Role of nitric oxide synthase 3 (NOS3) gene expression in patients of pre-eclampsia with special reference to cardiovascular and renal pathophysiology

Thesis submitted for the award of the degree of Doctor of Philosophy in Medical Physiology

By

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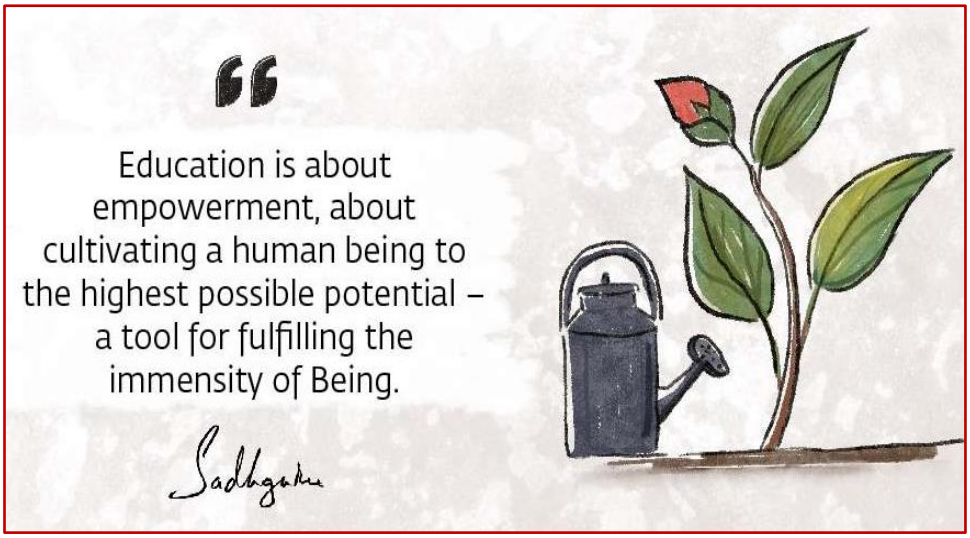

I dedicate this research work to my parents,

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who empowered us with good education

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INDEX

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	Full form
PE	Pre-eclampsia
NOS	Nitric oxide synthase
NO	Nitric oxide
MDA	Malondialdehyde
HRV	Heart rate variability
RT-PCR	Real time - Polymerase Chain Reaction
LF	Low frequency
HF	High frequency
BP	Blood Pressure
eNOS/NOS3	endothelial Nitric oxide synthase / Nitric oxide synthase 3 gene
iNOS/NOS2	inducible Nitric oxide synthase / Nitric oxide synthase 2 gene
RUPP	Reduced uterine perfusion pressure
ROS	Reactive oxygen species
mRNA	messenger Ribonucleic acid
ANS	Autonomic nervous system
FGR	Fetal growth restriction
RAS	Renin–angiotensin system
VEGF	Vascular endothelial growth factor
ECG	Electrocardiogram
SNS	Sympathetic nervous system
PNS	Parasympathetic nervous system

SNA	Sympathetic nervous activity
RNS	Reactive nitrogen species
O_2^-	Superoxide radicals
OH	Hydroxyl radicals
H_2O_2	Hydrogen peroxide
ONOO−	Peroxynitrite radicals
UPCR	Urine protein: creatinine ratio
AGT	angiotensinogen
ADMA	Asymmetric dimethylarginine
ACOG	American College of Obstetricians and Gynecologists
NICE	National Institute for Health and Care Excellence
sFlt-1	soluble fms-like tyrosine kinase-1
PP13	Placenta protein 13
PAPP-A	Pregnancy-associated plasma protein A
sEng	soluble endoglin
NF-κB,	nuclear factor-kappa B
Th	T helper cell
NK	Natural killer cell
dNK	Decidual natural killer cell
HLA-C	Human leukocyte antigen-C
MMP	Matrix metalloproteinases
C3	Complement 3
SNPs	Single nucleotide polymorphisms
PlGF	Placental growth factor

AT1-AA	Angiotensin II Type 1 receptor autoantibody
Tregs	T regulatory cells
IL	Interleukin
TNF-α	Tumor necrosis factor-α
ET	Endothelin
IUGR	Intrauterine growth restriction
HHcy	Hyperhomocysteinemia
L-NAME	NG-nitro-L-arginine methyl ester
SOD	Superoxide dismutase
TGFB1	transforming growth factor beta
TNF-α:	tumor necrosis factor alpha
DDAH	Dimethylarginine dimethylaminohydrolase
ACE	Angiotensin-converting enzyme
cGMP	Cyclic guanosine monophosphate
HUVEC	Human umbilical vein endothelial cell
NADPH	nicotinamide adenine dinucleotide phosphate
SDNN	Standard deviation of normal to normal intervals
SDANN	Standard deviation of the average NN intervals for each 5 min segment
RMSSD	Root mean square of successive RR interval differences
pNN50	Percentage of successive RR intervals that differby more than 50 ms
TBARS	Thiobarbituric acid reactive substances
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
ELISA	Enzyme-linked immunosorbent assay

T-AOC	Total antioxidant capacity
HRP	Horseradish peroxidase
OD	Optical density
Cq	Quantification cycles
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
PREVEND	Prevention of Renal and Vascular Endstage Disease
CRP	C-reactive protein
UPCR	Urine protein: creatinine ratio
UAGT	Urine angiotensinogen
UAGT/Cr	Urine angiotensinogen: Creatinine ratio
RAAS	Renin-angiotensin-aldosterone system
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

ABSTRACT

Role of nitric oxide synthase 3 (NOS3) gene expression in patients of preeclampsia with special reference to cardiovascular and renal pathophysiology

ABSTRACT

Background: Pre-eclampsia (PE) is a disorder that complicates pregnancy and is causing a rise in maternal and fetal morbidity and mortality. Oxidative stress affects patients of preeclampsia and is implicated in the pathogenesis. Gene expression of nitric oxide synthase (NOS) is altered and may contribute to cardiac and renal pathophysiology of pre-eclampsia.

Aim: The aim of the study was to determine the relationship between the gene expression of nitric oxide synthase and oxidative/nitrosative stress, which thereby could influence the cardiovascular and renal pathophysiology in pre-eclampsia patients.

Methods: Preeclampsia patients and normal pregnancy women were included in this case control study. Arterial blood pressure and heart rate were recorded. Frequency and time domain heart rate variability (HRV) analysis was done using the powerlab software. Levels of malondialdehyde (MDA), total antioxidant capacity and nitric oxide (NO) in the serum were estimated. Renal functions were determined by estimating serum proteins, urea, creatinine, uric acid and urine proteins and creatinine estimation. Urine angiotensinogen was also estimated. Urine protein/creatinine ratio and urine angiotensinogen/creatinine ratio were calculated later. Real-time polymerase chain reaction (RT-PCR) was used for nitric oxide synthase 3 (NOS3) and NOS2 gene profiling. Student's t-test was used for statistical analysis and p value <0.05 was considered statistically significant.

Results: A significantly higher ($p<0.0001$) mean arterial pressure was seen in the PE group. A significant increase was noted in LF/HF ratio, low frequency (LF) component, and a decrease in high frequency (HF) component of the HRV in pre-eclampsia. The time domain parameters, SDNN, SDANN, RMSSD and pNN50%, of HRV in preeclampsia patients, showed a reduction in their levels. Levels of serum malondialdehyde were increased ($p< 0.0001$), and on the other hand, the total antioxidant capacity was decreased in the PE group ($p=$

0.034). A significant decrease in the serum albumin (p=0.004) and an increase in the serum uric acid (p<0.0001) were seen. Urine protein and urine protein: creatinine ratio were significantly higher (p<0.0001). The urinary excretion of angiotensinogen was significantly reduced (p<0.0001). But, the serum levels of NO did not show a statistically significant reduction (p = 0.20). Gene expression profiling in the PE group showed a down regulation of NOS3 and NOS2 by about 8.49 and 51.05 times respectively.

Conclusion: Mean arterial pressure increase in preeclampsia may be due to endothelial dysfunction resulting from oxidative stress. Also, parasympathetic withdrawal along with sympathetic overactivity, noted in pre-eclampsia patients, may suggest cardiovascular risk in them, which may be detected early by HRV analysis. These changes may also trigger renal dysfunction. Nitric oxide could have played an important role as the mRNA expression of both NOS3 and NOS2 genes were reduced. However, cross-talk of NO with other vasoactive /biological substances cannot be overlooked.

Keywords: molecular genetics; preeclampsia/ eclampsia; vascular biology; heart rate variability

INTRODUCTION

Role of nitric oxide synthase 3 (NOS3) gene expression in patients of preeclampsia with special reference to cardiovascular and renal pathophysiology

CHAPTER 1.

INTRODUCTION

One disorder that complicates pregnancy very frequently is preeclampsia (PE), which may result in significant maternal and fetal morbidity as well as mortality[1]. Preeclampsia becomes evident after 20th week of pregnancy and an increase in blood pressure of more than or equal to 140/90 mmHg is its most characteristic feature. Protein is also seen in maternal urine (urinary albumin protein ≥300 mg/24 h)[2]. Preeclampsia is seen in 2–8% of all pregnancies over the world and it accounts directly for deaths of the mother and fetus[2,3]. Nearly one tenth of all maternal deaths are associated with hypertensive disorders of pregnancy in Asia and Africa[4]. The self-reported symptoms of pre-eclampsia during pregnancy, in India, was seen in 28.7% of the pregnant women in the past 5 years. It was 19.8% of the pregnant women in Karnataka in the past 5 years[5]. In India, preeclampsia is said to be found in 8-10% among pregnant women[6]. A study reports a prevalence of 7.8% for the hypertensive disorders seen in pregnancy and 5.4% of the study population in India was found to have preeclampsia[7].

Presentation of pre-eclampsia varies from hypertension and proteinuria to liver and cerebral involvement[8]. But, the most common complications of pre- eclampsia that needs in depth study are the cardiovascular and renal parameters due to the reason that there is an interaction between these systems. This may lead to cardio-renal syndrome in a pre-eclamptic woman later in her life[9]. Cardiovascular and renal systems play a major role in the pathophysiology and presentation of preeclampsia. Genetic, vasoactive, and immune factors are implicated in the vascular dysfunction associated with PE, which may lead to cardiovascular and renal pathology[9].

Systemic inflammation, oxidative stress and alterations in levels of angiogenic factors

and vascular reactivity is seen in normal pregnancy itself. This is exacerbated in pre-eclampsia with an associated breakdown of compensatory mechanisms, eventually leading to vascular dysfunction[10]. A modified two-stage model for the pathogenesis of preeclampsia was proposed. Poorly perfused placenta, along with maternal genetic and environmental factors, was implicated for the clinical features of preeclampsia[11].

The pathophysiological mechanisms of pre-eclampsia are not completely understood, but recent research has begun to unravel some of the potential mechanisms. Nitric oxide (NO) is a potent vasodilator and is thought to have a major effect on gestational vasodilation[12]. NO production from L-arginine is catalyzed by Nitric Oxide Synthase (NOS), which include neuronal NOS, endothelial NOS (eNOS/NOS3) and inducible NOS (iNOS/NOS2). There is evidence that an increase in NO production in maternal circulation occurs during pregnancy and a decrease during preeclampsia[13].

Placental ischemia due to reduced uterine perfusion pressure (RUPP) and may lead to endothelial and cardiovascular dysfunction. This could be due to increased production of cytokines which may trigger endothelial dysfunction by decreasing the bioavailability of nitric oxide (NO) and also increasing reactive oxygen species (ROS)[14].

The maternal vasculatures are major sources of reactive oxygen and nitrogen species which can interact to produce peroxynitrite, a powerful prooxidant that covalently modifies proteins by nitration of tyrosine residues, to possibly alter vascular function in pre-eclampsia[10]. Oxidative stress, an increase of reactive oxygen species above the normal scavenging by anti-oxidants may result in endothelial dysfunction and the clinical manifestations of pre-eclampsia[10,12]. Preeclampsia increases oxidative stress, both in placental as well as maternal systemic circulation[15].

NOS expression alters in preeclampsia; upregulation of eNOS expression has been demonstrated during normal pregnancy[16], but both messenger RNA (mRNA) and protein

expression for eNOS are decreased in endothelial cells from preeclampsia[17]. Some evidence suggests iNOS expressionis decreased, whereas eNOS expression is increased, probably as an adaptive or compensatory mechanism[18].

Blood pressure and vascular reactivity is influenced by autonomic nervous system (ANS) too. The interaction between the sympathetic and parasympathetic branches of ANS and their activity may be evaluated by heart rate variability (HRV)[19]. HRV analysis is a non-invasive diagnostic test that provides information about cardiovascular disease risk[20]. Hence, HRV analysis may be used in pre-eclampsia. A variance in HRV results were reported in pre-eclampsia studies. Reduced heart rate variability was observed by some authors[21] and increased variability by others[22].

An increase in the arterial and venous resistance by sympathetic sensitivity or by endothelial NO-mediated gestational vasodilatation inhibition is noted[23]. This may result in thrombotic microangiopathy in the kidneys[24]. Endothelial function disruption may impair renal function by podocyte dysfunction too[25]. There is also noticed a variation in the sensitivity for renin angiotensin system components during normal pregnancy and pre-eclampsia[26].

Although the above evidence suggests involvement of nitric oxide, oxidative stress in the pathophysiology of preeclampsia, there is a dearth of literature highlighting the influence of oxidative stress on the gene expression of NOS and also their effect on cardiovascular and renal pathophysiology. Hence, this study was taken up to understand the pathophysiology which could be of value in the management of pre-eclampsia.

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AIMS & OBJECTIVES

Role of nitric oxide synthase 3 (NOS3) gene expression in patients of preeclampsia with special reference to cardiovascular and renal pathophysiology

CHAPTER 2.

AIMS AND OBJECTIVES

AIM OF THE STUDY:

The aim was to study the relationship between the gene expression of nitric oxide synthase and oxidative/nitrosative stress, which thereby could influence the cardiovascular and renal pathophysiology in pre-eclampsia patients.

OBJECTIVES OF THE STUDY:

1. To determine NOS3 gene expression in pre-eclampsia and normal pregnancy.
2. To determine Oxidative/nitrosative stress (plasma nitric oxide, total oxidant and total antioxidant levels) in pre-eclampsia cases and normal pregnant controls.
3. To evaluate the renal functions (Blood urea, serum creatinine, plasma proteins, urinary proteins, urinary creatinine, urinary angiotensinogen), cardiovascular functions (Heart rate, blood pressure, heart rate variability) in pre-eclampsia cases and normal pregnant controls.

RESEARCH HYPOTHESIS:

There is a relationship between the gene expression of nitric oxide synthase and oxidative/nitrosative stress, which thereby could influence the cardiovascular and renal pathophysiology in pre-eclampsia patients.

REVIEW OF LITERATURE

Role of nitric oxide synthase 3 (NOS3) gene expression in patients of preeclampsia with special reference to cardiovascular and renal pathophysiology

CHAPTER 3.

REVIEW OF LITERATURE

Pregnancy is marked with significant physiological changes in every system, more so, in the cardiovascular system. These changes have been brought about to meet increased metabolic demands of mother and fetus and also to ensure good uteroplacental circulation for fetal growth and development. Insufficient hemodynamic adaptations can result in maternal and fetal morbidity, as seen in preeclampsia and intrauterine growth restriction. Maternal inability to adapt to these physiological changes can bring to light the underlying, previously silent, cardiac pathology[1,2].

Preeclampsia:

Pre-eclampsia is a pregnancy-related disorder that has a prevalence of 5-8% worldwide. It is responsible for considerable maternal and perinatal morbidity and mortality globally, with a predominance in the low- and middle-income countries. Pre-eclampsia occurs in 3% to 7% of nulliparous and 1% to 3% of multiparous women. Pre-eclampsia has also been a major cause of preterm birth, perinatal death, and intrauterine growth restriction[3].

Diagnostic criteria: Preeclampsia includes new-onset of hypertension plus new-onset of proteinuria ≥ 300 mg/24 h after 20 weeks of gestation. The criteria for pre-eclampsia have not changed over the past decade, despite changing scenario of the disease (systolic blood pressure >140 mmHg; diastolic blood pressure ≥90 mmHg; proteinuria ≥ 300mg/24h). But, preeclampsia is diagnosed as hypertension in association with liver function impairment, reduced platelet count, renal insufficiency, pulmonary oedema, or also cerebral / visual disturbances, in the absence of proteinuria, (Table 3.1). Clinical features of pre-eclampsia along with laboratory abnormalities determine the severity of the disease[4].

Table 3.1. Diagnostic criteria for pre-eclampsia[4]

Blood pressure	• Greater than or equal to 140 mm Hg systolic or greater than or equal to 90 mm Hg diastolic on two occasions at least 4 hours apart after 20 weeks of gestation in a woman with a previously normal blood pressure • Greater than or equal to 160 mm Hg systolic or greater than or equal to 110 mm Hg diastolic, hypertension can be confirmed within a short interval (minutes) to facilitate timely antihypertensive therapy
and	
Proteinuria	• Greater than or equal to 300 mg per 24 hour urine collection (or this amount extrapolated from a timed collection) or • Protein/creatinine ratio greater than or equal to 0.3* • Dipstick reading of 1+ (used only if other quantitative methods not available)
Or in the absence of proteinuria, new-onset hypertension with the new onset of any of the following:	
Thrombocytopenia	• Platelet count less than 100,000/microliter
Renal insufficiency	• Serum creatinine concentrations greater than 1.1 mg/dL or a doubling of the serum creatinine concentration in the absence of other renal disease
Impaired liver function	• Elevated blood concentrations of liver transaminases to twice normal concentration
Pulmonary edema	
Cerebral or visual symptoms	

* Each measured as mg/dL.

Risk factors: The risk factors for pre-eclampsia according to ACOG practice bulletin[4] are listed in table 3.2.

Table 3.2. Risk factors for pre-eclampsia[4]

	Risk factors
•	Chronic hypertension
•	Chronic renal disease
•	Diabetes Mellitus
•	Obesity
•	Primiparity
•	Multifetal pregnancy
•	In vitro fertilization
•	Advanced maternal age (older than 40 years)

Pathophysiology of Preeclampsia:

Although the etiology of preeclampsia is not clear, placental insufficiency due to inadequate remodeling of the maternal vasculature which perfuses the intervillous space plays an important role in the development of this disease. This may lead to a complex process of

ischemia-reperfusion in the placenta with the release of cytotoxic factors into the maternal circulation[5]. The placental hypoxia/reoxygenation phenomenon is linked to an imbalance in angiogenesis, vascular endothelium damage and cardiovascular complications. This phenomenon also increases the oxidative stress, which has ill consequences on the health of the mother and the fetus[6].

Pre-eclampsia has been explained as a two-stage disease with an imbalance between angiogenic and anti-angiogenic factors. The central hypothesis is that pre-eclampsia results from defective spiral artery remodeling, leading to cellular ischemia in the placenta, which in turn results in an imbalance between anti-angiogenic and pro-angiogenic factors. This imbalance in favor of anti-angiogenic factors leads to widespread endothelial dysfunction, affecting all the maternal organ systems. In addition, there is fetal growth restriction (FGR)[7].

The etio-pathogenesis of pre-eclampsia still remains a mystery, despite enormous research in this field. Evidence indicates that hypertension and proteinuria, which form the pillars to the diagnosis of pre-eclampsia, have several underlying causes, especially endothelial dysfunction and cardiovascular and renal pathology. But, the treatment of pre-eclampsia has not changed much in the past 50 years[8-13].

Pre-eclampsia is characterized by a rise in systemic vascular resistance with decrease in cardiac output and blood volume, combined with proteinuria. Although, there is a decrease in blood volume or hypovolemia, there is suppression of renin–angiotensin system (RAS) and also aldosterone secretion. This may suggest that the RAS may not be the cause of hypertension in PE or rather hypertension may lead to the suppression of RAS. Abnormal placentation early in pregnancy may result in the release of anti-angiogenic factors and cytokines, leading to vascular dysfunction. Elevated anti-angiogenic factors like sFlt-1 bind and inactivate vascular endothelial growth factor (VEGF). Research tells us that VEGF inhibition with drugs like sunitinib (anti-cancer drug) results in PE-like syndrome,

characterized by hypertension, proteinuria and renal damage. In such a condition, rise in endothelin-1 levels are also seen. Endothelin-1 is found to be an independent determinant of the hypertension and proteinuria in PE, and also a suppressor of renin. Studies in animal models of PE have shown that blockers of endothelin receptors prevent the development of this disease. Hence, endothelin system may be an important pathway leading to the clinical manifestations of PE[14].

Placenta is found to play an important part in the pathophysiology of preeclampsia. Abnormal cytotrophoblast invasion of spiral arterioles results in reduced uteroplacental perfusion, which triggers a cascade of events leading to this disorder. Soluble placental anti-angiogenic or pro-inflammatory factors are released due to placental ischemia. These factors reach the maternal circulation and cause dysfunction of the maternal vascular endothelium which results in increase in the formation of endothelin-1 and superoxide, increase in vascular sensitivity to angiotensin II and decrease in the formation of vasodilators such as nitric oxide[15].

As a result of increased vascular resistance and endothelial dysfunction in the mother, there is increased release of sFlt-1 from placenta, which decreases VEGF receptors and also of endothelial nitric oxide synthase. This affects the endothelial function and also diastolic relaxation. Cardiac function may be affected by its hypertrophy. Podocyte injury of glomerular cells and increased excretion of podocyte specific proteins in urine leads to proteinuria. Liver function may also be affected; sFlt-1 may cause a disturbance in thyroid hormone function too during or after pregnancy[16].

As pre-eclampsia has a great impact on maternal and perinatal morbidity and mortality, prediction of pre-eclampsia has been given great importance, which may depict the degree of abnormal placentation, endothelial dysfunction and feto-maternal unit perfusion. Various biomarkers help in early detection of the disease. Reduced maternal serum levels of PAPP-A and PP13 in early pregnancy may predict emerging pre-eclampsia. Also, increased levels of

homocysteine, ADMA, sEng, leptin and sFlt-1, in the first trimester, signal the onset of the disease, later in pregnancy. Higher levels of PAPP-A, ADMA, homocysteine and sFlt-1 in serum are associated with the severity of pre-eclampsia. Identification of such biomarkers may help in better surveillance and treatment of patients of pre-eclampsia[17].

Some of the important factors involved in the development of pre-eclampsia are immune maladaptation, inadequate trophoblast invasion, placental ischemia, oxidative stress and thrombosis. All these factors are genetically influenced and hence, the familial nature of pre-eclampsia is much evident. Therefore, extensive genetic research in the form of candidate gene studies and linkage analysis, interactions between fetal and maternal genotypes, the effect of environmental factors are being carried out and the results well documented[18].

Considering the above factors, Figure 3.1 may depict the possible pathways leading to the pathogenesis of pre-eclampsia[19]

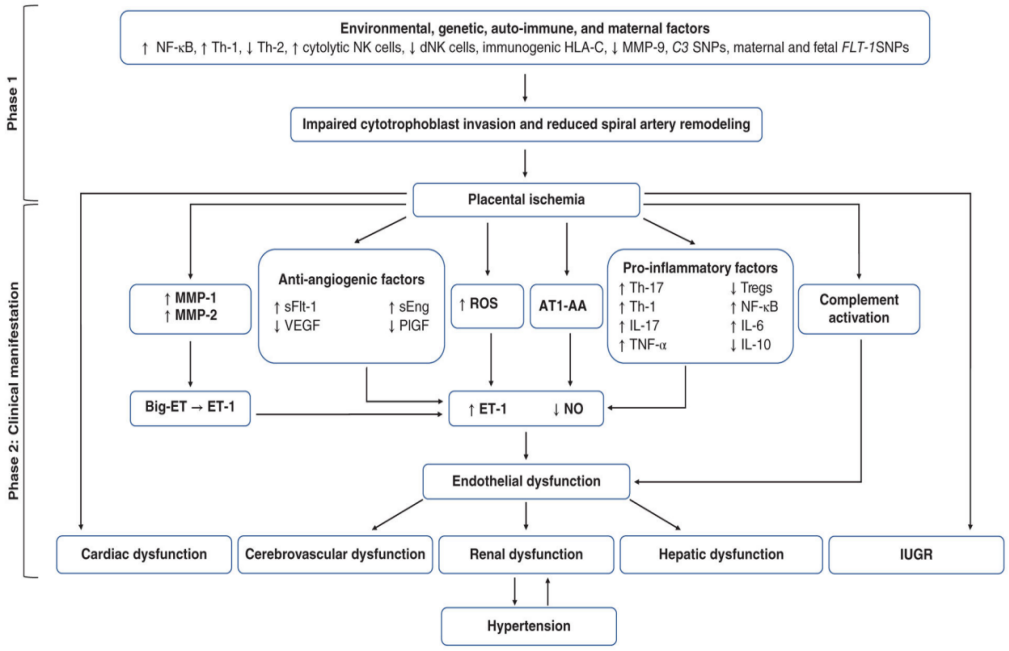

Figure 3.1. Possible pathways in the pathogenesis of pre-eclampsia[19]

NF-κB, nuclear factor-kappa B; Th, T helper cell; NK, natural killer cell; dNK, decidual natural killer cell; HLA-C, human leukocyte antigen-C; MMP, matrix metalloproteinases; C3, complement 3; SNPs, single nucleotide polymorphisms; FLT-1, fms-like tyrosine kinase-1; sFlt-1, soluble Flt-1; sEng, soluble endoglin; VEGF, vascular endothelial growth factor; ROS, reactive oxygen species; AT1-AA, angiotensin II Type 1 receptor autoantibody; PlGF, placental growth factor; Tregs, T regulatory cells; IL, interleukin; TNF-α, tumor necrosis factor-α; ET, endothelin; NO, nitric oxide; IUGR, intrauterine growth restriction.

Cardiovascular changes:

Maternal heart rate increases by 20% and cardiac output increases by 30%–40%, to compensate for the drop in systemic vascular resistance and also to increase the uterine artery blood flow in a healthy pregnancy. Vasodilation of maternal systemic vasculature is seen as early as around 5 weeks, in normal pregnancy. This is followed by full placentation and development of the circulation in placenta and uterus. A decrease in mean arterial pressure is seen during pregnancy. The arterial pressures decrease very much in the second trimester, but much of the decrease is seen early in pregnancy. As these changes occur very early in pregnancy, the importance of comparing hemodynamic measurements with preconception values rather than early pregnancy values is emphasized. Arterial pressures increase during the third trimester and assume their preconception levels by the end of postpartum period[20-22]. But, impaired spiral artery remodeling of placenta in pre-eclampsia leads to placental ischemia, which causes release of antiangiogenic and pro-inflammatory factors into maternal blood. These factors, in turn, cause endothelial dysfunction and vasoconstriction, which increases the arterial blood pressures in pre-eclampsia[19].

Heart rate variability (HRV) analysis: The basis for the HRV analysis is the RR time interval obtained from electrocardiogram (ECG) records. The RR interval is the time difference between two consecutive R peaks of the ECG wave complex, from which the heart rate can be obtained. The normal RR variability interval, and hence the heart rate is not stationary, which means, RR intervals or heart rate changes or varies across time due to several reasons. The sequences of these consecutives time differences (variability in the heart rate) generate a time series with several interesting properties. Heart rate variability (HRV) is mainly influenced by the interaction sympathetic and parasympathetic branches of ANS[23,24]. Analysis of HRV may be done using the time-domain indices of HRV (Table 3.3) or the frequency-domain indices of HRV (Table 3.4)[24,25].

Table 3.3. Time-domain indices of heart rate variability[24,25]

Parameter	Unit	Description
SDNN	ms	Standard deviation of NN intervals
SDRR	ms	Standard deviation of RR intervals
SDANN	ms	Standard deviation of the average NN intervals for each 5 min segment of a 24 h HRV recording
SDNN index (SDNNI)	ms	Mean of the standard deviations of all the NN intervals for each 5 min segment of a 24 h HRV recording
pNN50	%	Percentage of successive RR intervals that differ by more than 50 ms
HR Max – HR Min	bpm	Average difference between the highest and lowest heart rates during each respiratory cycle
RMSSD	ms	Root mean square of successive RR interval differences
HRV triangular index		Integral of the density of the RR interval histogram divided by its height
TINN	ms	Baseline width of the RR interval histogram

Interbeat interval, time interval between successive heartbeats; NN intervals, interbeat intervals from which artifacts have been removed; RR intervals, interbeat intervals between all successive heartbeats.

Table 3.4. Frequency-domain indices of heart rate variability[24,25]

Parameter	Unit	Description
ULF power	ms^2	Absolute power of the ultra-low-frequency band (≤0.003 Hz)
VLF power	ms^2	Absolute power of the very-low-frequency band (0.0033–0.04 Hz)
LF peak	Hz	Peak frequency of the low-frequency band (0.04–0.15 Hz)
LF power	ms^2	Absolute power of the low-frequency band (0.04–0.15 Hz)
LF power	nu	Relative power of the low-frequency band (0.04–0.15 Hz) in normal units
LF power	%	Relative power of the low-frequency band (0.04–0.15 Hz)
HF peak	Hz	Peak frequency of the high-frequency band (0.15–0.4 Hz)
HF power	ms^2	Absolute power of the high-frequency band (0.15–0.4 Hz)
HF power	nu	Relative power of the high-frequency band (0.15–0.4 Hz) in normal units
HF power	%	Relative power of the high-frequency band (0.15–0.4 Hz)
LF/HF	%	Ratio of LF-to-HF power

Autonomic nervous system governs the functioning of the cardiovascular system, which in turn has implications on cardiovascular health. Heart rate variability (HRV) is a measurable reflection of the balance between sympathetic and parasympathetic tone and may be used to

predict the diseases of cardiovascular system[26].

High levels of systolic as well as diastolic blood pressures in pre-eclampsia result from elevation of both peripheral resistance and sympathetic activity. Diastolic blood pressure is influenced by peripheral resistance, whereas systolic blood pressure is a direct function of cardiac output. The sympathetic nervous system (SNS) along with the parasympathetic nervous system (PNS) work unconsciously in opposite ways to regulate many functions of the body. The sympathetic nervous system is important for the regulation of arterial pressure, and its enhanced activity has been implicated in the causation of hypertension. Overactivation of the sympathetic nervous activity (SNA) leads to development of PE, but the exact mechanisms are unknown. Exaggerations in SNA and the inability of the uterine circulation to compensate for this could lead to the development of placental ischemia/reperfusion in PE. Placental ischemia and hypoxia result in the release of pro-hypertensive placental factors into the mother's circulation and hence, may trigger the onset of hypertension. These factors may induce hypertension by stimulating sympathetic nervous system and increasing its activity[27].

The increased SNA in PE can be noticed in the frequency domain parameters of heart rate variability (HRV), namely the LF component[28].

Oxidative stress:

An imbalance between the generation of reactive nitrogen species (ROS) or reactive nitrogen species (RNS) and the cellular antioxidant capacity is termed as oxidative stress. ROS include free radicals, such as hydroxyl radicals (OH^-), superoxide (O_2^-), and non-radicals, like hydrogen peroxide (H_2O_2) and singlet oxygen $^1O_2$. RNS include the nitric oxide (NO) and its derivative peroxynitrite (ONOO−). These radicals are part of normal cellular metabolism produced by enzymatic and non-enzymatic reactions taking place in the mitochondria predominantly[29].

Oxidative stress increases with high levels of ROS, resulting from a normal systemic

inflammatory response during normal pregnancy [30].

The major source of ROS during pregnancy is the placenta, which is the central organ that regulates it. Excessive oxidative stress in pregnancy could potentially damage the tissues (Figure 3.2)[31,32].

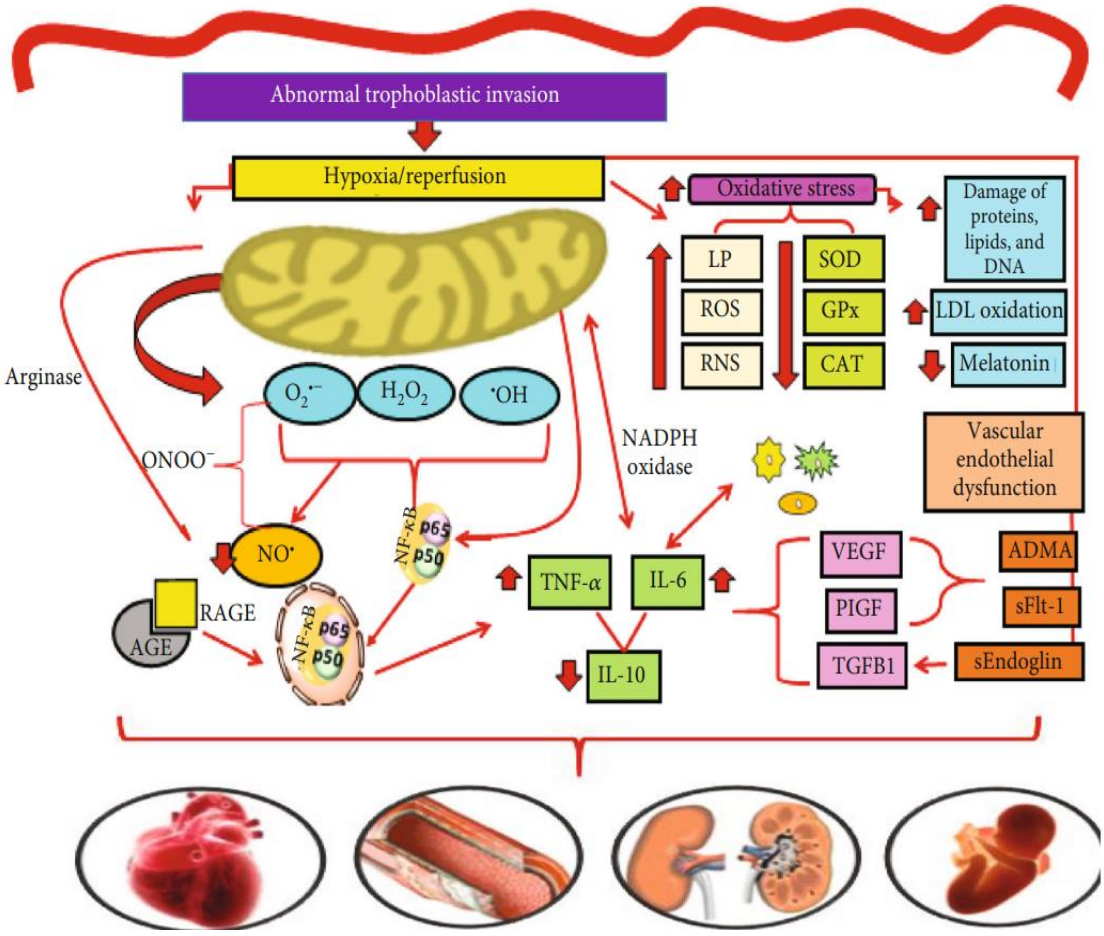

Figure 3.2. Oxidative stress and its consequences in pre-eclampsia[32]

ADMA: asymmetric dimethylarginine; AGE: advanced glycation end products; CAT: catalase; NADPH oxidase: nicotinamide adenine dinucleotide phosphate oxidase; IL: interleukin; VEGF: vascular endothelial growth; LP: lipid peroxides; ONOO− : peroxynitrite; SOD: superoxide dismutase; RNS: reactive nitrogen species; ROS: reactive oxygen species; NF-κB: nuclear factor kappa B; GPx: glutathione peroxidase; PIGF: placental growth factor; RAGE: advanced glycation end product receptors; sFlt-1: soluble Fms-like receptor tyrosine kinase; TGFB1: transforming growth factor beta; TNF-α: tumor necrosis factor alpha.

Increased oxidative stress is counter-balanced by an increase in antioxidants. But, when the oxidative stress exceeds the antioxidant defense in the placenta, the oxidative damage is transferred to the distal tissues[33].

Lipid peroxidation may result in free radical-mediated cytotoxicity and hence, lipid peroxide may be used as a marker of free radical-mediated cell / tissue injury. The initial oxidation of only a few lipid molecules can result in significant tissue damage, as lipid peroxidation is a self-propagating chain reaction[34].

Lipid peroxides in preeclampsia may arise from poorly perfused placental tissue, which may start-up the free radical formation and also generalized lipid peroxidation. Measurement of the lipid peroxides is difficult and therefore, malondialdehyde (MDA) is frequently analyzed as one of the end products of the lipid peroxidation[35].

Neutrophils are important in the eradication of invasive pathogens, but at the same time, they are also implicated in the inflammation and tissue damage seen in some cases of pregnancy[36]. Successful pregnancy demands a woman to have good immune-system adaptation so as to tolerate a fetus that is foreign to her. Normal pregnancy causes neutrophil activation and an increase in the number of granulocytes. The surface expression of adhesion molecules on granulocytes and also the intracellular reactive oxygen species in granulocytes were increased. Soluble factors, generated by placenta, may be responsible for such neutrophil activation[37,38].

The total leukocyte increase in PE is mainly due to neutrophil increase and not monocyte or lymphocyte. The increase in neutrophils is observed only in severe PE and not in mild PE or normal pregnancy. So, an increased neutrophil count may be associated with severity of the disease. [39,40].

Oxidative stress is not only associated with the pathogenesis of pre-eclampsia, but is also implicated in complications affecting the preterm infants such as intraventricular hemorrhage, respiratory distress syndrome, necrotizing enterocolitis and retinopathy of prematurity. Hence, dealing with oxidative stress becomes important in reducing the risk of preeclampsia in pregnant women and also preventing complications in the neonates[41].

Renal dysfunction:

Urinary protein excretion increases in normal pregnancy and protein excretion exceeding 300 mg in a 24 h urine collection is considered abnormal in pregnant women. Proteinuria may be regarded as one of the cardinal features of preeclampsia, although it is not included in the currently recommended diagnostic criteria of preeclampsia. Clinicians commonly use proteinuria levels to make clinical decisions regarding delivery of preeclamptic cases. It is seen that increased proteinuria worsens preeclampsia and is associated with poor perinatal outcomes too. NICE guidelines have not recommended repeating and following up of proteinuria once proteinuria is detected. ACOG also does not recommend requiring proteinuria for the diagnosis of preeclampsia if other severe preeclampsia features are present such as liver dysfunction and lower platelet count[4,42].

Excretion of ≥300 mg of protein in a 24-h urine specimen is considered as significant proteinuria. Therefore, a 24-h urine sample is the gold standard for diagnosis of significant proteinuria[43]. But, as it has been proved that the urine protein: creatinine ratio (UPCR) is highly predictive of proteinuria in preeclamptic patients, it may be used in order to hasten the treatment[44]. A quantitative analysis of a random urine sample for UPCR is better than the usage of dipsticks, and helps in proper diagnosis and treatment[45].

The intrarenal renin-angiotensin system (RAS) has functions of regulating blood pressure, renal cell growth, and hence, its derangement would affect the renal functions eventually. Levels of angiotensinogen (AGT) may depict the status of RAS as the sole precursor of all angiotensin peptides is AGT[46].

Glomerular endotheliosis signifies renal dysfunction and proteinuria, and they usually resolve after delivery of the fetus and placenta[47].

Endothelial dysfunction and nitric oxide: Endothelial cells of the blood vessels play critical role in cardiovascular homeostasis by regulating vascular tone, angiogenesis,

monocyte/leukocyte adhesion, platelet aggregation, blood fluidity and fibrinolysis. Vascular endothelium acts as a gatekeeper of cardiovascular health. Hence, its abnormality leads to cardiovascular diseases such as hypertension, atherosclerosis and myocardial infarction. Endothelial dysfunction may lead to improper vasodilation and vasoconstriction, high levels of ROS and proinflammatory factors, and also reduced NO bioavailability. Endothelial dysfunction disrupts the endothelial permeability as a part of the inflammatory response and results in cardiovascular diseases[48,49].

The vascular smooth muscle relaxant, nitric oxide, regulates blood flow and plays a vital role in the cardiovascular changes in pregnancy. Nitric oxide regulates feto-placental vascular permeability, vascular resistance and also platelet aggregation in the placenta. Hence, it is also implicated in abnormal feto-maternal vascular adaptations, such as in preeclampsia. Although the beneficial effects of nitric oxide in pregnancy are well-known, the serum levels of nitric oxide show high variability in pregnancy[32].

Research in animal models has shown that the reproductive tissues and blood vessels are more responsive to NO. Pregnancy is also associated with enhanced production of NO. Animal model of pre-eclampsia is developed in rats by infusion of A, a NO synthase inhibitor. In these pre-eclampsia models, there exists a state of NO deficiency. But, in humans, there are contradictions about the role of NO in maternal adaptation to pregnancy. NO may be one of several systems that act together to maintain a healthy adaptation in the mother. The functioning of each system may be determined genetically[50-57].

Research has thrown light on the primary and secondary roles of NO in the causation of preeclampsia. Few authors have reported the correlation between nitric oxide production dysfunction and various derangements seen in this disorder. A reduced bioavailability of NO and an excess of peroxynitrite is the primary dysfunction seen in pre-eclampsia[32] (Figure 3.3). An increase in $ONOO^-$ together with deficiency of NO initiate the changes seen in

preeclampsia, like the high blood pressure and proteinuria. Previous unsuccessful interventions point at the complexity of understanding the role of nitric oxide and help in formulating treatment strategies in the future[58].

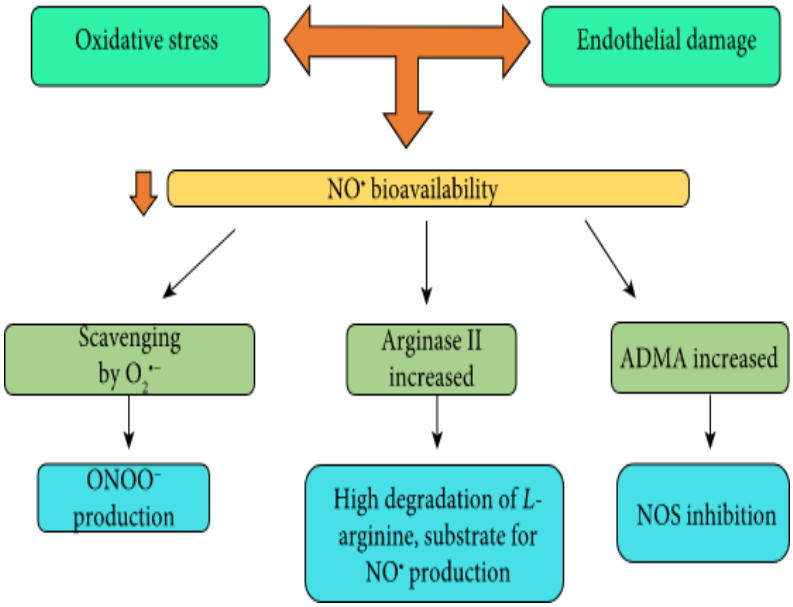

Figure 3.3. Reduced bioavailability of nitric oxide (NO) in pre-eclampsia[32]

Nitric oxide also suppresses proliferation of vascular smooth muscle cells. Nitric oxide synthases (NOSs) are extremely important for understanding of the pathophysiological mechanisms and also to plan therapeutic intervention[59].

Decreased production of NO by NO synthase or increased breakdown of NO may lead to diseases like hypertension, ischemic diseases, pre-eclampsia, premature delivery, and many others. Treatment with nitric oxide donors are tested in such conditions[60].

An increase in VEGF, asymmetric dimethylarginine (ADMA) and nitrite levels was observed in preeclampsia patients in a study when the authors attempted to compare these levels with those of normal pregnant women[61].

The nitric-oxide (NO) signaling cascade plays a vital role in the development of vascular network, in maintaining vascular tone and also maintaining normal placental function. A number of hormonal signals regulate this. Evidence suggests that the pathology of abnormal placental function involves specific molecules like hormone receptors, which could influence

NO release and later may have greater problems[62].

Pre-eclampsia and placental microvascular diseases may be associated with hyperhomocysteinemia (HHcy), a vascular risk factor. HHcy leads to accumulation of ADMA, which is an endogenous inhibitor of endothelial nitric oxide synthase (eNOS), which in turn causes endothelial dysfunction. Pre-eclampsia is marked by elevated maternal ADMA. In a study by Demir et al, it was noted that the levels of systolic and diastolic blood pressures, serum ADMA and homocysteine were significantly higher whereas levels of nitric oxide were lower in pre-eclampsia[63].

Levels of ADMA are elevated in cardiovascular and metabolic diseases, which act as a prognostic marker for major cardiovascular diseases. Elevated levels of ADMA are seen in early pregnancy who later develop preeclampsia. ADMA is also linked with uterine artery flow disturbances. The plasma concentration of ADMA is regulated by dimethylarginine dimethylaminohydrolase (DDAH). In preeclampsia, single nucleotide polymorphisms in the gene encoding for DDAH are identified. ADMA may be looked upon as a possible biomarker for early diagnosis preeclampsia[64].

Nitric oxide synthase, genetic variation and its expression in pre-eclampsia:

In a study, the pattern of nitric oxide biosynthesis was studied in normal and pre-eclamptic women at delivery. Plasma cGMP was elevated in both normal pregnant and pre-eclamptic women than in non-pregnant controls. cGMP levels were raised as early as 18–21 weeks and remained risen throughout pregnancy. Gene expression of EC-NOS and nitric oxide synthesis were found similar in HUVEC and in placenta from normal pregnancy and pre-eclampsia[65].

Impaired remodeling of uterine spiral arteries along with defective trophoblastic invasion causes abnormal placentation in pre-eclampsia, that leads to a poor adaptation of utero-placental circulation. This causes episodes of hypoxia/reoxygenation, that may lead to oxidative stress and resultant reduced nitric oxide bioavailability. This may be due to reaction

of NO with superoxide, that leads to production of peroxynitrite, which is a strong pro-oxidant as well as inflammatory agent. Oxidative stress leads to inhibition of placental eNOS and its uncoupling. This may be due to reduced levels of L-arginine due to enhanced arginase activity or tetrahydrobiopterin oxidation or post-translational modification of eNOS. This could further increase the oxidative stress by a NADPH oxidase-like system generating $O_2^{\bullet-}$, due to uncoupling of eNOS. (Figure 3.4)[52,66].

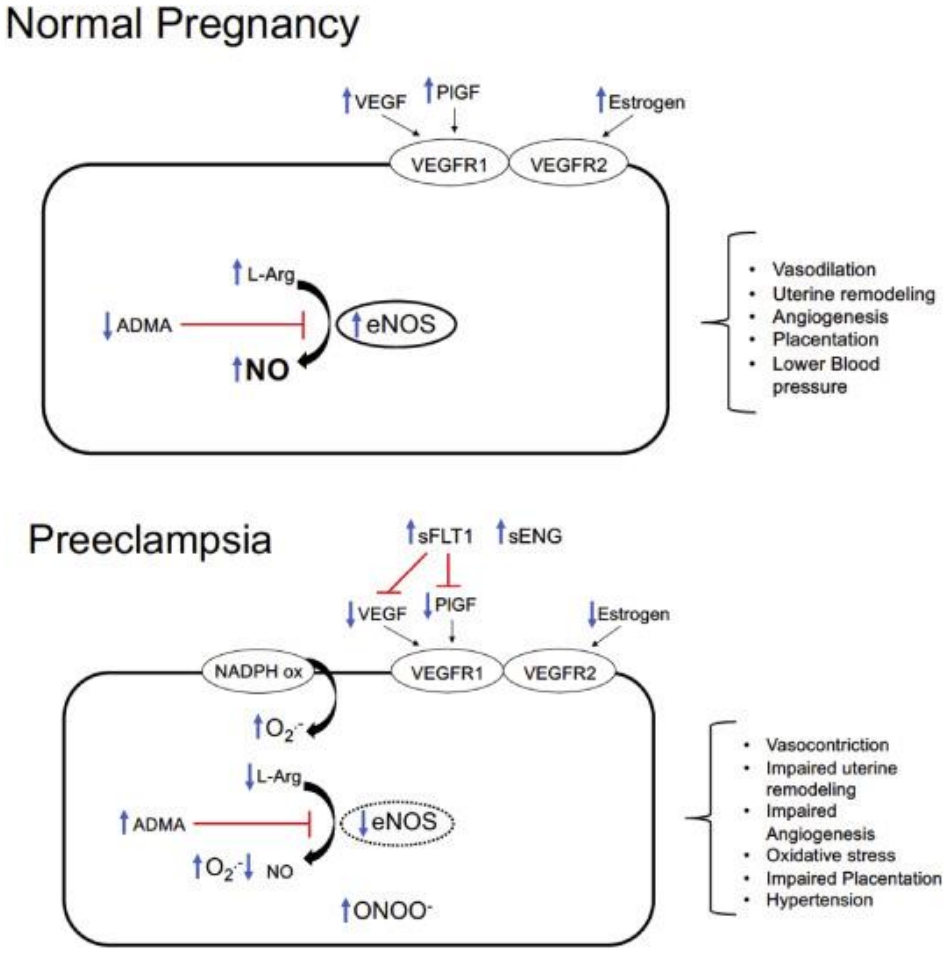

Figure 3.4. Nitric oxide signaling in normal pregnancy and in preeclampsia[52]
NO= nitric oxide, L-Arg= L-arginine, eNOS= endothelial nitric oxide synthase, VEGF= vascular endothelial growth factor, VEGFR1 & 2= vascular endothelial growth factor receptor 1 & 2, ADMA= asymmetric dimethylarginine, PlGF= placental growth factor, sFLT1= soluble fms-like tyrosine kinase 1 or soluble vascular endothelial growth factor receptor 1, sENG= soluble endoglin, O·−2= superoxide, ONOO− = peroxynitrite, NADPH ox= NADPH oxidase,.

Lipid peroxidation derived aldehydes could modify eNOS post-translationally, which would reduce NO production in pre-eclampsia[67].

Risk of preeclampsia may be affected by polymorphisms in endothelial nitric oxide synthase

(eNOS) gene. The association of eNOS G894T, T786C, and intron 4b/a polymorphisms with preeclampsia was studied using allele contrast, recessive, dominant and additive models. A significant association between the G894T variant and increased risk of preeclampsia was observed, but, there was no significant risk of preeclampsia either in T786C or intron 4b/a polymorphism. A significant association was also observed with Homozygosity TT in eNOS G894T variant which increased the risk of preeclampsia[68].

Nitric Oxide Synthase (NOS) enzyme synthesizes NO from L-arginine. NOS can exist either as a calcium-dependent or a calcium-independent isoform. Placental villi has both isoforms. In one of the studies, higher activity of NOS was seen in placental villi during first trimester than at term, but there was an impairment of NO metabolism in pre-eclamptic and growth-retarded pregnancies. Reduced activity of NOS was associated with smoking, and hence, problems of smoking during pregnancy may be attributed to NO metabolism[69].

The association of cardiovascular disease with endothelial nitric oxide synthase gene polymorphisms was studied in another study. The common variant encountered was Glu298Asp polymorphism within exon 7. These variants were associated with low plasma NO concentrations and low vascular reactivity[70].

The most functionally related polymorphisms of eNOS that are studied are intron 4 variable number tandem repeat, Glu298Asp (rs1799983) and −786T/C (rs2070744). These polymorphisms tend to increase the risk of cardiovascular disease, such as coronary artery disease, stroke, hypertension and pre-eclampsia[71].

Polymorphisms in eNOS gene might reduce the nitric oxide bioavailability and predispose to PE. This study by Fatini investigated the role of eNOS T-786C, G894T and 4a4b polymorphisms in the causation of pre-eclampsia and the recurrence of negative pregnancy events. The authors also studied the influence of angiotensin-converting enzyme (ACE) DD genotype on maternal–fetal blood flow. The risk of recurrence of negative events was

influenced by eNOS 894TT genotype, particularly in contemporary women homozygous for both *eNOS* 894TT and *ACE* DD genotypes. A progressive alteration of maternal–fetal flow indices were found in women carrying *eNOS* 894TT genotype throughout the pregnancy; this effect was seen more in women with *ACE* DD genotype[72].

In another study, the localization and intensity of eNOS was determined by immunohistochemistry. eNOS mRNA expression was determined by reverse transcription–polymerase chain reaction (RT-PCR) and the housekeeping gene used was β_2-microglobulin. Syncytiotrophoblast cells within the villi and decidual trophoblast cells showed staining of endothelial NOS, but the same was not seen in endothelium of terminal villous vessels. This study suggests that the amount of eNOS in the placenta was not deficient in pre-eclampsia, but eNOS may have a pathogenic role to play in this disease. Placental hypoxia, in this disease, did not lead to an upregulation of eNOS[73].

Genes modulating placental vascular development, blood pressure and fluid homeostasis may be involved in the etiopathogenesis of preeclampsia. Some of them are the angiotensinogen (*AGT*) gene variant Met235Threo and the endothelial nitric oxide synthase (*eNOS*) polymorphism Glu298Asp, which have been associated with pre-eclampsia. But, in this study, there was no association between the Met235Threo variant of the *AGT* gene and pre-eclampsia. However, the combined frequency of the *eNOS* variant genotypes (GT and TT) was significantly higher in the abruptio placentae (complication of pre-eclampsia) group than the control group. The *eNOS* GT genotype was found to be a greater risk factor for abruptio placentae development in patients of pre-eclampsia. These findings inform us that the presence of a Glu298Asp *eNOS* variant may lead to abruptio placentae in pre-eclamptic woman and may be used as a marker for its predisposition[61,74,75-81].

In a study conducted by Xiang et al, the expression of eNOS traffic inducer (NOSTRIN) was examined in the umbilical vessels of the patients with pre-eclampsia. Expression level of

NOSTRIN was significantly higher and eNos activity was reduced significantly in women with PE as compared to the normal group. Levels of NO2 −/NO3 − in PE patients were also significantly lower. There was a negative correlation between the expression of NOSTRIN and eNOS activity in PE[82].

eNOS Asp298 polymorphism influences the blood pressure response to exercise. Endothelial function is also affected by this variant and this becomes evident during vascular adaptation in pregnancy. Pre-eclampsia is more likely to occur in carriers of eNOS Asp298, more so, when their endothelium is exposed to adverse environmental influences. They may also develop atherosclerosis and cerebrovascular disease[83].

Association between inducible nitric oxide synthase (iNOS) genotypes and pre-eclampsia was studied. Four single nucleotide polymorphisms (SNPs) were observed, two of which, showed significant association with pre-eclampsia. G274T exon 16 SNP showed association with TNF-α levels and SOD activity too. Patients with pre-eclampsia, at 36 weeks of gestation, showed significantly increased serum NO levels, whereas SOD levels were decreased. A double-fold increase in TNF-α levels was also observed in them at 36 weeks, which later decreased significantly after delivery. All the above factors might play an intermingled role in the development of PE[84].

This study examined the effects of iNOS inhibitor, 1400 W, on the reduced uteroplacental perfusion pressure (RUPP). Study was done in placental ischemic animal models and in normal pregnant rats. Subcutaneously, N-[3-(Aminomethyl) benzyl] acetamidine was given in sham-operated and RUPP rats. Aortic reactive oxygen species (ROS) levels, nicotinamide adenine dinucleotide phosphate (NADPH)-dependent ROS production and plasma 8-isoprostane levels were evaluated. In RUPP rats, the mean arterial pressure increased by ~30 mmHg, and 1400 W attenuated this increase by ~50%. RUPP increased the levels of aortic ROS levels, NADPH-dependent ROS production and plasma 8-isoprostane levels. But,

treatment with 1400 W blunted these changes. RUPP also increased iNOS expression and aortic nitrotyrosine levels, and again, treatment with 1400 W attenuated these changes. These findings implicate the association of iNOS with RUPP[85].

In a study by Kao, 3% normotensive (NP) or PE plasma, collected from women, were incubated overnight in pregnant rat uterine and mesenteric arteries. High levels of superoxide and impaired endothelial dysfunction was seen in uterine arteries treated with PE plasma. It was normalized in the presence of antioxidants or inhibition of PG synthesis. In the presence of pan-NOS inhibitor in both NP- and PE-treated vessels, the uterine artery vasodilation was abolished. But, iNOS-dependent vasodilation was present only in NP-treated arteries. An increased eNOS expression and a decreased iNOS expression was noticed in uterine arteries exposed to PE plasma. Endothelial function in mesenteric arteries was not altered indicating the vascular-bed-specificity of the circulating factors[86].

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MATERIAL & METHODS

Role of nitric oxide synthase 3 (NOS3) gene expression in patients of preeclampsia with special reference to cardiovascular and renal pathophysiology

CHAPTER 4.

MATERIALS AND METHODS

This case control study was conducted in pregnant primigravida women aged 18-35 years with singleton pregnancies and diagnosed to have pre-eclampsia according to American College of Obstetricians and Gynecologists (ACOG) guidelines who were admitted in the department of Obstetrics and Gynecology at HSK Hospital, Bagalkot, Karnataka, India during March-2018 to February-2020.

- **Study Design**: Case-control study
- **Study Duration:** Two years (March 2018- February 2020)
- **Source of Data:** Pregnant women from the Department of Obstetrics and Gynecology, HSK Hospital, Bagalkot,Karnataka.
- **Sample Size:** 21 in each group, calculated using Open Epi software, based on a study by Madazli et al, 2002[1] (mean±SD of MDA between the groups and smallest meaningful difference of 1 was used) and 22 in each group, based on a study by Sakar et al, 2015[2] (mean ±SD of nitric oxide levels between the groups was used). Hence, it was rounded offto 25 in each group.

Calculation of sample size:

$$n_1 = \frac{(\sigma_1^2 + \sigma_2^2 / \kappa)(z_{1-\alpha/2} + z_{1-\beta})^2}{\Delta^2}$$

$$n_2 = \frac{(\kappa * \sigma_1^2 + \sigma_2^2)(z_{1-\alpha/2} + z_{1-\beta})^2}{\Delta^2}$$

The notation for the formulae are:

n_1 = sample size of Group 1

n_2 = sample size of Group 2

σ_1 = standard deviation of Group 1

σ_2 = standard deviation of Group 2

Δ = difference in group means

κ = ratio = n_2/n_1

$Z_{1-\alpha/2}$ = two-sided Z value (eg. Z=1.96 for 95% confidence interval).

$Z_{1-\beta}$ = power

Ethical Clearance: Institutional Ethics committee clearance was obtained from Shri B. M. Patil Medical College, BLDE (Deemed to be University), Vijayapur (BLDE(DU)/IEC/2017-18/242, 2019-20/352). Clearance was also obtained from S. Nijalingappa Medical College, Bagalkot, Karnataka (SNMC/IECHSR/2017-18/A-48/1.1,1.2).

Consent: Informed written consent was obtained from the participants before enrollment.

Inclusion criteria:

Cases: Pre-eclamptic pregnant primigravida women, diagnosed by American College of Obstetricians and Gynecologists (ACOG) guidelines[3] and aged 18-35 years with singleton pregnancies were included in the study as cases.

Controls: Healthy pregnant primigravida women aged 18-35 years with singleton pregnancies, matched for age and gestational weeks with the cases were included as controls.

Exclusion criteria:

Cases: Pregnant women diagnosed to have gestational hypertension or eclampsia according to ACOG guidelines; or having diabetes mellitus past history of systemic hypertension, cardiovascular or renal diseases were excluded.

Controls: Pregnant women with past history of diabetes mellitus, systemic hypertension, pre-eclampsia, cardiovascular or renal diseases were excluded from the control group.

Methodology: The required demographic information was collected from the participants according to a pre-designed proforma. They were examined clinically and the findings noted down. First trimester ultrasonography report was used when available, for calculation of gestational age as per ACOG guidelines[4].

Cardiovascular parameters:

Blood pressure: Enrolled pregnant women were asked to rest comfortably in a quiet room for15 minutes. Blood pressure was then recorded using a mercury sphygmomanometer (Diamond) and a stethoscope (Lifeline) by auscultatory method in lying down position. The

recording was done twice, with an interval of 15 minutes between each recording. The lower of two readings were considered. Systolic as well as diastolic blood pressures were noted. Pulse pressure was noted as the difference between systolic and diastolic blood pressures. The sum of diastolic blood pressure and one-third of pulse pressure was used to compute mean arterial pressure.

Heart rate and heart rate variability (HRV) analysis: Heart rate was recorded and heart rate variability (HRV) analyzed by PowerLab data acquisition system (AD instruments). Electrocardiography electrodes of the instrument were connected in lying down position for Lead II after application of electrode gel. The recording in the PowerLab was obtained for 15 minutes. The recording was observed carefully and any ectopics and artifacts were removed. HRV analysis in the frequency and time domains were considered[5]. Frequency domain indices such as relative power of the low-frequency band (0.04–0.15 Hz) in normalized units (LFnu), relative power of the high-frequency band (0.15–0.4 Hz) in normalized units (HFnu) and ratio of LF-to-HF power (LF/HF) were noted. Time domain indices such as standard deviation of the average NN intervals for each 5 min segment (SDANN), standard deviation of normal to normal intervals (SDNN), root mean square of successive RR interval differences (RMSSD) and percentage of successive RR intervals that differ by more than 50 ms (pNN50) were noted.

Blood sampling: Blood sample was drawn under aseptic precautions from the antecubital vein. 2.5mL of whole blood was transferred into PAXgene RNA tubes for nitric oxide synthase (NOS) gene expression by RT-PCR and another 2.5 mL was converted into serum for serum nitric oxide (ELISA kit method, Bioassay technology), serum malonaldehyde (MDA, TBARS method), total antioxidant capacity (ELISA kit method, Qayee-Bio), serum proteins, serum urea, serum uric acid and serum creatinine estimation.

Urine sampling: Spot urine sample was collected, centrifuged and urine protein, urine

creatinine and urine angiotensinogen (ELISA kit method, Bioassay technology) were estimated. Urine protein/creatinine ratio and urine angiotensinogen/creatinine ratio were calculated.

Oxidative stress markers:

Serum malondialdehyde estimation[6] (MDA, TBARS method):

Method: Thiobarbituric acid reactive substances (TBARS) method

Principle: A colored complex is formed when MDA reacts with TBA. Calculation of MDA values was done from absorbance co-efficient of MDA-TBA complex at 535nm.

Reagents: Thiobarbituric acid (TBA): 75mg of TBA dissolved in 15% trichloracetic acid (TCA). To this 2.08 mL of 0.2N HCl added and volume made up to 100 mL using 15% TCA.

Procedure:

Reagents	Blank (mL)	Test (mL)
Distilled water	0.75	-
Serum	-	0.75
TBA	3.0	3.0
Keep in boiling water bath for 15 minutes; Cool and centrifuge for 10 minutes; Take supernatant and measure absorbance.		
Absorbance at 535nm		

Calculation:

Concentration of serum MDA =

$$\frac{\text{Absorbance of test}}{\text{Nanomolar extinction co-efficient}} \times \frac{\text{Total volume}}{\text{Sample volume}}$$

$$= \frac{\text{Absorbance of test}}{1.56X10^5} \times \frac{3.75}{0.75}$$

$$= \frac{\text{Absorbance of test X } 3205.1}{100}$$

= ..... nmol/mL

Serum total antioxidant capacity (ELISA kit method, Qayee-Bio):

Assay principle: A double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) – one step, is used to assay the level of Total antioxidant capacity(T-AOC) in samples. Standard, test sample and HRP-labeled Total antioxidant capacity(T-AOC) antibodies are added to enzyme wells which are pre-coated with Total antioxidant capacity(T-AOC) antibody, later incubation and wash to remove the uncombined enzyme are carried out. Chromogen solution A and B are added - the color of liquid will change into blue. Then, the reaction with the acid will cause the color to become yellow. Depth of color and the concentration of the Total antioxidant capacity(T-AOC) sample are positively correlated.

Reagent Preparation: 20 × dilution of washing buffer: distilled water, diluted by 1:20, or 1 copy of the 20 × washing buffer plus 19 copies of the distilled water.

Standard is diluted with diluents in the method of multiple proportion dilution, and the concentrations achieved are: 500、200、125、62.5、31.2、0 ng/ml

Assay Procedure:

1. Each standard and blank well was duplicated. Every sample was made according to the required quantity.

2. Standard wells, blank wells and test sample wells were set respectively: (1) Blank well: sample and horseradish peroxidase (HRP) were not added, other steps were the same. (2) Standard wells: 50μl standard was added to standard wells. (3) Test sample wells: 40μl of special diluent was added and then 10μl of sample was added. (4) 50μl of horseradish peroxidase (HRP) into each well was added, except blank well. Then the plate was sealed, and gently shook, then incubated for 60 minutes at 37 ℃.

3. Excess liquid was discarded, dried, each well was filled with diluted washing liquid, mixed and shaken for 30 seconds, the washing liquid was discarded and the plate was tapped on to the absorbent paper. The process was repeated five times, and then pat dried.

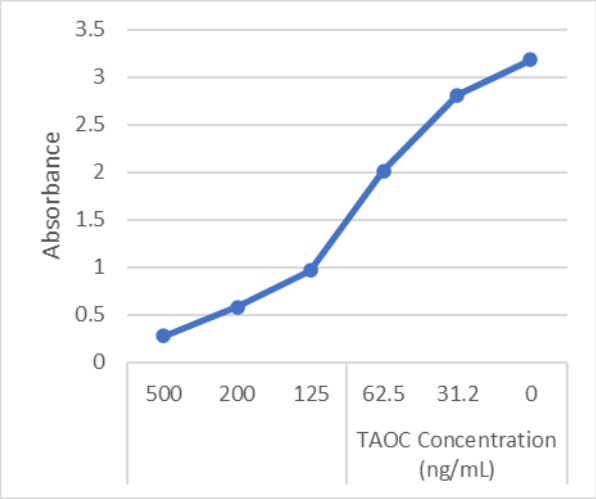
4. 50µl of chromogen solution A was added to each well, and then 50µl of chromogen solution B was added. Later, it was gently shaken and incubated for 10 minutes at 37℃ away from the light.

5. Stop Solution 50µl was added into each well to stop the reaction (the blue changes into yellow immediately).

6. Final measurement: Blank well was set at zero, the optical density (OD) was measured at 450 nm wavelength within 15 minutes after adding the stop solution.

7. According to standards' concentration and the corresponding OD values, the standard curve linear regression equation was calculated, and then the OD values of the sample were applied on the regression equation to calculate the corresponding sample's concentration.

Determining the Results: 1. The standard curve as used to determine the contents in an unknown sample. The standard curve was obtained by plotting the average OD (450 nm) obtained for each of the six standard concentrations on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis. 2. The OD value on the Y-axis was located and a horizontal line was extended to the standard curve in order to determine the amount in each sample. At the point of intersection, a vertical line was drawn to the X-axis and the corresponding concentration was read.



Renal functions:

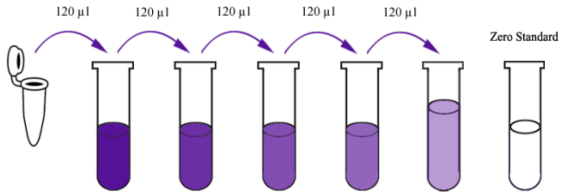
Serum and urine proteins (Bromcresol green method), Serum urea (Urease method), Serum uric acid (Uricase method), Serum and urine creatinine (Jaffe's method), and Urine proteins (Pyregolol method) were estimated by Biosystems BA 400 fully automated instrument with quality control check.

Urine angiotensinogen (ELISA kit method, Bioassay technology)

Assay Principle: This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human aGT antibody. aGT present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human aGT Antibody is added which binds to aGT in the sample. Then, Streptavidin-HRP is added and binds to the Biotinylated aGT antibody. Unbound Streptavidin-HRP is washed away during a washing step after incubation. Color develops in proportion to the amount of Human aGT after substrate solution has been added. Addition of acidic stop solution terminates the reaction. Absorbance is immediately measured at 450 nm.

Reagent Preparation: Room temperature was achieved for all the reagents. 120µl of the standard (1440ng/L) was reconstituted with 120µl of standard diluent to generate 720ng/L standard stock solution. Gentle agitation for 15 minutes was carried out for the standard to sit. Duplicate standard points were prepared by serially diluting the standard stock solution (720ng/L) 1:2 with standard diluent. Concentrations thus produced were 360ng/L, 180ng/L, 90ng/L and 45ng/L solutions. Dilution of standard solutions suggested were as follows:

720ng/L	Standard No.5	120µl Original Standard + 120µl Standard Diluent
360ng/L	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
180ng/L	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
90ng/L	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
45.ng/L	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
1440ng/L	720ng/L	360ng/L	180ng/L	90ng/L	45ng/L

- Wash Buffer: 20ml of Wash Buffer Concentrate was diluted to 30x into deionized or distilled water to yield 500 ml of 1x Wash Buffer.

Assay Procedure:

1. All reagents, standard solutions and samples were prepared. The reagents were brought to room temperature before use.

2. Strips were inserted in the frames for use.

3. 50µl standard was added to standard well.

4. 40µl sample was added to sample wells and then 10µl anti-aGT antibody was added to sample wells. 50µl streptavidin-HRP was added to sample wells and standard wells. Solutions were mixed well. The plate was covered with a sealer and incubated for 60 minutes at 37°C.

5. Sealer was removed and plate was washed 5 times with wash buffer. Wells were soaked with 0.35ml wash buffer for 30 seconds to 1 minute during each wash. The plate was blotted onto paper towels.

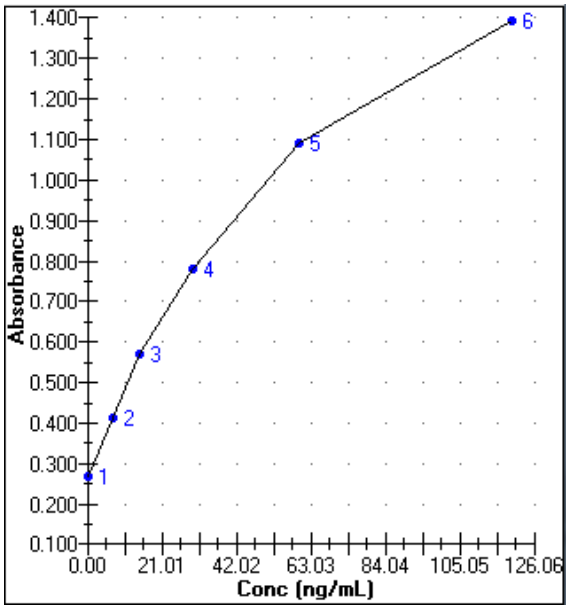
6. 50µl substrate solution A was added to each well and 50µl substrate solution B was added to each well. Plate was incubated in the dark, after covering with a new sealer for 10 minutes at 37°C.

7. 50µl of stop solution was put into each well. The blue color changed into yellow immediately.

8. Optical density (OD) value was determined within 10 minutes using a microplate reader at 450 nm.

Calculation of Result: A standard curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and a best fit curve was drawn through the points on the graph.

Calibration curve



Serum nitric oxide (ELISA kit method, Bioassay technology):

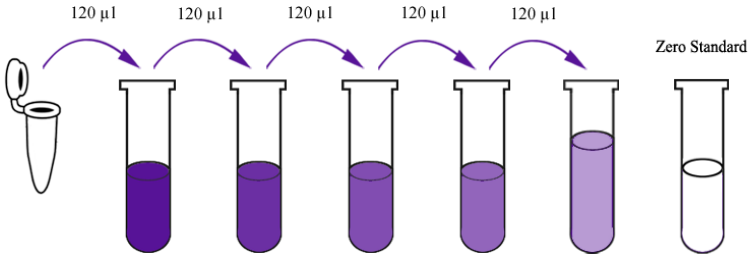
Assay Principle: This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with human NO antibody. NO present in the sample is added and binds to antibodies coated on the wells. And then biotinylated human NO Antibody is added and binds to NO in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated NO antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of human NO. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

Reagent Preparation

- Room temperature was achieved for all reagents before use.
- 120µl of the standard (640µmol/L) was reconstituted with 120µl of standard diluent to generate a 320µmol/L standard stock solution. Standard was allowed to sit for 15 mins with gentle agitation. Duplicate standard points were prepared by serially diluting the standard stock solution (320µmol/L) 1:2 with standard diluent to produce 160µmol/L, 80µmol/L, 40µmol/L and 20µmol/L solutions. Dilution of standard solutions suggested were as follows:

320µmol/L	Standard No.5	120µl Original Standard + 120µl Standard Diluent
160µmol/L	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
80µmol/L	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
40µmol/L	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
20µmol/L	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent

Standard	Standard	Standard	Standard	Standard	Standard
640µmol/L	320µmol/	160µmol/L	80µmol/L	40µmol/L	20µmol/L



- **Wash Buffer** 20ml of Wash Buffer Concentrate was diluted 30x into deionized or distilled water to yield 500 ml of 1x Wash Buffer.

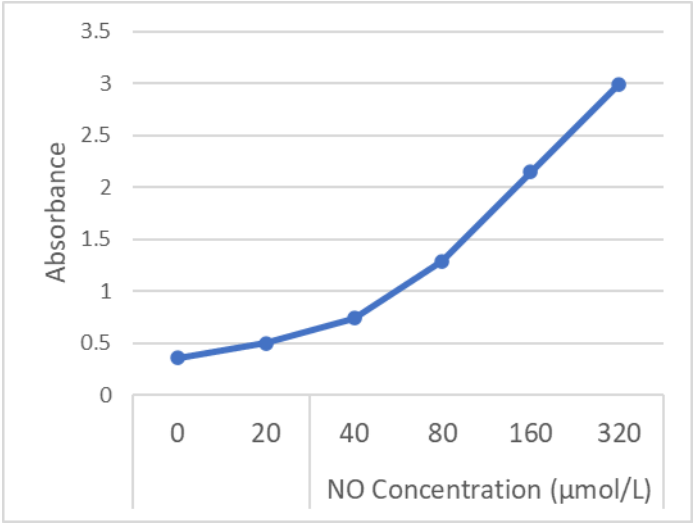
Assay Procedure

1. All the reagents, standard solutions and samples were prepared as instructed. Room temperature was achieved for all reagents before use.
2. The strips were inserted in the frames for use.

3. 50µl standard was added to standard well. Antibody was not added to standard well because the standard solution contains biotinylated antibody.
4. 40µl sample was added to sample wells and then 10µl anti-NO antibody was added to sample wells. 50µl streptavidin-HRP was added to both sample standard wells. Solutions were mixed well. Plate was covered with a sealer and incubated for 60 minutes at 37°C.
5. The sealer was removed and the plate was washed 5 times with wash buffer. Wells were soaked with at least 0.35ml wash buffer for 30 seconds to 1 minute during each wash. The plate was blotted onto paper towels.
6. 50µl substrate solution A was added to each well and then 50µl substrate solution B was added to each well. The plate was incubated in the dark for 10 minutes at 37°C, after covering with a new sealer.
7. Stop solution of 50µl was put into each well (blue color changed into yellow).
8. The optical density (OD) value was determined at 450nm immediately within 10 minutes using a microplate reader.

Calculation of Result

A standard curve was constructed by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and a best fit curve was drawn through the points on the graph.



Gene expression of NOS[7]:

Isolation of total ribonucleic acid (RNA) from human whole blood was carried by using PAXGene RNA isolation kit as per manufacturer (Qiagen) instructions:

1. PAXgene Blood RNA Tube was centrifuged for 10 minutes at 3600 x g using a swing-out rotor.
2. The supernatant was removed by decanting. 4 ml RNase-free water was added to the pellet, and the tube was closed using a fresh secondary BD Hemogard closure (supplied with the kit).
3. Vortexing was done until the pellet was visibly dissolved, and centrifugation was done for 10 minutes at 4000 x g using a swing-out rotor. The entire supernatant was removed and discarded.
4. 350 µl Buffer BR1 was added and vortexed until the pellet was visibly dissolved.
5. The sample was pipetted into a 1.5 ml microcentrifuge tube. 300 µl Buffer BR2 and 40 µl proteinase K were added. Mixing was done by vortexing for 5 seconds, and was incubated for 10 minutes at 55°C using a shaker–incubator atn400–1400
6. The lysate was pipetted directly into a PAXgene Shredder spin column, which was placed in a 2 ml processing tube, and centrifuged for 3 minutes at 14000 x g.
7. The entire supernatant of the flow-through fraction was transferred to a fresh 1.5 ml micro centrifuge tube without disturbing the pellet in the processing tube.
8. 350 µl 100% ethanol was added and mixed by vortexing. It was centrifuged briefly (1–2 seconds at 1000 x *g*) to remove drops from the inside of the tube lid.
9. 700 µl sample was pipetted into the PAXgene RNA spin column, placed in a 2 ml processing tube, and centrifuged for 1 minutes at 14,000 x *g*. The spin column was placed in a new 2 ml processing tube and the old processing tube containing flow-through was discarded.
10. The remaining sample was pipetted into the PAXgene RNA spin column, and centrifuged for 1 minutes at 14,000x *g*. The spin column was placed in a new 2 ml processing tube, and the old processing tube containing flow-through was discarded.
11. 350 µl Buffer BR3 was pipetted into the PAXgene RNA spin column and centrifuged for 1

minute at 14,000x *g*. The spin column was placed in a new 2 ml processing tube, and the old processing tube containing flow-through was discarded.

12. 10 µl DNase I stock solution was added to 70 µl Buffer RDD in a 1.5 ml microcentrifuge tube. Solution was mixed by gently flicking the tube, and centrifuged briefly to collect residual liquid from the sides of the tube.
13. The DNase I incubation mix (80 µl) was pipetted directly onto the PAXgene RNA spin column membrane, and placed on the benchtop (25°C) for 15 minutes.
14. 350 µl Buffer BR3 was pipetted into the PAXgene RNA spin column, and centrifuged for 1 minute at 14,000 x *g*. The spin column was placed in a new 2 ml processing tube, and the old processing tube containing flow- through was discarded.
15. 500 µl Buffer BR4 was pipetted into the PAXgene RNA spin column, and centrifuged for 1 minute at 14,000 x *g*. The spin column was placed in a new 2 ml processing tube, and the old processing tube containing flow-through was discarded.
16. Another 500 µl Buffer BR4 was added to the PAXgene RNA spin column. Later, it was centrifuged for 3 minutes at 14,000 x *g*.
17. The processing tube containing the flow-through was discarded and the PAXgene RNA spin column was placed in a new 2 ml processing tube. Later, it was centrifuged for 1 minute at 14,000 x *g*.
18. The processing tube containing the flow-through was discarded. The PAXgene RNA spin column was put into a 1.5 ml microcentrifuge tube and then, 40 µl Buffer BR5 was pipetted directly onto the PAXgene RNA spin column membrane. Later, it was centrifuged for 1 minute at 14,000 x *g* to elute the RNA.
19. The elution step (step 18) was repeated as described, using 40 µl Buffer BR5 and the same microcentrifuge tube.
20. The elute was incubated for 5 minutes at 65°C in the shaker–incubator (from step 5) without

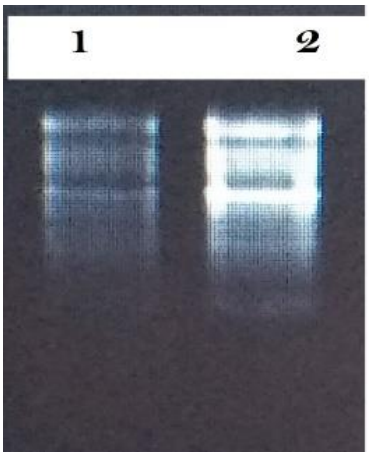
shaking. After incubation, it was chilled immediately on ice.

21. RNA samples were stored at –70°C.

Quality analysis of total RNA

Quality of total RNA was checked by using Agarose gel (1.5%) electrophoresis. 1.5g of agarose was dissolved in 100ml of 1XTAE bufffer by heating. The mixture was allowed to cool at 55^0c and 1µl of Ethidium Bromide was added. The melted Agarose was poured into gel casting tray with comb making sure that no air bubbles are trapped in the gel, after solidifying comb was removed. The tray was placed in electrophoretic tank contains 1X TAE tank buffer till the gel was completely submerged.

4µl of Total RNA was mixed with Bromophenol blue and loaded into the wells and run it at 50-100v for 2hours. After this, the gel was observed under UV documentation.



cDNA Synthesis

First Strand of cDNA was synthesised by using the following protocol:

1. The following reagents were added into a sterile, nuclease free tube on ice in the indicated order:

Total RNA	**10 µL**
Oligo (dT)18 primer	1 µL
Random Hexamer primer	1 µL

2. Mixing was done gently, centrifuged briefly and incubated at 65°C for 5 min. It was chilled on ice immediately.

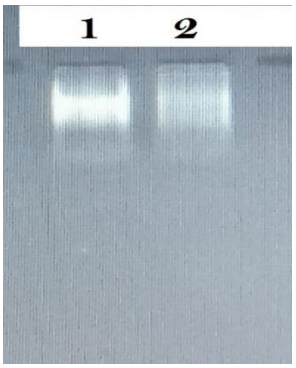
3. The following components were added in the indicated order:

5X Reaction Buffer	**4 µL**
RiboLock RNase Inhibitor	1 µL
10 mM dNTP Mix	2 µL
RevertAid M-MuLV RT	1 µL

4. Mixing was done gently and centrifuged briefly.
5. Incubation was done for 5 min at 25°C followed by 60 min at 42°C
6. The reaction was terminated by heating at 70°C for 5 min.
7. The reverse transcription reaction product was stored at -70°C.

Quality analysis of cDNA

Quality of cDNA was checked by using Agarose gel (1.5%) electrophoresis with standardised protocol.



Expression profiling of NOS3

Expression profiling analysis was done by using following reaction composition and thermo cyclic conditions:

Components	volume in µL (for 10µL)
Nuclease free water	3 µL
SYBR master mix	4µL
Forward Primer	1 µL
Reverse Primer	1 µL
Template	1 µL

Temperature	Time	
95.0 °C	10 min	
95.0 °C	15 sec	Repeat for 40 cycles
60.0 °C	30 sec	
95.0 °C	10 sec	
65.0 °C	5.0 sec	
95.0 °C	5.0 sec	

Forward and Reverse Primer for ***Unknown (Case and Controls)*** were NOS3, Forward and Reverseprimers for ***Positive control*** - were GAPDH and NOS3 primer without Template for ***negative control.*** The forward primer for NOS3 was CTGGCTTTCCCTTCCAGAT and the reverse primer was CTTAATCTGGAAGGCCCCTC. Each sample was analysed in triplicate along with one positive control and one negative control. The mean Cq (quantification cycles) value of sample (NOS3) and the mean Cq value of positive control (GAPDH, glyceraldehyde 3-phosphate dehydrogenase) was noted and then, the mean Cq value of triplet was used for expression analysis. ΔCq was calculated as the difference in Cq values between sample and positive control. ΔΔCq was then calculated as {Δcq (sample) - Δcq (Avg control group)}. 2^ - ΔΔCq was calculated later for final NOS3 gene expression.

Expression profiling of NOS2: The profiling of NOS2 was done as the above procedure (done for NOS3), except that the Forward and Reverse Primer for ***Unknown (Case and Controls)*** were NOS2, Forward and Reverse primers for ***Positive control*** - were GAPDH and NOS2 primer without Template for ***negative control.*** The forward primer for NOS2 was GATATCCCCCAGCCCTCAAGT and the reverse primer was GAGGCCCCAGTTTGAGAGAG.

Statistical Analysis:

Data was expressed as mean ± standard deviation and was entered in Microsoft Excel spreadsheet. Statistical analysis was done using SPSS 19.0 version software by Student's t test and a p value <0.05 was considered as statistically significant.

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RESULTS

Role of nitric oxide synthase 3 (NOS3) gene expression in patients of preeclampsia with special reference to cardiovascular and renal pathophysiology

CHAPTER 5.

RESULTS

In the present research work, 30 normal pregnant women as controls and 30 pre-eclampsia patients as cases were studied. However, gene expression could be correctly evaluated only in 25 samplesof each group.

The difference in the mean age (in years) of the participants was comparable and not significant (p=0.741) among both the groups. In normal pregnant women (controls), it was 22.97±3.12 and that in pre-eclampsia patients (cases), it was 22.67±3.83 (Figure 5.1). The mean gestational age (in weeks) was 37.63±3.16 in normal pregnancy controls and 36.10±4.29 in pre- eclampsia cases (Figure 5.2), which had no statistical significance (p=0.120).

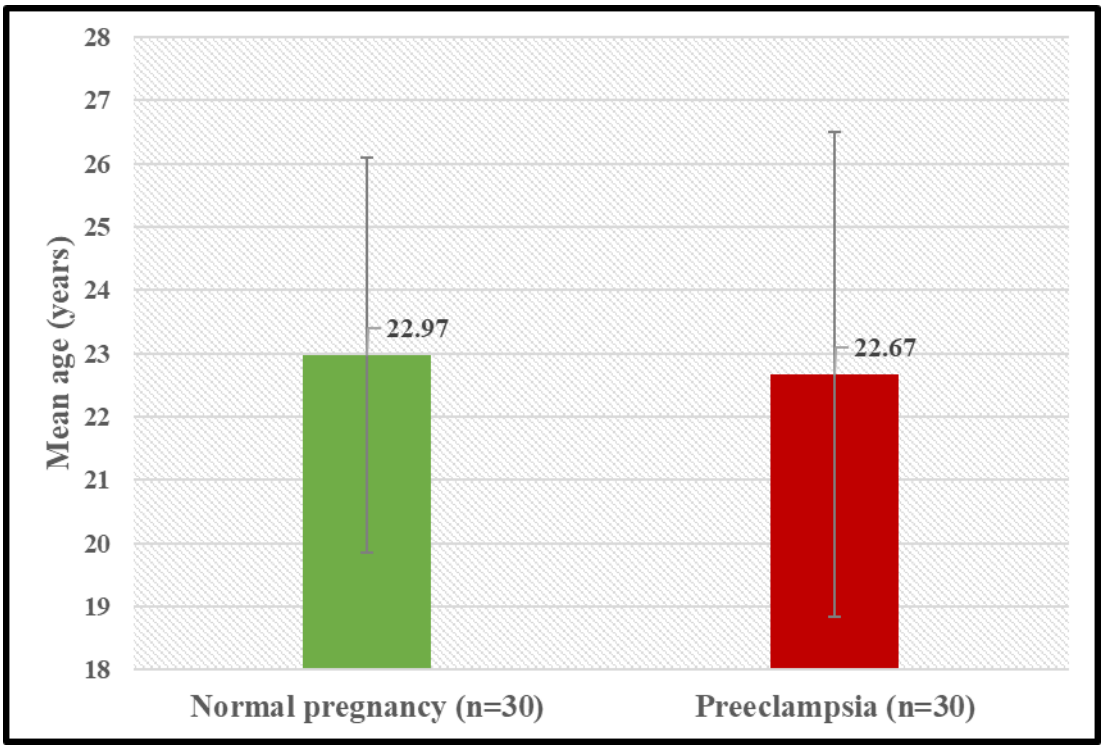


Figure 5.1. Comparison of maternal age in normal pregnancy versus preeclampsia

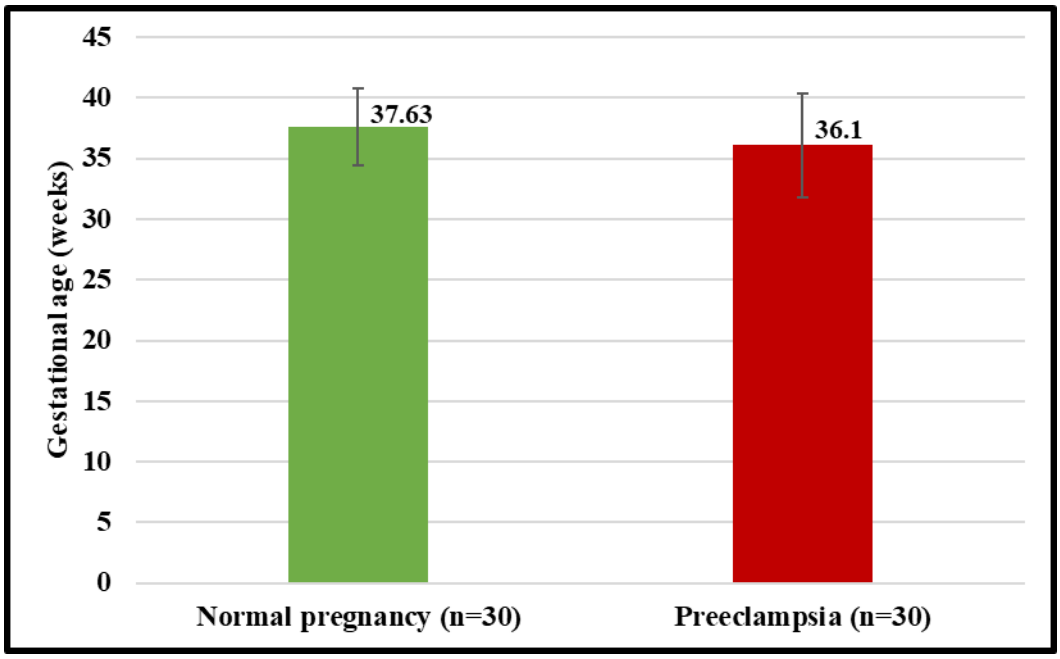


Figure 5.2. Comparison of gestational age in normal pregnancy versus preeclampsia

Cardiovascular functions:

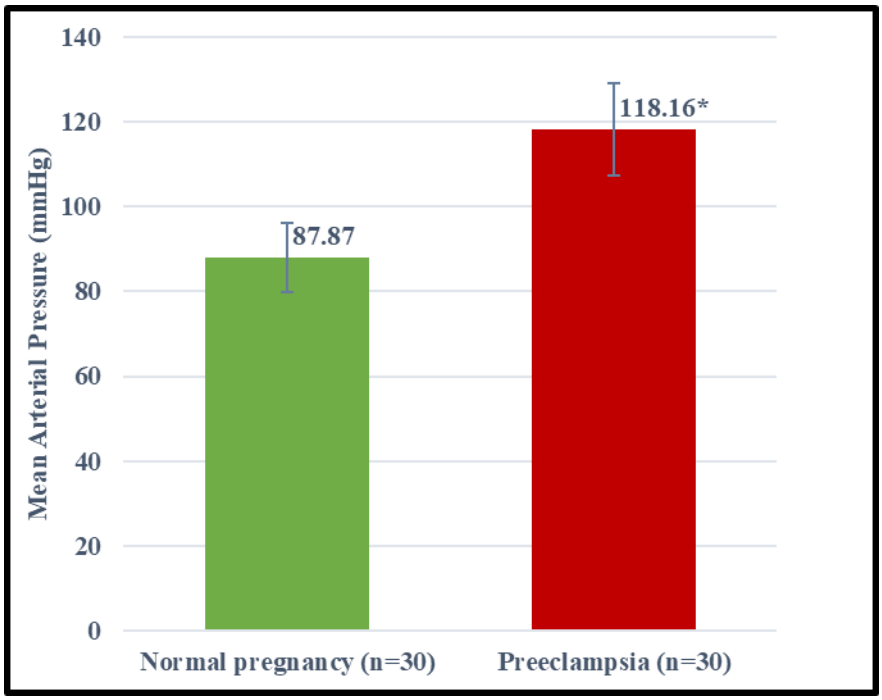
Heart rate: Table 5.1 shows that the mean heart rate (beats/min) was slightly lesser in cases (92.33 ± 12.04) than controls (93.10 ± 9.50), but not statistically significant.

Blood pressure: The systolic, diastolic and the mean blood pressures (mmHg) were significantly higher in cases as compared to controls (Table 5.1, Figure 5.3). They were significantly higher by 37.26, 26.80 and 30.28 mmHg respectively in cases of preeclampsia as compared to normal pregnancy healthy controls.

Table 5.1. Cardiovascular parameters in normal pregnancy versus preeclampsia

	Normal Pregnancy (Controls, n=30)	Pre-eclampsia (Cases, n=30)	t value	p value
Heart rate (beats/min)	93.10 ± 9.50	92.33 ± 12.04	0.274	0.785
SBP (mmHg)	116.80 ± 10.68	154.07 ± 17.10	10.122	**0.0001***
DBP (mmHg)	73.40 ± 8.60	100.20 ± 8.64	12.044	**0.0001***
MAP (mmHg)	87.87 ± 8.19	118.16 ± 10.95	12.134	**0.0001***

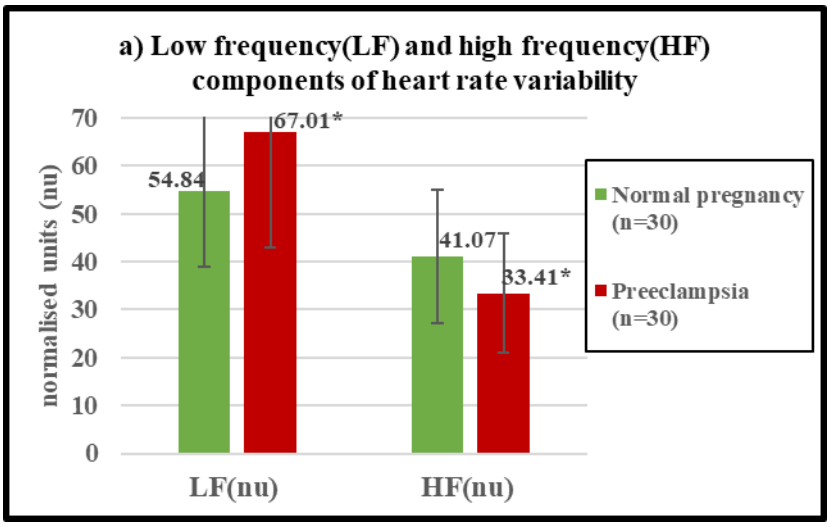
*Statistically significant; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; MAP: Mean arterial pressure

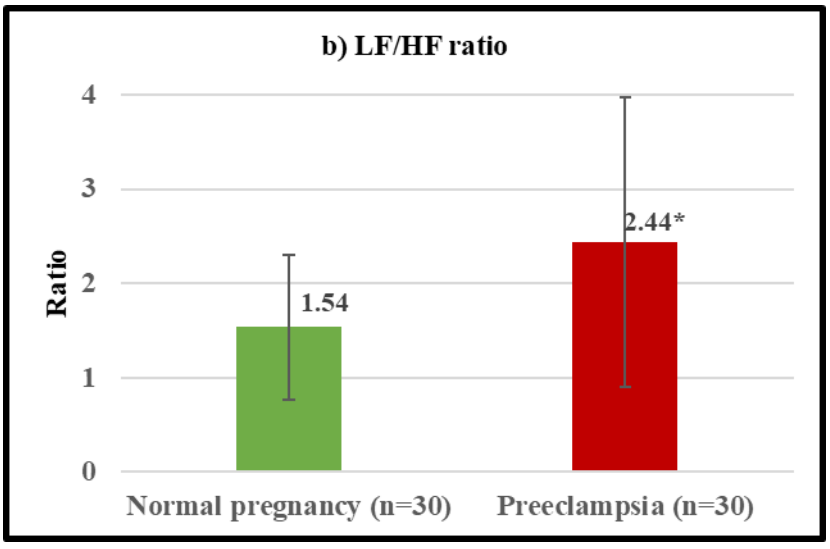


*Statistically significant

Figure 5. 3. Mean arterial pressure in normal pregnancy and preeclampsia

Heart rate variability: Heart rate variability analysis, in the frequency domain, as shown in figure 5.4 a and b, revealed that there was a significant increase (p=0.024) in the low frequency (LF, nu) component in preeclampsia cases (67.01 ± 23.95) as against normal healthy pregnant controls (54.84 ± 15.93). There was a significant decrease (p=0.029) in high frequency (HF, nu) component in pre-eclampsia group (33.41±12.50) as compared to the control group (41.07 ± 13.86). LF/HF ratio was increased significantly (p=0.005) in pre-eclampsia (2.44 ± 1.54) as compared to that in normal pregnancy (1.54±0.77).

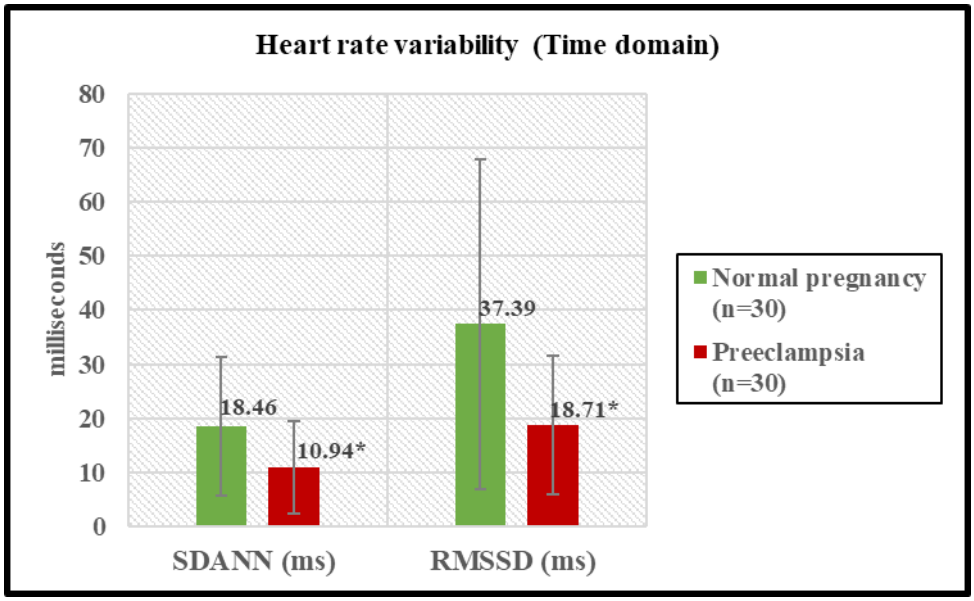




*Statistically significant

Figure 5.4. Frequency domain heart rate variability [a) LF and HF; b) LF/HF ratio] in normal pregnancy and preeclampsia

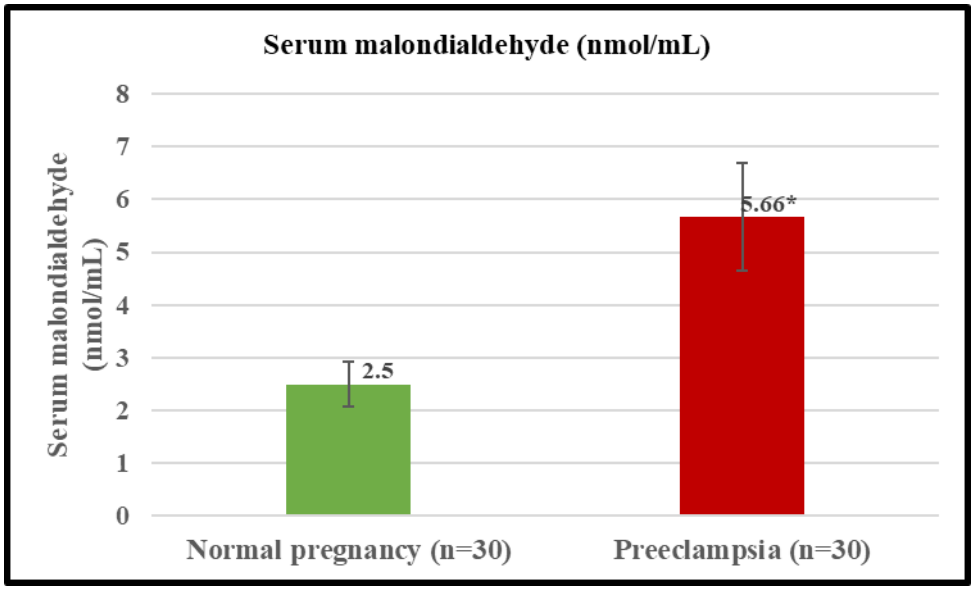
The time domain parameters, SDNN (ms), SDANN (ms), RMSSD (ms) and pNN50 (%), depicted in figure 5.5, showed a fall in preeclampsia (35.17 ± 20.20, 10.94 ± 8.49, 18.71 ± 12.80 and 2.37 ± 2.10 respectively), as compared to them in healthy pregnancy (50.24±23.62, 18.46±12.79, 37.39±30.58 and 4.49±3.97 respectively).



*Statistically significant; SDANN: standard deviation of average NN intervals for every 5 min segment; RMSSD: root mean square of successive RR interval differences

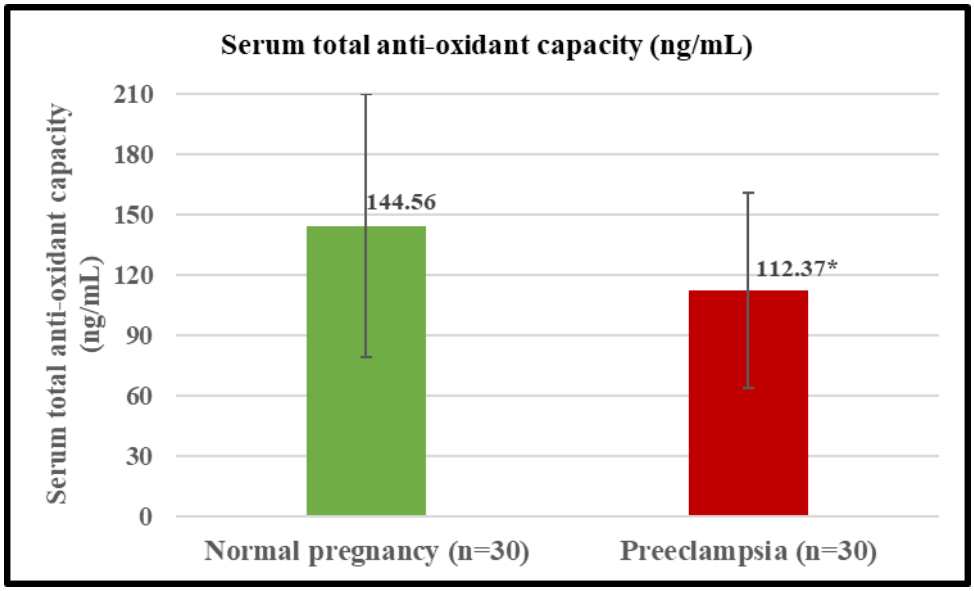
Figure 5.5. Time domain heart rate variability in normal pregnancy and preeclampsia

Oxidative stress markers: A significant state of oxidative stress was noted in cases of preeclampsia by an increase in the level of serum malondialdehyde (Figure 5.6) in nmol/mL, (5.66 ± 1.02) as compared to normal pregnancy (2.50 ± 0.42), which was statistically significant (p=0.0001) and a decrease in total anti-oxidant capacity (ng/mL) in pre-eclampsia (112.37 ± 48.36) as compared to normal pregnancy (144.56 ± 65.24), which was also significant (p=0.034) (Figure 5.7).



*Statistically significant

Figure 5.6. Oxidative stress marker (malondialdehyde) in normal pregnancy and preeclampsia



*Statistically significant

Figure 5.7. Total antioxidant capacity in normal pregnancy and preeclampsia

Renal functions: As shown in Table 5.2, an increase in the level of serum urea (mg/dL), serum creatinine (mg/dL) and urine creatinine (mg/L) was observed in pre-eclampsia (23.21± 5.12, 0.74±0.17 and 660.93 ± 433.14 respectively) as compared to those in normal

pregnancy (20.39±6.73, 0.73±0.13 and 626.03±319.86 respectively) but, were statistically not significant ($p>0.05$).

Serum uric acid (mg/dL) showed a statistically significant increase ($p<0.0001$) in pre-eclampsia (4.46 ± 1.07) as compared to normalpregnancy (3.71 ± 1.23).

A decrease in total proteins (g/dL) was observed in pre-eclampsia (6.06 ± 0.68) as compared to normal pregnancy (6.28 ± 0.71), but was not significant ($p=0.217$). However, serum albumin (g/dL) was significantly reduced ($p=0.004$) in preeclampsia (3.27 ± 0.55) as compared to normal pregnancy (3.64 ± 0.41). A significant increase ($p<0.0001$) in urine proteins (mg/L) was observed in preeclampsia (2098.03 ± 1765.22) when compared with healthy pregnantcontrols (51.66 ± 29.08).

A significant increase ($p<0.0001$) in urine protein: creatinine ratio was also observed in preeclampsia(3.52 ± 2.93) when compared with healthy pregnant controls (0.106 ± 0.08).

Table 5.2. Renal functions in normal pregnancy and preeclampsia

	Normal Pregnancy (n=30)	**Preeclampsia (n=30)**	**t value**	**p value**
S. Urea (mg/dL)	20.39 ± 6.73	23.21 ± 5.12	1.83	0.072
S. Creatinine (mg/dL)	0.73 ± 0.13	0.74 ± 0.17	0.167	0.868
S. Uric acid (mg/dL)	3.71 ± 1.23	4.46 ± 1.07	4.126	**0.0001***
S. Total Plasma proteins (g/dL)	6.28 ± 0.71	6.06 ± 0.68	1.248	0.217
Serum albumin (g/dL)	3.64 ± 0.41	3.27 ± 0.55	2.956	**0.004***
Urine protein (mg/L)	51.66 ± 29.08	2098.03 ± 1765.22	6.349	**0.0001***
Urine creatinine (mg/L)	626.03 ± 319.86	660.93 ± 433.14	0.355	0.724
Urine Protein/Creatinine ratio	0.106 ± 0.08	3.52 ± 2.93	6.38	**0.0001***

*Statistically significant

Table 5.3 depicts statistically significant decrease in urine angiotensinogen levels (ng/L) and urine angiotensinogen: creatinine ratio (ng/mg) in preeclampsia cases (2252.7 ± 405 and 5.39 ± 4.9 respectively) when compared with healthy pregnant controls (4467.8 ± 1246.5 and 9.38 ± 6.6 respectively).

Table 5.3. Urinary angiotensinogen and angiotensinogen/creatinine ratio in normal pregnancy and preeclampsia

	Normal Pregnancy (Controls, n=30)	**Pre-eclampsia (Cases, n=30)**	**t value**	**p value**
Urine angiotensinogen (ng/L)	4467.8 ± 1246.5	2252.7 ± 405	9.25	**0.0001***
Urine angiotensinogen/creatinine ratio (ng/mg)	9.38 ± 6.6	5.39 ± 4.9	2.64	**0.011***

*Statistically significant

Nitric oxide: A decrease in serum nitric oxide levels (µmol/L) was noted in pre-eclampsia (30.09 ± 10.08) as compared to normal pregnancy (35.43 ± 20.37), which was not significant (p=0.204), as depicted in table 5.4.

Table 5.4. Serum nitric oxide levels in normal pregnancy and preeclampsia

	Normal Pregnancy (Controls, n=30)	**Pre-eclampsia (Cases, n=30)**	**t value**	**p value**
Serum Nitric Oxide (µmol/L)	35.43 ± 20.37	30.09 ± 10.08	1.285	0.204

mRNA expression of NOS3: As depicted in table 5.5, the RT-PCR mean difference in the Cq values of NOS3 as against the positive control (GAPDH) was higher in cases (9.10 ± 2.74) as compared to controls (7.96 ± 3.30), and consequently when the gene expression of NOS3 was calculated (by **2^ - ΔΔCq),** it was found that NOS3 gene was down-regulated in cases as compared to controls (Figure 5.8) by a relative difference of 8.49 times.

Table 5.5. mRNA expression of NOS 3 in preeclampsia

	Cq of sample (NOS3) 1	**Cq of Positive Control (GAPDH) 2**	**ΔCq 1 - 2**	**ΔΔCq {Δcq (sample) - Δcq (Avg control group)}**	**2^ - ΔΔCq**
Normal Pregnancy (Controls, n=25)	23.14 ± 3.43	15.11 ± 1.82	7.96 ± 3.30	0.32 ± 3.63	14.63
Preeclampsia (Cases, n=25)	24.73 ± 2.56	15.63 ± 3.19	9.10 ± 2.74	1.14 ± 2.74	**6.14**

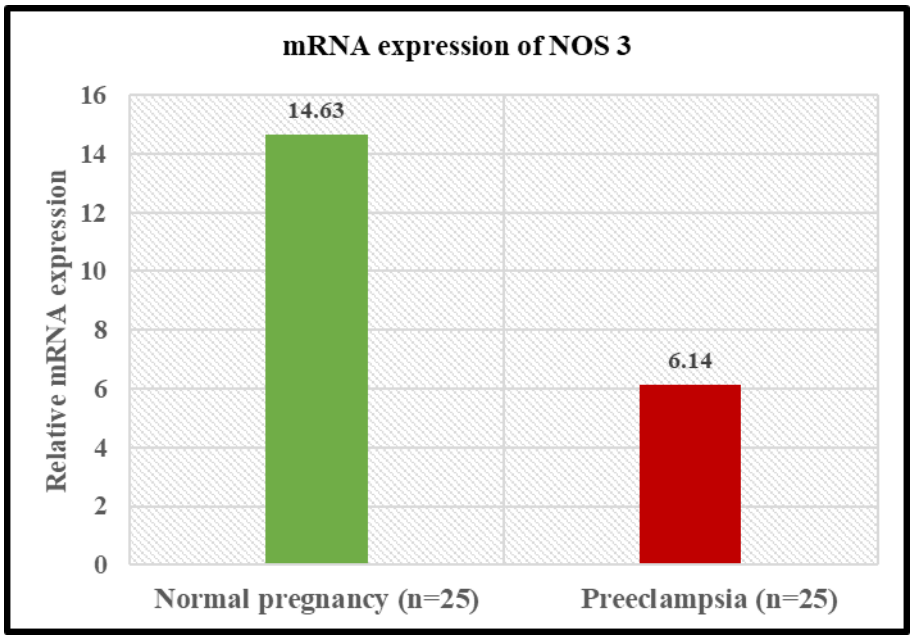


Figure 5.8. Relative mRNA expression of NOS 3 in preeclampsia

mRNA expression of NOS2: The mean difference in the Cq values of NOS2 as against the positive control (GAPDH) was higher (Table 5.6) in cases (11.16±2.42) as compared to controls (10.15±3.49). Consequently, when the gene expression of NOS2 was calculated (by **2^ - ΔΔCq),** it was found that NOS2 gene was down-regulated by a difference of 51.05 times in cases as compared to controls.

Table 5.6. mRNA expression of NOS 2 in preeclampsia

	Cq of sample (NOS2) 1	**Cq of Positive Control (GAPDH) 2**	**ΔCq 1 - 2**	**ΔΔCq {Δcq (sample) - Δcq (Avg control group)}**	**2^ - ΔΔCq**
Normal Pregnancy (Controls, n=25)	26.63 ± 1.79	16.47 ± 3.34	10.15 ± 3.49	0.004 ± 3.49	51.07
Preeclampsia (Cases, n=25)	27.40 ± 1.27	16.24 ± 2.59	11.16 ± 2.42	10.38 ± 3.19	**0.02**

DISCUSSION

Role of nitric oxide synthase 3 (NOS3) gene expression in patients of preeclampsia with special reference to cardiovascular and renal pathophysiology

CHAPTER 6.

DISCUSSION

In the present research, two groups of thirty cases of preeclampsia and thirty controls of normal pregnancy were included. These two groups were comparable in their maternal age as well as their gestational age, without any statistically significant difference in these parameters. This is important to decide on the relationship of study variables among the defined cases and also to identify if a particular variable is evidently more common in cases as compared to controls.

Cardiovascular functions and pathophysiology:

In the present study, there was an increase in systolic, diastolic and mean blood pressures. This was also evidenced in many other studies[1-3], as it is one of the diagnostic criteria for pre-eclampsia. But, there was no significant change in the heart rate in the present study.

Normal pregnancy promotes wide range of cardiovascular adjustments. During normal pregnancy, spiral arteries undergo trophoblast-mediated remodeling to be converted into high capacitance and low resistance vessels which is necessary for proper growth of the feto-placental unit. Impaired cytotrophoblast migration and invasion is seen in preeclampsia which results in impaired spiral artery remodeling. This leads to placental ischemia and hence generation of oxidative stress, which affects the endothelial function, which in turn reduces the bioavailability of nitric oxide. Endothelial dysfunction results in cardiovascular and renal dysfunction too. Increase in the peripheral resistance and a decrease in renal pressure natriuresis, results in elevation of blood pressure[1].

Endothelial dysfunction has been characterized by imbalanced vasodilation and vasoconstriction. There is also evidence of elevated reactive oxygen species (ROS) and

proinflammatory factors, as well as deficiency of nitric oxide (NO) bioavailability. Endothelial dysfunction is known to disrupt the endothelial barrier permeability too, that forms a part of the inflammatory response which favors cardiovascular disease development[4,5]. All these factors lead to an increase in peripheral resistance.

Another factor that would influence the peripheral resistance is the autonomic nervous system activity. High levels of systolic and diastolic blood pressures in preeclampsia depict an enhanced sympathetic activity and a resultant high peripheral resistance[6]. Diastolic blood pressure is influenced by peripheral resistance, whereas systolic blood pressure depends on cardiac output. Stroke volume times the heart rate renders the cardiac output[7]. In the present study, as there was no statistically significant difference in heart rate, the stroke volume may be responsible for a significant difference in the systolic blood pressure. Myocardial contractility and venous return determine the stroke volume[7]. It is a well-known fact that there is a reduction in the plasma volume in pre-eclampsia[8] and hence, an increase in stroke volume may not contributed by venous return. From these facts, it is evident that myocardium contractility would rather determine the systolic blood pressure. Enhanced sympathetic nervous activity may suggest a rise in contractility of the myocardium as well as peripheral resistance. This may result in increase in both systolic and diastolic blood pressures.

Frequency domain analysis of the heart rate variability in the present study revealed a significant rise in LF/HF ratio and the low frequency (LF) component, whereas a dip in high frequency (HF) component in cases of preeclampsia. Other authors also reported similar results[9-11]. Sympathetic tone is depicted by LF component, parasympathetic tone by HF component and sympatho-vagal balance by LF/HF ratio. Sympathetic predominance is suggested by an increase in LF/HF ratio whereas parasympathetic predominance by its decrease[10,12]. In the present study, SDANN, SDNN, RMSSD and pNN50 (time domain parameters) were lowered in cases of preeclampsia. Similar findings were observed by other

authors too[9]. Parasympathetic activity is suggested by these time domain parameters of HRV[10,12]. In the present study, sympathetic overactivity as well as parasympathetic withdrawal was evident, which could have resulted in an increase in blood pressures, as stated earlier. The outcome of pre-eclampsia may be influenced by these parameters. The amount of variability in the heart rate found at present may indicate the risk of cardiovascular disease development, even after the pregnancy is terminated[13].

Oxidative stress: Pregnancy itself is a state of oxidative stress as a result of increased maternal metabolism and the metabolic activity of the placenta. The hypoxic or poorly perfused placenta resulting from inadequate trophoblast invasion exhibits increased oxidative stress in pre-eclampsia as manifested by increased free radical formation, increased lipid peroxides and reduced antioxidant defenses[5,14]. In the present study too, there was an increase in the oxidative stress marker, serum malonaldehyde and decrease in the serum total antioxidant capacity. Other authors also reported that the mean plasma levels of malondialdehyde were higher whereas the levels of glutathione and superoxide dismutase levels were significantly lower in pre-eclampsia[15,16]. A number of studies have reported that plasma levels of MDA and other lipid peroxidation products are elevated in pregnancy, and all further increased in preeclamptic pregnancies[17-19]. This provides further evidence that inappropriate or excessive lipid peroxidation may play an important role in the pathophysiology of preeclamptic pregnancies.

Lipid peroxidation may be a causative factor in pregnancy induced hypertension. It may lead to protein modification/dysfunction, inflammatory response, release of cytokines, endothelial dysfunction and cell apoptosis[20]. Oxidative stress causing endothelial dysfunction may lead to increase in peripheral resistance, which may result from either direct vasoconstriction or indirectly due to decrease in NO bioavailability[21]. This would again be responsible for elevated blood pressure in pre-eclampsia.

Although the etiology of preeclampsia is unknown, it is known that preeclampsia is associated with an imbalance of increased lipid peroxides and decreased antioxidants[18]. Besides the determination of oxidative damage, many investigators have evaluated antioxidant capacity in the maternal circulation by estimating the total antioxidant capacity, the concentration of specific antioxidants, or the activity of antioxidant enzymes[19,22-24]. The current study also reported a decrease in the serum total antioxidant capacity. This observation was also reported by several studies and the authors concluded that higher MDA/total antioxidant capacity ratio in women with preeclampsia is indicative of oxidative stress[25-29]. Cellular integrity in normal pregnancy is maintained by antioxidants by inhibiting peroxidation reactions[30]. Cellular and extracellular enzymes such as superoxide dismutase, glutathione reductase, catalase and free-radical scavengers form antioxidant defense mechanisms. Vitamins C and E, carotenoids, glutathione, serum albumin, and metabolites such as bilirubin and uric acid also act as antioxidants. Free radicals are scavenged by Vitamin C in the aqueous phase. The lipid-soluble vitamin E prevents the formation of lipid peroxides and protects the cell membranes[31].

Normal pregnancy is associated with an increase in oxidative stress and lipid peroxidation, but antioxidant protection also increases[32]. In pre-eclampsia there is a further increase in lipid peroxidation and also an insufficient increase in antioxidants to combat the increase in oxidative stress[33-35].

Maternal oxidative stress may reach the brain stem sites that are involved in regulation of sympathetic vasomotor tone, mainly the rostral ventrolateral medulla (RVLM) and nucleus tractus solitarii (NTS). This may lead to their dysfunction and augmentation of vasomotor tone and hence rise in blood pressure[36,37]. Rise in blood pressure affects the cerebral circulation where it leads to loss of autoregulatory capacity. This causes blood-brain barrier (BBB) disruption and also vasogenic edema. Edema may also be due to a decrease in

serum albumin, as seen in the present study, which may cause a decrease in oncotic pressure, which may, in turn, result in edema. Edema may cause dysfunction of vasomotor area, contributing again to a rise in blood pressure[38].

However, supplementation with antioxidants did not show any difference in terms of reduction in the incidence of pre-eclampsia[39].

Renal pathophysiology: Endothelial dysfunction has been reported to result in renal dysfunction too. The immensely popular Prevention of Renal and Vascular End stage Disease (PREVEND) study[40] proved that the endothelium synthesizes many inflammatory molecules. These molecules have been associated with subtle reductions in creatinine clearance. Another study has attributed that a dysfunctional endothelium seems to be a key factor in the risk for renal insufficiency in individuals with hypertension[41]. In the present study, an increase in the urinary protein excretion and a decrease in serum albumin was noted, which was also quoted by other authors[42-44] The evident vasoconstriction either due to endothelial dysfunction or increased sympathetic nervous activity has its effects on the renal mechanisms[42]. Two mechanisms for proteinuria were proposed by Drumond et al. First, broadened epithelial foot processes lead to complete disruption of the slit diaphragms, allowing for a high loss of protein across the glomerular wall. Second, reduced charge selectivity could increase proteinuria despite a reducedarea available for ultrafiltration[43]. Proteinuria may also be due to swelling of the endothelial cells and also the distortion of the fenestrae. It may also be due to podocyte injury and podocyte-specific protein excretion[44]. Glomerular endotheliosis was evidenced in normal pregnancy as well as pre-eclampsia, although the degree of endotheliosis was higher in patients of preeclampsia[45,46]. Adecrease in serum albumin causing a dip in oncotic pressure may cause exaggeration of endothelial cell swelling and dysfunction of the factors responsible for effective glomerular filtration, thereby leading to proteinuria[46].

There was an increase in the urine protein: creatinine ratio (UPCR) in the present

study, which was also seen by other authors[47,48]. This ratio is given by spot urine protein excretion to creatinine excretion and therefore normalizes protein excretion to glomerular filtration rate, so that a better picture of the amount of proteinuria is obtained[47]. The urine protein: creatinine ratio (UPCR) may be used as a screening test as it is highly predictive of proteinuria in preeclamptic patients so as to hasten the treatment[48].

There was a significant increase in serum uric acid levels in the present study. Serum uric acid levels are noted to have increased very early in pre-eclampsia. It may be possible that women destined to develop preeclampsia come into pregnancy with elevated uric acid as part of the metabolic syndrome. Furthermore, the ability of uric acid to promote inflammation, oxidative stress and endothelial dysfunction affects placental development, its function, and also maternal vascular function. Ischemic injury and oxidative stress promote a feed-forward cycle of uric acid production[49]. Increasing evidence suggest that an elevated serum uric acid in pregnancy is a valuable biomarker for preeclampsia[50].

Angiotensinogen secretion by proximal tubular epithelia is reflected in the levels urine angiotensinogen and hence, urinary angiotensinogen may be a useful biomarker for detecting the functioning of intrarenal RAS[51]. Urine angiotensinogen (UAGT) levels and Urine angiotensinogen: Creatinine ratios (UAGT/Cr) in pregnancies with pre-eclampsia were found to be lower than in normal pregnancies in the present study and similar results were observed by other authors too[52,53]. In normal pregnancy, increase in progesterone and oestrogen levels will activate the renin-angiotensin- aldosterone system (RAAS), but a reduced sensitivity to angiotensin II is noted with a resultant increased excretion of urinary angiotensinogen. But, in pre-eclampsia, there is an upregulation of angiotensin receptors and hence, more sensitivity for angiotensin II and hence, decrease in the excretion of angiotensinogen[52,53]. Other authors claim that the expression of RAS components in the kidney may have been downregulated or not changed in preeclampsia. The authors also

suggest that the pathophysiology of preeclampsia may be independent of angiotensin II/RAAS[54].

Nitric oxide: Endothelial damage due to oxidative stress significantly reduce the levels of NO[55]. Bioavailability of nitric oxide is reduced due to oxidative stress or under production of NO or reduced activity/expression of eNOS[56]. Low levels of L-arginine and high levels of asymmetric dimethylarginine (ADMA), which is an endogenous eNOS inhibitor might also be responsible for low levels of NO in preeclampsia[21]. Serum nitric oxide levels, in the present study, were reduced insignificantly. Similar results as the present study were noted by Hodzic et al[57]. However, an insignificant reduction in NO levels may be due to the influence of dietary nitrates or reduced excretion of nitrates due to renal dysfunction[58]. The reason for this observation could also be that serum nitric oxide levels may be trying to increase to maintain homeostasis.

Nitric oxide synthase gene expression: A glance into the mRNA expression of NOS gene revealed a down- regulation of both NOS3 and NOS2 genes in the present study. Gene expression may portray the link between oxidative stress, sympathetic activity, renal mechanisms and nitric oxide bioavailability.

Increased production of ROS seems to inhibit the expression and functions of eNOS[55]. Lipid peroxidation products may reduce the activity of eNOS and also decrease NO bioavailability[59,] Increased ROS leads to lipid peroxidation which increases the levels of MDA, as seen in the present study, and lipid peroxidation also decreases the calcium/ATPase activity, which in turn, may decrease the eNOS expression, as it is calcium dependent[57]. Uncoupling of eNOS may further lead to dysfunction of eNOS activity[60].

Upregulation of proinflammatory genes like iNOS occurs due to oxidative stress. iNOS is stimulated in normal pregnancy and produces excess of NO temporarily through activation of proinflammatory transcription factor nuclear factor kappa-light-chain-enhancer

of activated B cells (NF-κB)[61]. In a study, authors observed that iNOS expression was maximum in preterm but, not-in-labor patients. This may be attributed to increased cytokine levels seen during pregnancy that would increase the expression of iNOS. On the other hand, iNOS expression was seen to be reduced by 75%, in preterm in-labor or term in-labor patients. iNOS expression was more during pregnancy and less towards term or in-labor [62]. In another study too, there was a decrease in both eNOS and iNOS expression in platelets of patients with preeclampsia, although it was not statistically significant[63]. Similarly, low expression of iNOS was seen in preeclampsia, in the present study, where most of the participants were towards term. The possible explanation for this could be that, towards term or in labour, there is a decrease in the number of progesterone receptors and hence less cytokines and therefore, a decline in iNOS expression[62].

Finally, reduced expression of both NOS3 and NOS2 may lead to insufficient NO, which is not sufficient to normalize the blood pressure. Although the present study could explain most of the pathophysiology on the grounds of increased oxidative stress, some authors have reported that antioxidant supplementation failed to produce any positive outcomes. Hence, other molecules like VEGF, PIGF, heat shock proteins, which may interact with NO signaling have to be researched for further understanding of the pathophysiology of preeclampsia.

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SUMMARY & CONCLUSION

Role of nitric oxide synthase 3 (NOS3) gene expression in patients of preeclampsia with special reference to cardiovascular and renal pathophysiology

CHAPTER 7.

SUMMARY AND CONCLUSION

In the present study,

- Pre-eclampsia shows a significant increase in the mean arterial pressure.
- Sympathetic overactivity in addition to parasympathetic withdrawal is seen in pre-eclampsia may result in an increase in MAP.
- Oxidative stress may lead to endothelial dysfunction and reduced NO bioavailability, which could be the reason for increased mean arterial pressure.
- Renal functions have been impaired and the handling of angiotensinogen is also affected as a result of oxidative stress and sympathetic overactivity – decrease in UAGT excretion; increase in UPCR.
- NO is reduced insignificantly and also the gene expression of NOS3 and NOS2 have been reduced.

Research hypothesis that there is a relationship between the gene expression of nitricoxide synthase and oxidative/nitrosative stress, which thereby could influence the cardiovascular and renal pathophysiology in pre-eclampsia has been **proved**.

Clinical implications: Tools which could be used for early diagnosis, management and prevention of future cardiovascular and renal diseases in pre-eclampsia women are -

- Heart rate variability analysis to test the cardiovascular autonomic functions
- Urinary P/C ratio
- Urinary angiotensinogen/creatinine ratio
- Pharmacologically active molecules which increase the genetic expression of nitric oxide synthase may be contemplated.

Research implications: A better understanding of the pathophysiology of pre-eclampsia and hence, transition of research for early diagnosis and better clinical outcomes could be possible.

Limitations of the study: Although NOS mRNA expression was done, protein expressionquantification was difficult in terms of feasibility with the available resources and hence, could not be done.

Future perspective: Follow up study of preeclampsia patients with serial NOS3 and NOS2 gene expression and also evaluation of the role of heat shock proteins in preeclampsia is contemplated.

ANNEXURES

Role of nitric oxide synthase 3 (NOS3) gene expression in patients of preeclampsia with special reference to cardiovascular and renal pathophysiology

CHAPTER 8. ANNEXURES

Informed consent form

CONSENT FORM I
INFORMATION FOR PARTICIPANTS OF THE STUDY

Title of the project:
Role of nitric oxide synthase 3 (NOS3) gene expression in patients of pre-eclampsia with special reference to cardiovascular and renal pathophysiology

1. **Name, Designation, Address, Phone No. and Email ID of the Investigator:**

Dr. Anita Herur
Professor of Physiology, S. Nijalingappa Medical College,
Bagalkot.
Phone: 9844268316
Email: dranitaherur@yahoo.co.in

2. **Name of Guide with designation, Department, Phone No. and Email ID:**

Dr. Manjunatha Aithala
Professor
Department of Physiology
Shri B. M. Patil Medical College, Vijayapura
Phone: 9902103620
Email: bharatimanju@ymail.com

3. **Name of Co-guide with designation, Department, Phone No. and Email ID:**

Dr. Kusal K. Das
Professor
Department of Physiology
Shri B. M. Patil Medical College, Vijayapura
Phone: 9448194257
Email: kusaldas@gmail.com

Dr. Ashalata Mallapur
Professor and HOD
Department of OBG
S. Nijalingappa Medical College,
Bagalkot.
Phone: 9945699986
Email: drashalatamallapur@gmail.com

3. Purpose/ Objectives of this project /study:

1. To determine NOS3 gene expression in pre-eclampsia and normal pregnancy.
2. To determine Oxidative/nitrosative stress (plasma nitric oxide, total oxidant and total antioxidant levels) in pre-eclampsia cases and normal pregnant controls.
3. To evaluate the renal functions (Blood urea, serum creatinine, plasma proteins, urinary proteins, urinary creatinine, urinary angiotensinogen), cardiovascular functions (Heart rate, blood pressure, heart rate variability) in pre-eclampsia cases and normal pregnant controls.

4. **Procedure/Methods of the study:** Preeclampsia patients and normal pregnancy women were included in this case control study. Arterial blood pressure and heart rate were recorded. Frequency and time domain heart rate variability (HRV) analysis was done using the powerlab software. Levels of malondialdehyde (MDA), total antioxidant capacity and nitric oxide (NO) in the serum were estimated. Renal functions were determined by estimating serum proteins, urea, creatinine, uric acid and urine proteins and creatinine estimation. Urine angiotensinogen was also estimated. Urine protein/creatinine ratio and urine angiotensinogen/creatinine ratio were calculated later. Real-time polymerase chain reaction (RT-PCR) was used for nitric oxide synthase 3 (NOS3) and NOS2 gene profiling.

5. **Expected duration of the subject participation:** 25 minutes
6. **Expected benefits from the research to the participant:** The results of the present study will help us to understand the pathophysiology of pre-eclampsia and its genetic basis, which in-turn will help in appropriate management of pr-eclampsia.
7. **Any risks expected from the study to the participant**: There is more than minimal risk as 7ml of blood shall be drawn for the above mentioned tests.
8. **Maintenance of confidentiality of records:**
   The study records will be kept confidential. Your personal identity will not be revealed in any publication or release of results. Study record will be kept indefinitely for analysis.
9. **Provision of free treatment for research related injury:**
   Although the study procedure itself carries more than minimal risk, treatment of any unforeseeable event will be provided free of cost by the institute to you.
10. **Compensation of the participants for disability or death resulting from such injury:**
    Compensation for any unforeseeable research-related injury or death resulting from such injury will be duly given to you through hospital insurance policy number 68040236170200000011
11. **Freedom to withdraw from the study at any time during the study period without the loss of benefits that the participant would otherwise be entitled:**
    It is entirely your decision to participate in the study. If you want to discontinue from the study, you are free to leave without stating any reason. Your withdrawal would in no way result in SNMC withholding goodwill or normal medical care**.**

12. **Possible current and future uses of the biological material and of the data to be generated from the research and if the material is likely to be used for secondary purposes or would be shared with others, this should be mentioned**
    All the data and materials obtained from you will be used only for research purposes. It will not be used for secondary purposes nor will it be shared with others**.**

13. Address and telephone number of the Investigator and Co-Investigator/Guide:

Dr. Anita Herur
Professor of Physiology, S. Nijalingappa Medical College,
Bagalkot.
Phone: 9844268316
Email: dranitaherur@yahoo.co.in

2. Name of Guide with designation, Department, Phone No. and Email ID:

Dr. Manjunatha Aithala
Professor
Department of Physiology
Shri B. M. Patil Medical College, Vijayapura
Phone: 9902103620
Email: bharatimanju@ymail.com

14. Name of Co-guide with designation, Department, Phone No. and Email ID:

Dr. Kusal K. Das
Professor
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Dr. Ashalata Mallapur
Professor and HOD
Department of OBG
S. Nijalingappa Medical College,
Bagalkot.
Phone: 9945699986
Email: drashalatamallapur@gmail.com

15. Contact details of Chairman of the IEC, SNMC for appeal against violation of rights.

Dr. S. L. Hoti
Scientist G, Director,
ICMR regional center, Belgaum.
Phone: 8105536970

CONSENT FORM II
PARTICIPANT CONSENT FORM

Participant's name:

Address:

Phone No.

Email ID:

Title of the project: **Role of nitric oxide synthase 3 (NOS3) gene expression in patients of pre-eclampsia with special reference to cardiovascular and renal pathophysiology**

The details of the study have been provided to me in writing and explained to me in my own language. I confirm that I have understood the above study and had the opportunity to ask questions. I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without the medical care that will normally be provided by the hospital being affected. I agree not to restrict the use of any data or results that arise from this study provided. Such a use is only for scientific purpose(s). I have been given an information sheet giving details of the study. I fully consent to participate in the above study.

Signature of the Participant: _____ Date: _____

Signature of the Witness: _____ Date: _____

Role of nitric oxide synthase 3 (NOS3) gene expressions in patients of pre-eclampsia with special reference to cardiovascular and renal pathophysiology

PROFORMA FOR COLLECTION OF DATA

PATIENT ID:	**IP/OP NUMBER:**
NAME:	**AGE:**
Ht:	**Wt:**
OCCUPATION:	**ADDRESS:**
PHONE NUMBER:	**CLINICAL FEATURES:**
OBSTETRIC HISTORY:	**PAST HISTORY:**
GESTATIONAL AGE:	
PULSE:	**BLOOD PRESSURE:**
UREA:	**CREATININE:**
TOTAL PLASMA PROTEINS:	**ALBUMIN:**
GLOBULIN:	**A/G RATIO:**
NO:	**MDA**
TOTAL ANTI-OXIDANT STATUS:	**URINE PROTEIN:**
URINARY ANGIOTENSINOGEN:	**URINE CREATININE:**
HRV PARAMETERS:	**HEART RATE:**
LF:	**HF:**
LF/HF ratio:	**SDANN**
RMSSD	**PNN50**
NOS 3 gene expression:	**NOS 2 gene expression:**

BLDE
(DEEMED TO BE UNIVERSITY)
PLAGIARISM VERIFICATION CERTIFICATE

1.Name of Student/Faculty: **Dr. ANITA HERUR** Reg. No:**16PHD001**

2. Title of the Thesis: **Role of nitric oxide synthase 3 (NOS3) gene expression in patients of preeclampsia with special reference to cardiovascular and renal pathophysiology**

3. Department: **PHYSIOLOGY**

4. Name of Guide & Designation: **Dr. Manjunatha Aithala, Professor of Physiology**

5. Name of Co Guide & Designation: **Dr. Kusal K. Das, Professor of Physiology and Dr. Ashalata Mallapur, Professor & HOD of OBG**

The above thesis was verified for similarity detection. The report is as follows:

Software used: **URKUND** Date: 09-05-2022
Similarity Index (%): 1% [ONE PERCENT] Total word count: 10166

The report is attached for the review by the Student and Guide.
The plagiarism report of the above thesis has been reviewed by the undersigned.
The similarity index is below accepted norms.
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The thesis may be considered for submission to the University. The software report is attached.

Signature of guide	Signature of co-guide	Signature of co-guide	Signature of student
Dr. Manjunatha Aithala	**Prof. Kusal K. Das**	**Dr. Ashalata Mallapur**	**Dr. Anita Herur**
Professor Physiology BLDE(DU) Shri B.M.Patil Medical College, Hospital & R.C. Vijayapur-586103	Prof. Kusal K. Das PhD Laboratory of Vascular Physiology & Medicine Department of Physiology BLDE(DU) Shri B M Patil Medical College Vijayapur-586103 Karnataka India	Professor & Head Dept. of Gynecology S. Nijalingappa Medical College & HSK. Hospital & Research Centre BAGALKOT - 587 102	

Verified by -
Signature: 10/05/22
Name & Designation: Dr. Prasanna Kumara BM
University Librarian
BLDE (Deemed to be University)
Shri B M Patil Medical College
Vijayapura - 586103

Ethical clearance certificate

BLDE
(DEEMED TO BE UNIVERSITY)
Declared as Deemed to be University u/s 3 of UGC Act, 1956
The Constituent College
SHRI B. M. PATIL MEDICAL COLLEGE, HOSPITAL & RESEARCH CENTRE, VIJAYAPURA

BLDE (DU)/IEC/ 352 /2019-20 28th June, 2019

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The ethical Committee of this University met on 28th June, 2019 at 11.00 a.m. scrutinizes the Synopsys/ Research Projects of Post Graduate Student / Under Graduate Student /Faculty members of this University /Ph.D. student College from ethical clearance point of view. After scrutiny, the following original/ corrected and revised version synopsis of the thesis/ research projects has been accorded ethical clearance.

Title: "*Role Of Nitric Oxide Synthase 3 (NOS3) Gene Expression In Patients Of Pre-Eclampsia With Special Reference To Cardiovascular And Renal Pathophysiology*"

Name of the Principal Investigator: Dr. Anita Herur,Ph.D. Student.

Name of the Guide: Dr. Manjunath Aithal, Professor & HoD, Dept. of Physiology.

Dr. Sharada Metgud
Chair person
IEC, BLDE (DU),
VIJAYAPURA

Dr.Akram A. Naikwadi
Member Secretary
IEC, BLDE (DU)
VIJAYAPURA
MEMBER SECRETARY
Institutional Ethics Committee
BLDE (Deemed to be University)
Vijayapura-586103. Karnataka

Following documents were placed before Ethical Committee for scrutinization.

- Copy of Synopsys/Research Projects
- Copy of inform consent form
- Any other relevant document

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(Recognized by Medical Council of India and Affiliated to Rajiv Gandhi University of Health Sciences, Karnataka.)
SNMC-INSTITUTIONAL ETHICS COMMITTEE ON HUMAN SUBJECTS RESEARCH
☎08354-235340 Fax: 08354-235360 Website: www.snmcbgk.in
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Office of the Institutional Ethics Committee

The Ethical Committee of SNMC reviewed the following documents:

1. **Research Protocol entitled** Role of nitric oxide synthase 3 (NOS3) gene expression in patients of pre-eclampsia with special reference to cardiovascular and renal pathophysiology
2. **Information sheet for participants of the study (Consent Form –I) and (Consent Form –II)** Role of nitric oxide synthase 3 (NOS3) gene expression in patients of pre-eclampsia with special reference to cardiovascular and renal pathophysiology

NOTE: It is to be noted that neither PI nor any of the proposed study team members were present during the decision-making procedures of the Ethics Committee, and members who are independent of the Investigator, have voted/ provided opinion on the trial.

Dr Anita Herur abstained from the voting process

Discussion points:
After reviewing the documents submitted by the Principal Investigator, the Committee has decided to grant approval for conducting the above mentioned study.

You are requested to report to the Ethics Committee the Following:
1. Progress of the study at the end of 4 months.
2. Provide a report to the Ethics Committee on completion of the study.

The Ethics Committee of SNMC follows procedures that are in compliance with the requirements of ICH (International Conference on Harmonization) related to GCP (Good Clinical Practice), schedule Y and all other applicable Indian regulations.

If you have any Questions concerning the above, please feel free to contact the undersigned.

Thanks & Regards,

vijayamahantesh 18/5/19

(Dr. Vijayamahantesh SN)
Member Secretary

Member Secretary,
IEC
S.N. Medical College
BAGALKOT

Paper presentation certificates

KSOGA Conference 2018

29th Annual Conference of Karnataka State Obstetrics and Gynecology Association

Organized by
BVV Sangha's S Nijalingappa Medical College and HSK Hospital & RC, Bagalkot
Bagalkot Obstetric and Gynecological Society

Certificate

We for She

This is to certify that **Dr. Anita Herur** has

presented a free paper titled **Heart rate variability in pre-eclampsia- a case control study** in

the 29th KSOGA Conference held on 6th and 7th October 2018 at

S. Nijalingappa Medical College, Bagalkot.

Dr M B Bellad, President, KSOGA
Dr Kasthuri Dhonimath, Hon. Secretary Gen KSOGA
Dr Ashalata Mallapur, Organizing Chairperson
Dr Nagarathna Gourakkal Kuntoji, Organizing secretary
Dr Ashok S Mallapur, Dean, SNMC

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64th Annual National Conference of
Association of Physiologists & Pharmacologists of India

APPICON 2018

'ADVANCING HORIZONS OF MEDICAL PHYSIOLOGY & PHARMACOLOGY IN RESEARCH AND TEACHING'

CERTIFICATE OF PARTICIPATION

This is to certify that

Prof./Dr./Mr./Ms. Anita Herur

bearing Register No. 43959 Registered with KARNATAKA Medical Council has attended the Conference as a ~~Keynote Speaker~~/~~Plenary & Invited Plenary Speaker~~/~~Symposium Speaker~~/~~Chairperson~~/Delegate and **Presented Paper (Oral/E-poster)** organized by the Departments of Physiology & Pharmacology, Melaka Manipal Medical College and Kasturba Medical College-Manipal, Manipal Academy of Higher Education, Karnataka, India held on 29th, 30th November & 1st December 2018.

APPICON 2018 is accredited with 6 credit hours by Karnataka Medical Council, vide letter number KMC/CME/276/2018 dated 7.7.2018

Dr Kirtana R Nayak, Organizing Secretary, Prof. & Head, Dept. of Physiology, Kasturba Medical College Manipal MAHE, Manipal
Dr Kiranmai S Rai, Organizing Chairperson, Prof. & Head, Dept. of Physiology, Melaka Manipal Medical College MAHE, Manipal
Dr Nagaraj Annegowda, Zonal Chairman (KMC CME) Accreditation Committee
Dr Ullas Kamath, Dean, Melaka Manipal Medical College MAHE, Manipal
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Dr Ramji Singh, General Secretary, Association of Physiologists & Pharmacologists of India

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CERTIFICATE

Type of Conference-Multi speciality

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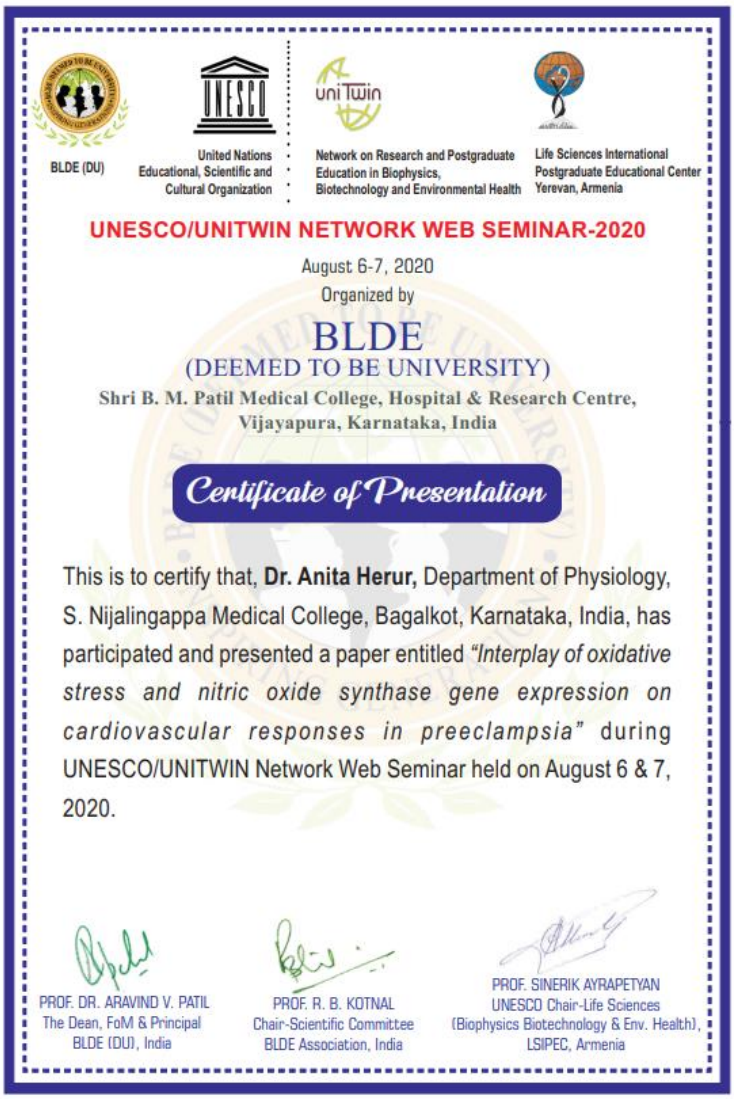
This is to certify that

Dr/Mr/Ms Anita Herur

has Participated as Delegate / Delivered an Oral / ~~Poster~~ presentation in the 9th Annual International Conference of Indian Academy of Biomedical Sciences.

He / She is granted 4 CPD credit points by Maharashtra Medical Council.

Prof. (MS) Syed Mudassar, President, IABS | Prof. Abbas Ali Mehadi, Secretary, IABS | Prof. Bipin M. Tiwale, Organizing Secretary | Dr.R.K.Sharma, Organizing Chairman | Dr.Sandeep S. Kadam, MMC Observer (MMC/MAO-01898/2018) | Dr.Shailesh Kore, MMC Observer (MMC/MAO-00603/2015)



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United Nations Educational, Scientific and Cultural Organization

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Life Sciences International Postgraduate Educational Center Yerevan, Armenia

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August 6-7, 2020

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Shri B. M. Patil Medical College, Hospital & Research Centre,
Vijayapura, Karnataka, India

Certificate of Presentation

This is to certify that, **Dr. Anita Herur,** Department of Physiology, S. Nijalingappa Medical College, Bagalkot, Karnataka, India, has participated and presented a paper entitled *"Interplay of oxidative stress and nitric oxide synthase gene expression on cardiovascular responses in preeclampsia"* during UNESCO/UNITWIN Network Web Seminar held on August 6 & 7, 2020.

PROF. DR. ARAVIND V. PATIL
The Dean, FoM & Principal
BLDE (DU), India

PROF. R. B. KOTNAL
Chair-Scientific Committee
BLDE Association, India

PROF. SINERIK AYRAPETYAN
UNESCO Chair-Life Sciences
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Publications

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Autonomic functions in pre-eclampsia and normal pregnancy

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Keywords:
Pre-eclampsia; heart rate variability; sympatho-vagal balance; cardiovascular disease

ABSTRACT

Pre-eclampsia (PE) is a major complication of pregnancy that could lead to maternal and fetal morbidity and mortality. The analysis of heart rate variability (HRV) is a noninvasive diagnostic tool that provides important information about autonomic functioning and individual's risk of developing cardiovascular disease. The aim of the present study was to compare the frequency and time domain heart rate variability parameters in pre-eclampsia (PE) with that of normal healthy (NP), as a measure of the autonomic functions. This was a case-control study. Pregnant women diagnosed to have pre-eclampsia according to ACOG guidelines and aged 18-35 years were included as cases. Healthy pregnant women, matched for age, gravida and gestational weeks were included as controls. Gestational age was calculated from first trimester USG. Blood pressure was recorded using a mercury sphygmomanometer (Diamond). Heart rate and heart rate variability (HRV) was recorded by Powerlab (AD instruments). HRV analysis was done in the frequency and time domains. Statistical analysis was done using Student's t test. There was a significant increase in the systolic and diastolic blood pressures, low frequency (LF) domain, LF/HF ratio and a decrease in high frequency (HF) components of the HRV analysis in pre-eclampsia. The time domain parameters of HRV, SDNN, SDANN, RMSSD and pNN50%, showed a fall in preeclampsia. Sympathetic over activity combined with parasympathetic withdrawal is seen in pre- eclampsia, which may suggest cardiovascular risk in these patients. Autonomic function testing of heart rate variability may be used as a tool for early diagnosis, management and prevention of future cardiovascular diseases in pre-eclampsia women.

1. INTRODUCTION

One among the many major disorders that complicate pregnancy is pre-eclampsia (PE), which may significantly affect maternal and fetal morbidity and mortality [1]. Pre-eclampsia manifests after 20th week of pregnancy and is characterized by an increase in blood pressure (BP≥140/90 mmHg) and protein in maternal urine (urinary albumin protein ≥300 mg/24 h) [2]. Pre-eclampsia occurs in 2–8% of pregnancies

worldwide, and is the second leading cause of direct maternal and fetal deaths [2], [3] Blood pressure is mainly influenced by the autonomic nervous system. The interaction of the two branches of the autonomic nervous system (ANS): sympathetic and parasympathetic and their activity may be assessed by heart rate variability (HRV) [4]. The analysis of heart rate variability (HRV) is a non-invasive diagnostic tool that provides important information about the risk for cardiovascular disease [5]. Hence, HRV analysis tells us about the involvement of the sympathetic and parasympathetic system in pre-eclampsia. HRV studies in pre-eclampsia have shown varied results. Some have observed a reduced variability [6] and others an increase in variability [7]. The aim of the present study was to compare the heart rate variability parameters in pre-eclampsia (PE) with that in normal pregnancy (NP), as a measure of the autonomic functions.

2. Materials and Methods

This was a case-control study for which Institutional Ethics committee clearance was obtained and subjects were recruited from the department of Obstetrics and Gynaecology. Pregnant women diagnosed to have pre-eclampsia according to ACOG guidelines [8] and aged 18-35 years were included as cases (PE). Healthy pregnant women, matched for age, gravida and gestational weeks were included as controls (NP). Pregnant women with past history of hypertension, diabetes mellitus, or heart disease were excluded from both the groups. Informed consent was taken from all the participants. The sample size was 30 in each group. Gestational age was calculated from first trimester USG and according to ACOG guidelines [9]. After 15 minutes of rest, blood pressure was recorded using a mercury sphygmomanometer (Diamond) in lying down position twice with a gap of 15 minutes between each recording. Heart rate and heart rate variability (HRV) was recorded and analyzed by Powerlab (AD instruments). ECG electrodes were connected in lying down position for Lead II and recording was obtained for 15 minutes. Ectopics and artifacts were removed from the recording. HRV analysis was done in the frequency and time domains [4]. Frequency domain indices such as relative power of the low-frequency band (0.04–0.15 Hz) in normalized units (LFnu), relative power of the high-frequency band (0.15–0.4 Hz) in normalized units (HFnu) and ratio of LF-to-HF power (LF/HF) were noted. Time domain indices such as standard deviation of normal to normal intervals (SDNN), standard deviation of the average NN intervals for each 5 min segment (SDANN), root mean square of successive RR interval differences (RMSSD) and percentage of successive RR intervals that differ by more than 50 ms (pNN50) were also noted. Statistical analysis was done using SPSS 19.0 version software by Student's t test and a p value <0.05 was considered statistically significant.

3. Results

Thirty subjects were recruited in each group and as one sample HRV could not be analyzed due to many errors, analysis was done for the rest twenty-nine samples. The mean age and the gestational age of the mothers in both NP and PE groups were comparable and did not show any statistical difference (Table 1).

Table 1. Mean maternal and gestational age in NP and PE groups

	Normal pregnancy (NP) (n=29)	**Preeclampsia (PE) (n=29)**	**t value**	**P value**
Maternal age (years)	22.93 ± 3.17	22.62 ± 3.89	0.355	0.740
Gestational Age (weeks)	37.59 ± 3.20	35.97± 4.30	1.668	0.109

Both the systolic and diastolic blood pressures were significantly higher in preeclampsia as compared to normal pregnancy (Figure 1).

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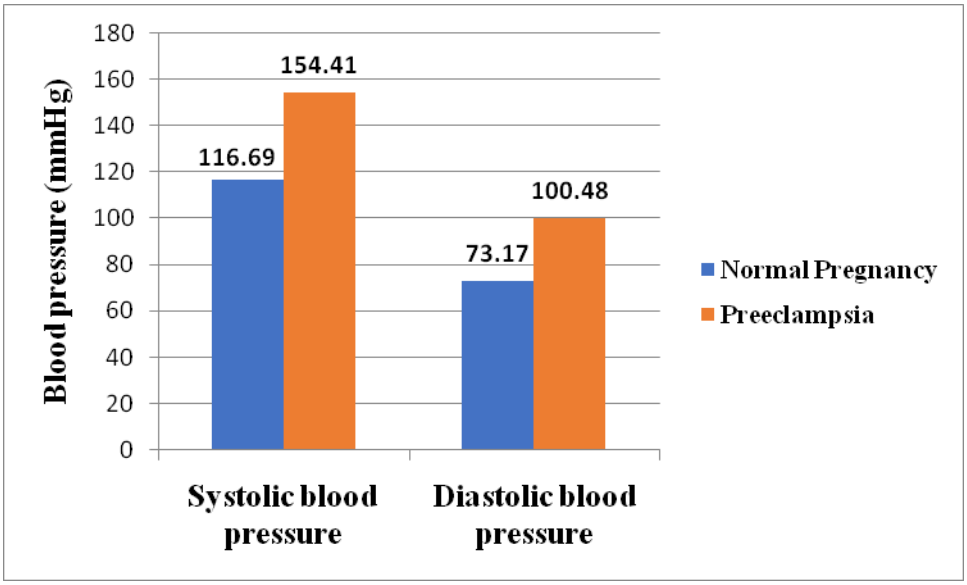

Figure 1. Blood pressures in normal pregnancy and preeclampsia

The mean heart rate (beats/min) was slightly lower (92.69 ± 12.10) in preeclampsia as compared to normal pregnancy (93.41 ± 9.51), but was not statistically significant (p=0.801).

Heart rate variability analysis revealed that there was a significant increase in the low frequency (LF) component, LF/HF ratio and a decrease in high frequency (HF) component in pre-eclampsia. The time domain parameters, SDNN, SDANN, RMSSD and pNN50, showed a fall in preeclampsia (Table 2).

Table 2. Heart rate variability analysis in NP and PE groups

	Normal Pregnancy (NP) (n=29)	**Preeclampsia(PE) (n=29)**	**t value**	**p value**
Frequency domain				
LF(nu)	54.84 ± 15.93	67.01 ± 23.95	2.318	**0.024**
HF(nu)	41.07 ± 13.86	33.41 ± 12.50	2.245	**0.029**
LF/HF ratio	1.54 ± 0.77	2.44 ± 1.54	2.910	**0.005**
Time domain				
SDNN (ms)	50.24 ± 23.62	35.17 ± 20.20	2.655	**0.010**
SDANN (ms)	18.46 ± 12.79	10.94 ± 8.49	2.684	**0.009**
RMSSD (ms)	37.39 ± 30.58	18.71 ± 12.80	3.087	**0.003**
pNN50 (%)	4.49 ± 3.97	2.37 ± 2.10	2.576	**0.013**

4. Discussion

Elevated levels of systolic and diastolic blood pressure in pre-eclampsia may depict an increase in

sympathetic activity in pre-eclampsia, with a resulting increase in the peripheral resistance [10]. Peripheral resistance influences the diastolic blood pressure, whereas cardiac output influences the systolic blood pressure, and cardiac output, in turn, is given by heart rate and stroke volume11 (Guyton). As there was no significant change in heart rate in the present study, the stroke volume would be responsible for a significant change in systolic blood pressure. Stroke volume would again depend on venous return and myocardial contractility [11] (Guyton). Reduction in the plasma volume is seen in pre-eclampsia [12] and hence, contractility of the myocardium would determine the systolic blood pressure. An increase in the sympathetic activity would thus explain the increase in myocardial contractility and peripheral resistance and hence, increase in both systolic and diastolic blood pressures. There was a significant increase in the low frequency (LF) component, LF/HF ratio and a decrease in high frequency (HF) component in pre-eclampsia, on frequency domain analysis of the heart rate variability in the present study and the same was also reported by other authors [13- 15]. LF component reflects sympathetic tone and HF component reflects parasympathetic tone; whereas the LF/HF ratio depicts the sympatho-vagal balance, an increase indicates sympathetic predominance and a decrease signifies parasympathetic predominance [4], [14]. The time domain parameters, SDNN, SDANN, RMSSD and pNN50, showed a fall in preeclampsia in the present study as observed by others too13. These time domain parameters of HRV indicate parasympathetic activity [4], [14]. In the present study, there was sympathetic over activity combined with parasympathetic withdrawal, which would influence the outcome of pre-eclampsia. This and even the magnitude of variability may also determine the risk of developing cardiovascular diseases even after the termination of pregnancy. Hence, autonomic function testing in the form of heart rate variability may be used as a tool for early diagnosis, management and prevention of future cardiovascular diseases in pre-eclampsia women.

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Interplay of Oxidative Stress and Nitric Oxide Synthase Gene Expression on Cardiovascular Responses in Preeclampsia

Interação do estresse oxidativo e da expressão dos genes das óxido nítrico sintases nas respostas cardiovasculares na pré-eclâmpsia

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Abstract

Objective To assess the influence of oxidative stress on the gene expression of nitric oxide synthases (NOS 3 and NOS 2) and, hence, the cardiovascular responses in preeclampsia.

Methods This was a case control study in which patients with preeclampsia (PE group) and normal pregnancy controls (NP group) were included according to the guidelines of the American College of Obstetricians and Gynecologists (ACOG). The serum levels of malondialdehyde (MDA), total antioxidant capacity, and nitric oxide (NO) were estimated, and the heart rate and mean arterial pressure were recorded. The gene profiling of NOS3 and NOS2 was performed through real-time polymerase chain reaction (RT-PCR). The statistical analysis was performed using the Student *t*-test, and values of $p < 0.05$ were considered statistically significant.

Results The serum levels of malondialdehyde were increased ($p < 0.0001$), and the total antioxidant capacity was reduced in the PE group ($p = 0.034$), indicating oxidative stress. In the PE group, the mean arterial pressure was significantly higher ($p < 0.0001$), but the serum levels of NO did not show a statistically significant reduction ($p = 0.20$). The gene expression profiling of NOS3 and NOS2 revealed a down regulation in the PE group by 8.49 and 51.05 times respectively.

Conclusion Oxidative stress may lead to endothelial dysfunction, which could result in increased mean arterial pressure. Nitric oxide may play a role in this mechanism,

Keywords
- molecular genetics
- preeclampsia/ eclampsia
- vascular biology

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but interactions with other vasoactive /biological substances cannot be overlooked, as the gene expression of NOS3 and NOS2 has been reduced.

Resumo

Objetivo Avaliar a influência do estresse oxidativo na expressão genética das óxido nítrico sintases (*nitric oxide synthases*, NOS, em inglês; NOS 3 e NOS 2) e, consequentemente, nas respostas cardiovasculares na pré-eclâmpsia.

Métodos Este foi um estudo caso-controle no qual pacientes com pré-eclâmpsia (grupo PE) e controles com gravidez normal (grupo GN) foram incluídos de acordo com as diretrizes do American College of Obstetricians and Gynecologists (ACOG). Foram estimados os níveis séricos de malondialdeído (MDA) da capacidade antioxidante total, e de óxido nítrico (*nitric oxide*, NO, em inglês). A frequência cardíaca e a pressão arterial média foram registradas. O perfil genético da NOS3 e da NOS2 foi feito por reação em cadeia de polimerase em tempo real (*real-time polymerase chain reaction*, RT-PCR, em inglês). A análise estatística foi feita utilizando-se o teste *t* de Student, e valores de $p < 0,05$ foram considerados estatisticamente significativos.

Resultados Os níveis séricos de malondialdeído sérico estavam aumentados ($p < 0,0001$), e a capacidade antioxidante total, reduzida no grupo PE ($p = 0,034$), o que indicava estresse oxidativo. No grupo PE, a pressão arterial média era significativamente maior ($p < 0,0001$), mas os níveis séricos de NO não demostraram redução estatisticamente significativa ($p = 0,20$). O perfil de expressão genética da NOS3 e da NOS2 revelou uma regulação negativa no grupo PE de 8,49 e 51,05 vezes, respectivamente.

Conclusão O estresse oxidativo pode levar à disfunção endotelial, o que pode resultar em aumento da pressão arterial média. O NO pode desempenhar um papel neste mecanismo, mas as interações com outras substâncias vasoativas/biológicas não podem ser negligenciadas, uma vez que a expressão genética da NOS3 e da NOS2 foi reduzida.

Palavras-chave
- genética molecular
- pré-eclâmpsia/eclâmpsia
- biologia vascular

Introduction

Normal pregnancy is marked by systemic inflammation, oxidative stress, and changes in angiogenic factors and vascular reactivity. This phenomenon is very much increased in pre-eclampsia (PE), with an impairment of compensatory mechanisms, eventually leading to vascular dysfunction.[1] The pathophysiology of PE is not completely understood. Nitric oxide (NO) may be responsible for gestational vasodilation due to its vasodilator action.[2] The production of NO from L-arginine is catalyzed by nitric oxide synthases (NOSs), which include neuronal NOS, endothelial NOS (eNOS/NOS3) and inducible NOS (iNOS/NOS2).[3]

Reduced uterine perfusion pressure (RUPP) and placental ischemia may lead to endothelial and cardiovascular dysfunction through increased production of cytokines, which may trigger endothelial dysfunction by decreasing the bioavailability of NO and increasing that of reactive oxygen species (ROS).[4]

The maternal vasculature is a major source of reactive oxygen and nitrogen species, which can interact to produce peroxynitrite, a powerful prooxidant that alters vascular function in PE.[1] Preeclampsia increases oxidative stress in the placental and maternal systemic circulations.[5]

The expression of NOSs alters in PE; upregulation of eNOS expression has been demonstrated during normal pregnancy,[6] but the expressions of messenger ribonucleic acid (mRNA) and protein for eNOS are decreased in endothelial cells in cases of PE.[7]

However, there is a dearth of literature highlighting the role of oxidative stress on NOS gene expressions and the impact of these on the systemic circulation. Hence, the present study aims to assess the influence of oxidative stress on NOS 3 and NOS 2 gene expressions and, hence, the cardiovascular responses in PE.

Methods

This was a case-control study conducted in a tertiary care centre in North Karnataka, India. Clearance was obtained from the Ethics Committee at S. Nijalingappa Medical College, Bagalkot, Karnataka, India (SNMC/IECHSR/2017-18/A-48/1.1). Informed consent was obtained from all the participants. The size of the sample would have to be 23 participants in each group, which was calculated using the OpenEpi software, based on a study by Madazli et al., 2002[8] (the mean SOD between the groups was used), and 22 participants in each group, based on a study by Kashinakunti et al., 2010[9] (the mean MDA levels between the groups were used). Hence, the sample size was rounded off to 30 participants in each group.

Primigravidas aged between 18 and 35 years with singleton pregnancies and diagnosed with PE according to the guidelines of the American College of Obstetricians and

Gynecologists (ACOG)[10] were included in the study as the cases (PE group). Pregnant women diagnosed with gestational hypertension or eclampsia according to the ACOG guidelines, and those with diabetes mellitus, history of systemic hypertension, and cardiovascular or renal diseases were excluded from the study.

Healthy primigravidas aged between 18 and 35 years with singleton pregnancies, matched for age and gestational week with the cases were included as controls (normal pregnancy [NP] group), and pregnant women with a history of diabetes mellitus, systemic hypertension, PE, and cardiovascular or renal diseases were excluded from the control group.

The required demographics were collected from the participants according to a predesigned proforma. They were clinically examined, and the findings were recorded. Gestational age was calculated from first trimester ultrasonography report and as per the ACOG guidelines.[11] Blood pressure was recorded using a mercury sphygmomanometer (Diamond, Maharashtra, India). The heart rate was caculated from lead II of the electrocardiogram (ECG), which was recorded using Powerlab (AD instruments, Sidney, Australia). The blood samples were drawn from the antecubital vein following aseptic precautions. Whole blood (2.5 mL) was transferred to PAXgene (QIAgen, Venlo, Netherlands) ribonucleic acid (RNA) tubes for NOS gene expression by real-time polymerase chain reaction (RT-PCR). Another 2.5 mL of blood was converted into serum for the estimation of the serum levels of NO (enzyme-linked immunosorbent assay [ELISA] kit method) and serum malondialdehyde (MDA, using the thiobarbituric acid reactive substance [TBARS] method), and the total antioxidant capacity (ELISA kit method).

Gene Expression Profiling

Isolation of the total RNA from human whole blood was performed using the PAXGene RNA isolation kit and following the manufacturer instructions. The quality of the total RNA was checked by agarose gel (1.5%) electrophoresis, and the gel was observed with an ultraviolet (UV) transilluminator (Cleaver Scientific, Rugby, Warwickshire, United Kingdom). Complementary deoxyribonucleic acid (cDNA) was synthesised, and its quality was checked with agarose gel (1.5%) electrophoresis with a standardized protocol.

Expression Profiling of NOS3

The gene expression profiling for NOS3 in samples from both the groups (NP and PE) was analysed using forward primer *5' CTGGCTTTCCCTTCCAGAT 3'* and reverse primer *5' CTTAATCTGGAAGGCCCCTC 3'*, along with the GAPDH gene as a positive control.

Expression Profiling of NOS2

The gene expression profiling for NOS2 in samples from both the groups (NP and PE) was analysed using forward primer *5' GATATCCCCCAGCCCTCAAGT 3'* and reverse primer *5' GAGGCCCCAGTTTGAGAGAG 3'*, along with the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as a positive control.

Each reaction was analysed in triplicate along with a negative control (without sample). Quantification cycles (Cq) values were recorded using the CFX Real-Time PCR (Bio-Rad, Hercules, CA, United States). The gene expressions of NOS3 and NOS2 were calculated using 2^ - ΔΔCq formula.

The serum levels of nitric oxide and total antioxidant capacity were estimated using ELISA kits as per the manufacturer's guidelines, and the serum levels of MDA were estimated by the TBARS method.[12]

Statistical Analysis

Data was analyzed with the Statistical Package for the Social Sciences (IBM SPSS Statistics for Windows, IBM Corp., Amonk, NY, United States) software, version 19.0, using the Student *t*–test, and values of $p < 0.05$ were considered statistically significant.

Results

►**Table 1** depicts the mean age and the gestational age of the mothers in both groups, and the results showed that there was no significant difference between them.

Table 1 Maternal and gestational ages in normal pregnancy and preeclampsia

	Normal pregnancy (NP) group (n = 30)	Preeclampsia (PE) group (n = 30)	t-value	*p*-value
Maternal age (years)	22.97 ± 3.12	22.67 ± 3.83	0.333	0.215
Gestational age (weeks)	37.63 ± 3.16	36.10 ± 4.29	1.578	0.120

Table 2 Oxidative stress markers in normal pregnancy and preeclampsia

	Normal pregnancy (NP) group (n = 30)	Preeclampsia (PE) group (n = 30)	t-value	*p*-value
Serum malondialdehyde (nmol/mL)	2.50 ± 0.42	5.66 ± 1.02	12.682	0.0001
Serum total antioxidant capacity (ng/mL)	144.56 ± 65.24	112.37 ± 48.36	2.171	0.034

Table 3 Cardiovascular responses in normal pregnancy and preeclampsia

	Normal pregnancy (NP) group (n = 30)	Preeclampsia (PE) group (n = 30)	t-value	*p*-value
Systolic blood pressure (mmHg)	116.80 ± 10.68	154.07 ± 17.10	10.122	0.0001
Diastolic blood pressure (mmHg)	73.40 ± 8.60	100.20 ± 8.64	12.044	0.0001
Mean arterial pressure (mmHg)	87.87 ± 8.19	118.16 ± 10.95	12.134	0.0001
Heart rate (beats/min)	93.10 ± 9.50	92.33 ± 12.04	0.274	0.785

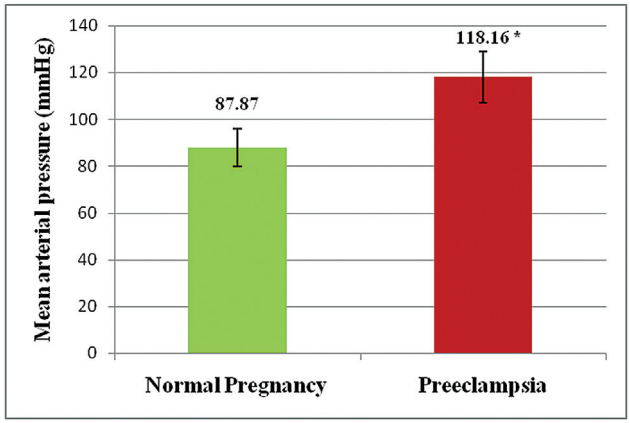

Fig. 1. Mean arterial pressure of patients with preeclampsia.

A significant oxidative stress was noted in the PE group by an increase in the serum levels of MDA and a decrease in total antioxidant capacity (►**Table 2**).

The systolic, diastolic, and mean arterial pressures (in mmHg) were found to be significantly higher, by 37.26, 26.80, and 30.28 respectively in the PE group (►**Table 3**) (►**Figure 1**). The mean heart rate was slightly lower in the PE group as compared to the NP group, but not statistically significant.

There was no significant difference in the serum levels of NO in both the groups (NP group: 35.43 ± 20.37 µmol/L; PE group: 30.09 ± 10.08 µmol/L; t = 1.285; $p = 0.204$). The gene expression profiling was only performed in 25 suitable samples from each group. The mean difference in the Cq values of NOS3 in the RT-PCR, as against the positive control (GAPDH), was higher in the PE compared to the NP group. Consequently, when the gene expression of NOS3 was calculated, a down-regulation was observed in the PE group compared to the NP group by a difference of 8.49 times (►**Table 4**).

The mean difference in the Cq values of NOS2 in the RT-PCR as against the positive control (GAPDH) was higher in the PE group; consequently, when the gene expression of NOS2 was calculated, a down-regulation was oberved in the PE group compared to the NP group, by a difference of 51.05 times (►**Table 5**).

Discussion

Pregnancy is a state of oxidative stress due to increased metabolism in the mother and the metabolic activity of the placenta. The ischemic or poorly-perfused placenta resulting from inadequate trophoblast invasion exhibits increased oxidative stress in PE, evident from the increased formation of free radicals, lipid peroxides, and reduced antioxidant defenses.[1,13] The mean plasma levels of MDA were higher, and the levels of glutathione and superoxide dismutase were significantly lower in PE.[8,14] In the present study, we also observed an increase in the oxidative stress marker, serum MDA, which was accompanied by a decrease in the serum levels of total antioxidant capacity. In a study by Lee et al.,[15] the neutrophils from women with PE produced significantly more ROS than those of the age-matched normotensive controls. Antioxidants protect against lipid peroxidation mediated by free radical. Normal pregnancy is associated with an increase in oxidative stress and lipid peroxidation, but antioxidant protection also increases.[16] In PE, there is still a further increase in lipid peroxides as well as an insufficient increase in antioxidants to combat the increase in oxidative stress and lipid peroxidation.[17] An increase in oxidative stress and a decrease in antioxidants may be

Table 4 Gene expression of endothelial nitric oxide synthase (NOS3) by real-time polymerase chain reaction

	Cq of the sample (NOS3) 1	Cq of the positive control (GAPDH) 2	ΔCq 1 - 2	ΔΔCq {Δcq of sample – average Δcq of control group}	2^ - ΔΔCq
Normal pregnancy group (n = 25)	23.14 ± 3.43	15.11 ± 1.82	7.96 ± 3.30	0.32 ± 3.63	14.63
Preeclampsia group (n = 25)	24.73 ± 2.56	15.63 ± 3.19	9.10 ± 2.74	1.14 ± 2.74	6.14

Abbreviations: Cq: Quantification cycles; GAPDH: Glyceradehyde 3-Phosphate dehydrogenase.

Table 5 Gene expression of inducible nitric oxide synthase (NOS2) by real-time polymerase chain reaction

	Cq of the sample (NOS2)	Cq of the positive control (GAPDH)	ΔCq	ΔΔCq {Δcq of sample – average Δcq of control group}	2^ - ΔΔCq
Normal pregnancy group (n = 25)	26.63 ± 1.79	16.47 ± 3.34	10.15 ± 3.49	0.004 ± 3.49	51.07
Preeclampsia group (n = 25)	27.40 ± 1.27	16.24 ± 2.59	11.16 ± 2.42	10.38 ± 3.19	0.02

Abbreviations: Cq: Quantification cycles; GAPDH: Glyceradehyde 3-Phosphate dehydrogenase.

responsible for the subsequent pathophysiology of PE. However, in one study,[18] supplementation with antioxidants like vitamins C and E did not show any difference in terms of reduction in the incidence of PE.

Reactive oxygen species seem to play an important role in the endothelial dysfunction associated with PE. Oxidative stress induces the adhesion of leukocytes and platelets to the endothelium as well as the release of cytokines and anti-angiogenic factors, which suggest an inflammatory state, as observed in PE.

Vascular endotheliosis, an increase in the inflammatory vasoconstrictors, and a decrease in the nitric oxide may have been responsible for an increase in the mean arterial pressure, as observed in the present study. A previous study[19] also revealed an increment of 13.3% in the mean arterial pressure in PE as against 5.2% in the normotensive group. But the decrease in serum NO was not statistically significant. Similarly, studies show an increase in NO production during pregnancy, and a decrease during PE,[3] or an increase in NO levels in PE,[20] or no change in NO levels.[21]

Through the intrinsic synthesis of NO, eNOS is expressed constitutively in the vascular endothelium and maintains the vascular tone, hence inhibiting the adhesion of leukocytes and platelets to the endothelium and preventing the proinflammatory state.[22] Increased ROS production seems to suppress the expression and function of eNOS.[23] In the present study, a decrease in the expression of NOS3 was observed. Reduced expression of eNOS and oxidative stress could play a role in the pathology of PE, both in the placenta and in the maternal endothelium.[24] Increased ROS leads to lipid peroxidation, which increases the levels of MDA, as observed in the present study, and lipid peroxidation also decreases the calcium/adenosine triphosphatase (ATPase) (ATPase) activity, which in turn, may decrease the eNOS expression, as it is calcium dependent.[14]

Oxidative stress leads to up-regulation of proinflammatory genes like iNOS, which is stimulated in a proinflammatory condition, such as NP, and produces a temporary excess of NO, maybe through activation of proinflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).[18] The literature also reports that the expression of iNOS is decreased, and that of eNOS is increased, as a result of the compensatory mechanism at a later stage in PE.[14] In the present study, the gene expressions of iNOS or NOS2, as well as of eNOS or NOS 3, were reduced. But our understanding is that a decrease in antioxidants as the cause has not been well supported, as supplementation of vitamins C and E failed to produce any positive response in PE. Hence, other mechanisms involved have to be contemplated. In PE, the inflammatory condition and excessive oxidative stress may stimulate the production of heat shock proteins (HSPs), which may suppress iNOS; and the role of VEGF, s-Flt or HIF-alpha should also be considered. The exact mechanisms underlying the pathophysiology of PE need to be researched in depth for the proper understanding, diagnosis, and therapeutic options. Although the gene expression was done in the present study, the quantification of the expression of proteins was not feasible with the available resources. A follow-up study on PE patients with serial NOS2 and NOS 3 gene expression is contemplated. The authors also intend to study the role of HSPs in PE.

Conclusion

Oxidative stress may lead to endothelial dysfunction, which could result in increased mean arterial pressure, and NO may play a role in this mechanism, but interactions with other vasoactive /biological substances cannot be overlooked, as the gene expressions of NOS3 and NOS2 have been reduced.

Contributions

All the authors have contributed equally to this paper, namely to the conception and design, data collection or analysis, and interpretation of data, writing of the article, and review of the intellectual content. Therefore, all authors approved the final version to be published.

Conflict of Interests

The authors have no conflict of interests to declare.

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