (4.89 ± 2.18 ng/mL), NO (5.93 ± 2.25μmoles/L) concentrations were significantly reduced in preeclampsia compared with healthy pregnant (p=0.000).

Table 3 illustrates subgroup data of preeclampsia with respect to eNOS (4.33 ± 2.03 ng/mL) levels were significantly reduced in severe preeclampsia compared to mild preeclampsia (5.80 ± 2.15). However, nitric oxide (5.43 ± 2.14 μmoles/L) levels reduced in severe preeclampsia, but not showing any significance between the groups (p=0.052).

Table 4 shows, eNOS negatively correlated with systolic r =-0.229), diastolic (r =-0.178) and mean arterial blood pressure (r=-0.197). Nitric oxide levels were negatively correlated with systolic blood pressure r =-0.250), diastolic r =-0.208) and mean arterial pressure (r =-0.229) in preeclampsia.

Table 1: Demographic Details of Preeclampsia and Normotensive Healthy Pregnant Women

Parameters	Preeclampsia (n=120)	Normotensive healthy pregnant women (n=120)	*P*
Age (years)	22.99 ± 3.50	23.34 ± 3.21	0.230
Primigravide (*n*, %)	99 (82.5%)	105 (87.5%)	-
Multigravide (*n*, %)	21 (17.5%)	15 (12.5%)	-
Gestational age at sampling (wks)	36.89 ± 3.27	38.77 ± 1.70	0.000
BMI (kg/m^2)	27.28 ± 3.85	25.66 ± 3.93	0.005
Systolic blood pressure (mmHg)	157.3 ± 15.06	115.65 ± 8.01	0.000
Diastolic blood pressure (mmHg)	101.31 ± 10.67	74.06 ± 6.66	0.000
Mean arterial pressure (mmHg)	120.08 ± 11.18	87.84 ± 6.39	0.000
Presence of proteinuria (*n*,%)	120 (100%)	Nil	-
Pulse rate (bpm)	88.00 ± 5.65	86.10 ± 7.91	0.002

BMI: Body mass index, Values expressed in Mean ± SD

Table 2: Hematological and Biochemical Parameters of Preeclampsia and Normotensive Healthy Pregnant Women

Parameters	Preeclampsia (n=120)	Normotensive healthy pregnant women (n=120)	*P*
Hematological parameters			
Hemoglobin (g%)	11.10 ± 2.03	11.24 ± 1.78	0.866
Platelet count x (10^9/L)	234.18 ± 78.46	243.74 ± 65.71	0.583
Biochemical parameters			
RBS (mg/dL)	84.46 ± 20.86	83.60 ± 16.41	0.643
Serum Urea (mg/dL)	16.26 ± 9.37	14.33 ± 4.63	0.210
Serum creatinine (mg/dL)	0.53 ± 0.20	0.49 ± 0.12	0.067
Serum uric acid (mg/dL)	5.83 ± 1.86	4.62 ± 1.34	0.000

Continued...

Parameters	Preeclampsia (n=120)	Normotensive healthy pregnant women (n=120)	*P*
Serum AST (IU/L)	25.04 ± 11.61	20.65 ± 7.68	0.001
Serum ALT (IU/L)	18.92 ± 10.16	14.80 ± 7.00	0.000
Serum eNOS (ng/mL)	4.89 ± 2.18	6.56 ± 3.36	0.000
Serum Nitric Oxide (µmoles/L)	5.93 ± 2.25	11.20 ± 4.73	0.000

RBS: Random blood sugar, AST: Aspartate transaminase, ALT: Alanine transaminase, eNOS: Endothelial nitric oxide synthase, Values expressed in Mean ± SD

Table 3: Comparison of eNOS and NO Levels between Mild vs Severe Preeclampsia

Parameters	Mild Preeclampsia (n=44)	Severe preeclampsia (n=76)	*P*
eNOS (ng/mL)	5.80 ± 2.15	4.33 ± 2.03	0.001
Nitric Oxide (µmoles/L)	6.38 ± 2.10	5.43 ± 2.14	0.052

eNOS: Endothelial nitric oxide synthase, Values expressed in Mean ± SD

Table 4: Correlation of eNOS and NO with Blood Pressure

Parameters	*r* - value	*P*
Systolic blood pressure		
eNOS	-0.229*	0.012
Nitric Oxide	-0.250**	0.006
Diastolic blood pressure		
eNOS	-0.178	0.052
Nitric Oxide	-0.208*	0.022
Mean arterial pressure		
eNOS	-0.197*	0.031
Nitric Oxide	-0.229*	0.012

eNOS: Endothelial nitric oxide synthase
**. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed).*

Discussion:

In preeclampsia, placenta is considered as a source of inflammation and release of vaso-constrictor molecules to initiate endothelial cell injury and endothelial dysfunction [4, 18]. Endothelial dysfunction in pregnancy contributes to hypertension; however, the mechanisms involved are still unclear [7].

In the present study, we measured maternal serum eNOS and nitric oxide concentrations in preeclampsia and in healthy pregnant. The results indicated reduced concentrations of eNOS and NO levels in preeclampsia in comparison with healthy pregnant women. These findings were similar with other studies [8, 9, 13, 15]. Based on the severity of preeclampsia, study subjects were sub-classified into mild and severe preeclampsia. Serum eNOS and NO levels were reduced significantly in severe group than mild preeclampsia group. However, statistical significance associated with only eNOS but not NO levels. The eNOS and NO concentrations were inversely related with respect to blood pressure; indicate the functional role of nitric oxide. This observation is similar with few study reports and in contradiction with other study reports [8, 15, 18]. It has been reported that eNOS in the mother and in the fetus contribute to utero-placental vascular changes and increased uterine arterial blood flow, whereas in preeclampsia reduced due to placental abnormalities, particularly with respect to the eNOS/NO pathway [8]. Kim *et al*. *in-vivo* model reported that lower degree of placental eNOS expression in syncytiotrophoblasts of pre-eclampsia and control samples. This characteristic finding substantiates placental cellular function of eNOS [10]. Carolina *et al*. reported that *invivo*-derived Syncytiotrobhoblast Extracellular Microvesicles (STBMV) and Syncytiotrobhoblast Extracellular Exosomes (STBEX) isolated from placental perfused lobes to have less eNOS activity in preeclampsia compared with controls and *invivo*-derived plasma STBMV analyzed by flow cytometry showed less STBMV bound eNOS expression in preeclampsia compared with normal pregnancy. This may contribute to the decreased levels of nitric oxide in preeclampsia, which may affect the vascular functions [11]. Zawiejska *et al*. reported that decreased levels of maternal serum eNOS in severe preeclampsia [19]. The decreased nitric oxide levels in preeclampsia may be due its low production or increased breakdown and may have an adverse effect on placental hemodynamics and linked to vascular endothelial dysfunction, in the current study as well as other studies [7, 18, 20].

Preeclampsia is a multi-organ disorder evinced hyperuricemia and elevated liver enzymes. The increased serum uric acid levels may be due to increased xanthine oxidase activity and reduced excretion of uric acid in the proximal tubules secondary to hypovolemia, an early change in preeclampsia development [21]. In addition to this, preeclampsia is associated with oxidative stress, which may also contribute to the hyperuricemia in preeclampsia [22]. Hyperuricemia leads to reduction in nitric oxide synthesis in endothelial cells [23-24].

In the current study, elevated liver enzymes such as AST and ALT levels in preeclampsia may be explained by the effect of hypoxia on the hepatic cells in preeclampsia. Dysfunctional endothelium leads to reduced prostacyclins and elevated

thromboxane levels, which causes vasoconstriction of blood vessels of the liver. Hypoxic liver cells undergo necrosis and degeneration and would increase the AST levels [25-26]. In preeclampsia, liver cells and blood vessels produce different mediators such as fibronectin, endothelin-1, thromboxane, which cause vasoconstriction and hypoxia of the liver, which finally result in elevated liver enzymes [27].

Many etiological factors have been involved in the pathogenesis of preeclampsia which leads to insufficient placentation, endothelial cell injury and altered endothelial function [28-29]. Dysfunctional endothelium results in elevated systemic vascular resistance, and reduced placental perfusion, which is aggravates the placental ischemia-reperfusion injury, stimulates increased generation of Reactive Oxygen Species (ROS), lipid peroxidation and reduced antioxidant status, and further exacerbates endothelial dysfunction [30]. In addition to this, mitochondria also could be considered as a source of ROS generation, which might trigger the endothelial dysfunction, also trigger redoxsignaling process to induce cell apoptosis and contributing to pathogenesis of preeclampsia [31].

Conclusion:

The present study results conclude that reduced maternal serum eNOS, nitric oxide levels and increased serum uric acid, liver transaminases in preeclampsia. The eNOS and nitric oxide were negatively correlated with blood pressure and reflect the endothelial dysfunction and therefore, estimation of these molecules in maternal circulation may serves as indicators of vascular dysfunction in preeclampsia.

Acknowledgements:

We would like to thank the authorities of Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka, India for supporting this doctoral study.

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How to cite this article:
Gandham R, Dayanand CD, Sheela SR. Evaluation of Endothelial Nitric Oxide Synthase and Nitric Oxide Levels in Preeclamptic and Normotensive Pregnant Women. *J Krishna Inst Med Sci Univ* 2021; 10(1):27-36

Submitted: *11-Aug-2020* ***Accepted:*** *23-Nov-2020* ***Published:*** *01-Jan-2021*