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C-786T (Promoter) Allelic Variants Of Nitric Oxide Synthase Gene And Its Association With Nitric Oxide In Preeclampsia-Case Control Study.

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ABSTRACT

Pregnancy is a hyper coagulable state with increased tendency for thrombus formation. The etiology of preeclampsia is multifactorial and regulated by multiple genetic pathways. preeclampsia and endothelial nitricoxide synthase (eNOS)gene polymorphism was studied. To find out the distribution of allelic variant in promoter T-786C of eNOS Gene and the association between NOS gene polymorphism and preeclampsia. To assess nitric oxide level and correlate its level with eNOS gene polymorphism in our population. This study included 50 preeclampsia women and 50 healthy pregnant women in the age group of 20- 40 years with the gestational age of 24- 36 weeks of pregnancy. DNA was extracted from whole blood samples. The PCR products for promoter T-786 CofeNOS gene of polymorphism were separated electrophoretically using 2 %agarose gel and the serum nitric oxide level was estimated by Griess method. The C allele carrier which is represented by CC + CT genotypes and the Callele of promoter T-786C of eNOS gene of polymorphism were significantly associated with increased risk of preeclampsia. The mean (SD) levels of serum nitric oxide among the cases was lower than the controls and this difference was found to be statistically significant. The C allelic carrier (CC+ CT) was found to have lower level of serum nitric oxide and eNOS C-786T(Promoter)polymorphism may exert an effect on serum nitric oxide level by altering the transcriptional efficiency. So this eNOS C-786T(Promoter)polymorphism and lower level of serum nitric oxide are the possible risk factor for preeclampsia.

Keywords: Preeclampsia, Polymorphism of eNOS gene promoter C-786T, Nitric Oxide and Genotype (CC/CT/TT)

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INTRODUCTION

Preeclampsia, a pregnancy related disorder that is characterized by hypertension, proteinuria, and end –organ damage in the mother. As per the estimates of World Health Organisation, the global incidence of preeclampsia is 5% to 7% and in India it's reported in 8-10% of all pregnancies. In developing and developed countries, preeclampsia is one of the leading causes of maternal mortality and morbidity. These are explained by generalized vascular endothelial cell dysfunction. Essential in the pathogenesis of preeclampsia is endothelial dysfunction due to impaired trophoblast invasion and spiral artery remodelling, resulting in abnormal implantation and placental hypo-perfusion [1, 2]. As a potent vasodilator, circulating nitric oxide (NO) plays a crucial role in endothelial function regulation, blood pressure control and cardiovascular homeostasis [3, 4]. Endothelial NO availability is largely regulated by its synthesis by eNOS. The gene that encodes eNOS3, is therefore considered as a candidate gene for preeclampsia [5]. The endothelial nitric oxide synthase gene (NOS3), located at the 7q35-q36 region, has emerged as a logical candidate gene in the development of preeclampsia. Variants (polymorphisms) of the NOS3 gene have been investigated for their association with preeclampsia and other disorders such as hypertension. The three most common variants examined for clinical relevance, based on their potential functional effects are (i) a G894T substitution in exon 7 resulting in a Glu to Asp substitution at codon 298 (rs1799983), (ii) an insertion-deletion in intron 4 (4a/b) consisting of two alleles (the a*-deletion which has four tandem 27-bp repeats and the b*-insertion having five repeats), and (iii) a T786C substitution in the promoter region (rs2070744).The presence of mutated homozygous CC genotype and C allele of -786T/C polymorphism of eNOS gene influences the higher susceptibility to develop severe Preeclampsia development [6].

Aim Of The Study

To find an association between eNOS gene polymorphism and preeclampsia.

Objectives

To find out the distribution of allelic variant in promoter T-786C of Enos Gene and the association between gene polymorphism and preeclampsia.

To assess nitric oxide level and correlate its level with eNOS gene polymorphism in our population.

MATERIALS AND METHOD

It is a case control study conducted in patients attending Department of obstetrics and Gynaecology of Kilpauk Medical college and Hospital for a period of six months with a total of 100 sample size with Cases: 50 Preeclampsia women in the age group of 20-40 years and Controls: 50 healthy pregnancy women.

Inclusion Criteria

Pregnant women age group of 20-40 years (Gestational age of 24 to 36 weeks of pregnancy) Blood pressure higher than 140/90 mmHg on two occasions more than 6hrs apart and proteinuria-300mg/24hrs, after the 20th week of pregnancy.

Exclusion Criteria

Patients with Renal disease, Chronic illness, Diabetes mellitus Systemic hypertension, Acute/Chronic infection, Ischaemic stroke &Coronary artery disease, Fever, Previous H/O Recurrent pregnancy loss, Previous H/O preeclampsia , Anti Phospholipid Antibody Syndrome, Multiple pregnancy, Hypothyroidism were all excluded from the study. After obtaining consent, under aseptic precautions, 5ml of venous blood sample was collected after an overnight fasting from all subjects in serum tube and EDTA tube and mixed by gentle shaking. Buffy coat/whole blood used for DNA extraction and serum used for biochemical analysis.

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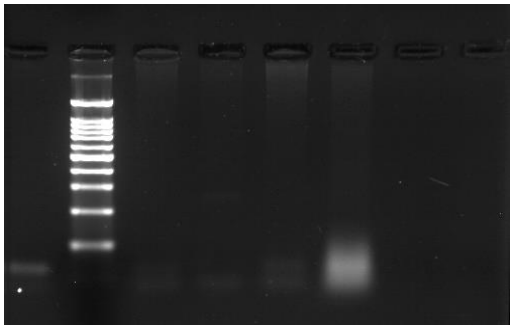
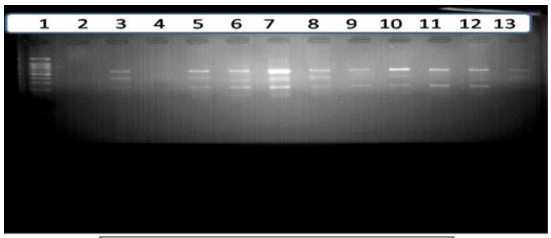
DNA Extraction was done by DNA Mini preparation Kit (From helini biomolecules, Chennai) according to the manufacturer's instruction. Extracted DNA was identified by 1% agarose gel electrophoresis and comparison with a known molecular weight 1kb DNA (Lambda DNA) ladder. C allele was F:CCTCCACTGCTTTTCAGAGG and R:CTGAGGCAGGGTCAGACG (365bp) T allele was F:CATCAAGCTCTTCCCTGTCT and R:TGACATTAGGGTATCCCTTCC (211bp). 2×PCR Master mix was used. Amplification of the extracted DNA was carried out in CYBERLAB SMART PCR-PRO, thermal cycler. PCR product was run on 2.5% agarose gel. Measurement of serum nitricoxide was done by cadmium based reduction of nitrite to nitrate followed by Greiss method in Spectrophotometer.





Lane 1 – 100 bp DNA ladder
Lane 2,4 – Negative control
Lane 3,5,6,7,8,10 – CT genotype
Lane 9,11,12-TT genotype
Lane 13 – CC genotype

Statistical Analysis

The student *t test* was used to analyse clinical and laboratory data, and the χ2 test wherever required. The frequency of genotypes (TT, CT, CC) at -786 promotor region of the eNOS gene by using the χ2 test.

Allele frequency by using the equation p + q = 1 where p and q are the frequencies of each allele at the particular locus. Hardy Weinberg equation equilibrium as shown by p2 + q2 + 2pq = 1.

The unpaired Student t *test* and analysis of variance to analyse the significance of difference in values of nitric acid in different genotypes at 786 promotor regions.

RESULTS

Table 1: Shows the baseline characters of variables such as age, gestational age, gravida and genotype.

Variable		Cases Mean (SD)	Control Mean (SD)	P value
Age		26.00 (0.48)	25.04 (0.51)	0.17 ^
Gestational age(weeks)		32.86 (0.61)	33.80 (0.42)	0.21^
Variable	Category	Cases No (%)	Control No (%)	P value
Gravida	1	28 (48.3%)	30 (51.7%)	0.42*
	2	22 (52.4%)	20 (47.6%)	
Genotype	CC genotype	12 (75.0%)	4 (25.0%)	0.06*
	CT genotype	23 (59.0%)	16 (41.0%)	
	TT genotype	15 (33.3%)	66.7%)	

^ - independent t test * - Chi square test

Table 2: Genotype

	Cases	Control	Odds ratio	P value
CC + CT	35 (63.6%)	20 (36.4%)	3.5 (1.529 – 8.012)	0.003
TT	15 (33.3%)	30 (66.&%)		

The presence of C gene in cases is 3.5 times more than that of the controls. The 95% confidence interval is 1.529 – 8.012 ($p<0.01$).

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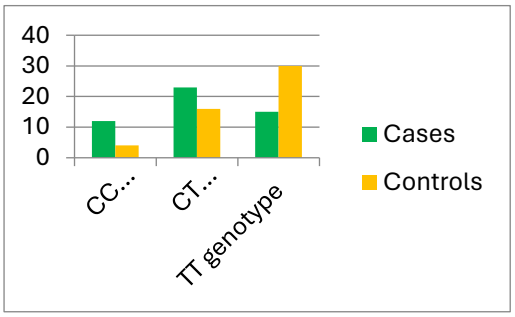
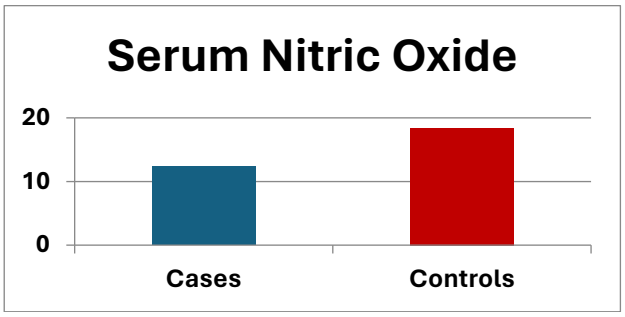
Bar Diagram 1: Genotype

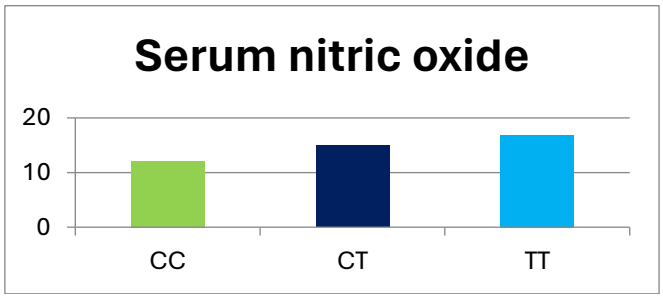


Bar Diagram 2: Serum nitric oxide



The mean (SD) levels of Serum nitric oxide among the cases was 12.32(4.79) which was lower than the mean (SD) Serum nitric oxide levels among the controls, 18.40(6.85). This difference was found to be statistically significant by t test ($p < 0.001$).

Bar Diagram 3



The mean (SD) levels of Serum nitric oxide among the persons with CC genotype was 12.08(4.61) ; for CT genotype was 14.95(7.31) and that of TT genotype was 16.88(6.25) . ANOVA test for the difference in the values of was found to be statistically significant ($p<0.05$). On post hoc test the statistically significant difference was found between CC genotype and TT genotype.

CC and CT genotype is more among cases in comparison to controls which was also statistically significant

In Serum nitric oxide the difference was found to be statistically significant by t test ($p < 0.001$).

DISCUSSION

Preeclampsia is an important clinical problem that has been extensively studied, but the causes and treatment have not been fully resolved [6]. Preeclampsia affects about 1-5% of women who conceive [7] and accounts for about 20% of clinically recognized pregnancy losses [8]. Despite extensive research to explain the causative effects of preeclampsia, about 50% - 60% of cases are still idiopathic. Endothelial damage, impaired placental vascularization, and resultant oxidative stress have been proposed to play a role in the pathophysiology of preeclampsia.

In a normal pregnancy, the NO pathway is activated, leading to increased NO availability and levels, which are further responsible for maternal vasodilation required to accommodate the increase in circulating volume during pregnancy without a rise in blood pressure [9-11]. Endothelial NO synthase (eNOS) has been regarded as the source of endothelial NO, which plays a critical role in vascular physiology and impaired placental vascularization [12].

In recent years, much attention has been paid to determining the association between the eNOS gene 786 T (Promoter) allelic variant and preeclampsia. The rapid accumulation of advanced knowledge in genetics and molecular biology, as well as fast progress in diagnostic methods, has enabled the development of a new research field called molecular medicine. A key method used in that kind of diagnostics is polymerase chain reaction (PCR)–based applications, which are characterized by much

higher sensitivity and specificity than classical diagnostics [13]. The pathogenesis of preeclampsia is poorly understood, and the search for low-penetrance genes by hypothesis-driven candidate-gene studies (genetic association study-GAS) and hypothesis-free genome-wide association studies is ongoing [14].

In our study the mean (SD) levels of Serum nitric oxide among the persons with CC genotype was 12.08(4.61) ; for CT genotype was 14.95(7.31) and that of TT genotype was 16.88(6.25) . ANOVA test for the difference in the values of was found to be statistically significant ($p<0.05$). On post hoc test the statistically significant difference was found between CC genotype and TT genotype. Results of the present study showed that the -786 T-allele of the promoter polymorphism is associated with lower NO levels. Reporter gene studies have shown that promoter -786T (Promoter) substitution markedly blunts the transcription rate of the eNOS gene, and hence NO production. These findings confirm our results that women with preeclampsia are associated with a high frequency of the promoter-786T (Promoter) allelic variant, which might explain why this polymorphism is associated with low serum NO levels.

Our finding also shows the promoter -786T polymorphism of the eNOS gene, namely "allele -786T," is associated with preeclampsia in Chennai women residing in South India. T allele carriers, represented by (CC + CT+TT) genotypes, and the T allele of the promoter -786T polymorphism are possible risk factors for preeclampsia, as they were presented with a high frequency in women with preeclampsia and were associated with decreased serum NO levels in this group. Alpoim et al [15] states that the distribution of T786C alleles was proportional between preeclamptic and normotensives women. Ben Ali Gannoun et al [16]. also found higher frequencies of homozygous -786T/-786T (rs2070744) in Preeclampsia. g.-786T > C is also associated with increased risk for developing severe PE according to Seremak- Mrozikiewicz et al [17]. Mechanistic [18] studies suggest that inflammation and oxidative stress resulting from decreased NO production related to eNOS polymorphisms can play a critical role in Preeclampsia. For example, Zhuge et al [19] proposed that eNOS deficiency leads to decreased red blood cell-derived NO bioactivity, subsequent vascular oxidative stress, endothelial dysfunction and PE. Most recently, it is reported that preeclamptic women had reduced plasma eNOS concentrations emphasizing importance of eNOS in pregnancy

We recommend testing for the promoter -786T (Promoter) allelic variant polymorphism of the eNOS gene in all Indian women experiencing preeclampsia or unexplained hypertension during pregnancy. Since the NO pathway plays an important role in the pathophysiology of preeclampsia, any factors balancing NO metabolism could be useful in the treatment of preeclampsia, consequently reducing substantial morbidity and associated maternal and fetal mortality.

CONCLUSION

In summary, despite extensive research, preeclampsia remains a significant clinical challenge, affecting a notable percentage of pregnant women and contributing to pregnancy losses. While progress has been made in understanding its pathophysiology, much remains idiopathic, with hypotheses centred on endothelial damage, impaired placental vascularization, and oxidative stress.

Recent investigations into endothelial nitric oxide synthase (eNOS) gene polymorphisms, particularly the -786 T variant, have revealed potential mechanisms underlying preeclampsia. The association of the -786 T allele with reduced nitric oxide (NO) levels highlights the role of NO pathway dysregulation in its development.

These findings suggest a genetic predisposition to preeclampsia, notably among certain ethnic groups like Chennai women in South India. Screening for eNOS gene polymorphisms, especially the -786 T variant, holds promise as a diagnostic tool for identifying at-risk women.

Moreover, these discoveries have broader implications in molecular medicine, leveraging advancements in genetics and molecular biology, particularly through polymerase chain reaction (PCR) based techniques, to explore new research avenues and potential therapies.

Further studies are warranted to fully elucidate the intricate interplay between genetic predisposition, NO metabolism, and preeclampsia. Understanding these mechanisms may lead to more targeted treatments, ultimately reducing the substantial morbidity and mortality associated with this condition.

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