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**INTERNATIONAL JOURNAL OF
 ADVANCED RESEARCH (IJAR)**

Article DOI:10.21474/IJAR01/8748
 DOI URL: <http://dx.doi.org/10.21474/IJAR01/8748>



RESEARCH ARTICLE

HIGH PLASMA MITOCHONDRIAL DNA EARLY IN PREGNANCY IS A RISK FACTOR FOR DEVELOPMENT OF PREECLAMPSIA.

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Manuscript Info

Manuscript History

Received: 19 January 2019

Final Accepted: 21 February 2019

Published: March 2019

Key words:-

Preeclampsia, Primigravida,
 mitochondrial DNA, Early prediction.

Abstract

Objectives: Estimation of plasma mitochondrial DNA (mtDNA) levels in primigravida during 1st trimester and evaluation of its relation to development and severity of preeclampsia (PE).

Patients & Methods: The study included 44 PE women (PE group) and 10 women free of PE (Control group). At the 1st visit, all women underwent complete clinical examination and gave fasting blood sample for investigations. All women were asked to attend 4-weekly till delivery for evaluation of PE manifestations including increased systolic (SBP) and diastolic blood pressure (DBP) with proteinuria. SBP ≥ 160 mmHg and DBP ≥ 110 with proteinuria $>2+$ indicates severe PE and early-onset PE was diagnosed if manifestations appeared at <34 gestational weeks. Plasma levels of mtDNA were estimated using real-time PCR.

Results: Seventeen women developed early-onset PE and 35 women had mild PE. Mean plasma levels of mtDNA were significantly higher in PE women than controls, in women developed early-onset PE than women developed late-onset PE and in women developed severe PE than those developed mild PE. Statistical analyses defined high 1st trimester plasma mtDNA as significant early predictor for development of PE and when combined with high SBP during 1st trimester as significant early predictor for severe PE. However, high plasma mtDNA during 1st trimester in young women as significant early predictor for development of early-onset PE

Conclusion: High 1st trimester plasma level of mtDNA could be considered as a measure for mitochondrial dysfunction with subsequent liability to develop PE especially the early-onset type and severe manifestations.

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Introduction:-

Preeclampsia (PE) is a devastating pregnancy disorder that severely harms the health of pregnant women and infants⁽¹⁾. PE is a disorder with wide variance of severity and subsequent management, while women with severe manifestations requires pre-term delivery, women with mild PE may reach term with minor interventions⁽²⁾.

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PE is a multisystem disorder with poorly understood etiology until nowadays and lack of reliable diagnostic tests, so it is very unlikely that a single or a small group of biomarkers will accurately predict PE⁽³⁾.

Human placenta has a central role in pregnancy through the syncytio-trophoblast cells, which are the main components of placenta that support the relationship between the mother and fetus⁽⁴⁾. Mitochondrial oxidative phosphorylation is the key energy source for placental functions and fetal growth, so impaired placental mitochondria respiratory chain complex activities can lead to adverse pregnancy outcomes⁽⁵⁾.

Structural and ultrastructure of hypertensive placentae was extensively studied, where chorionic villi in hypertensive placentae were found to have thickened basement membrane with patchy necrosis, gross thinning of the syncytium and distorted microvilli⁽⁶⁾.

Pregnancy significantly affects function of mitochondria of the placenta up to 11th gestational week and when maternal blood flow had increased both placental content of mitochondria and mitochondrial respiratory function are increased since the 12th GW and onward, so metabolic plasticity of placental mitochondria has relevance to placenta-mediated diseases⁽⁷⁾ and growing evidence indicates that mitochondrial impairment may contribute to pathogenesis of PE⁽⁸⁾.

Mitochondria in female germ line undergo dynamic quantitative and qualitative changes to maintain intact template of mitochondrial genome from one generation to another, and the quality of mitochondria determines the meiotic divisions and fertilization abilities and activation after fertilization or sustaining embryo development. Also, normal number of functional mitochondria is crucial for proper implantation and pregnancy maintenance⁽⁹⁾.

Objectives:-

Estimation of plasma mitochondrial DNA (mtDNA) levels in primigravida and evaluation of its relation to development and severity of PE

Design

Prospective comparative clinical trial

Setting

Tertiary referral hospital, KSA

Patients & Methods:-

The current study was conducted since June 2016 till March 2018 and targets to evaluate all primigravida presenting to Antenatal Care Unit (ACU) for inclusion and exclusion criteria. Exclusion criteria included multiple pregnancy, fetal abnormalities, pre-conception diabetes, essential hypertension, renal, hepatic or cardiac diseases. Also, women with body mass index (BMI) >35 kg/m², endocrinopathy, family history of essential hypertension, metabolic syndrome, or gestational hypertension were excluded from the study. Women presenting after the 12th gestational week (GW), refused to sign the consent, or lost during follow-up were also excluded. All women fulfilling the inclusion criteria and presented prior to the 12th GW and signed the written fully informed consent were included in the study. Such protocol was approved by the Local Ethical Committee.

At time of the 1st visit all women underwent complete clinical and gynecological examination and gestational age (GA) was calculated since the 1st day of the last menstrual period and was confirmed on US examination. Systolic and diastolic blood pressures (SBP & DBP) were measured to assure for being normotensive and estimation of fasting blood glucose level to assure normoglycemia. All women were asked to attend the ACU regularly 4-weekly till delivery.

Preeclampsia (PE) was diagnosed according to the American Society of Hypertension as gestational hypertension and proteinuria, defined as a qualitative 1+ dipstick reading⁽¹⁰⁾. Severity of PE was stratified according to The American College of Obstetrics and Gynecology bulletins as mild or severe which is characterized by SBP \geq 160 mmHg and DBP \geq 110, proteinuria >2+ on a voided random urine and/or presence of systemic clinical manifestation⁽¹¹⁾. PE was categorized according time of onset into early PE onsets at <34 GW and late PE that onsets at >34 GW^(12, 13). Women who developed manifestations of PE during pregnancy course were collected as PE group, while

women who completed their pregnancy free of hypertensive manifestations were considered as controls and data of ten of control women of cross-matched age and body mass index to women of PE group were collected as control group.

Laboratory investigations

Blood sampling

At time of 1st antenatal visit, 5 ml blood sample was obtained withdrawn under complete aseptic conditions and collected into EDTA-containing tubes, centrifuged at 1600g for 10 min, and plasma was removed and stored at -80°C till be processed.

Preparation and Quantification of Plasma DNA

Sample processing:

Plasma mitochondrial DNA (mtDNA) was extracted using of QIAamp Blood Kit (Qiagen GmbH) according to manufacturer's instruction⁽¹⁴⁾. Frozen blood samples were thawed on ice and mixed by vortex, and 100 µl of plasma was mixed with 100 µl of phosphate-buffered saline (PBS), followed by brief vortex. Then, diluted plasma was centrifuged for 5 min at 700g at 4°C and 190 µl of supernatant was carefully pipetted without touching the pellets or bottom of tubes with pipette tips. Thereafter, the supernatant was centrifuged at for 15 min at 18,000g at 4°C and 170 µl of the supernatant was taken without any contamination.

DNA isolation:

Briefly, supernatant samples were incubated with lysis buffer (included in the kit) and proteinase K at 56°C for 15 min and DNA was eluted in 200 µl of elution buffer (included in the kit). Then, for the quantitative real-time polymerase chain reaction (qPCR) assay, DNA solution was further diluted 10 times with nuclease-free deionized, distilled H₂O.

Primers and qPCR

The prepared diluted samples were used for measurement of DNA level using SYBR Green dye-based qPCR assay using a PRISM 7300 sequence detection system (Applied Biosystems). The primer sequences were as follows:

1. human NADH dehydrogenase 1 gene (mtDNA):
2. forward 5'-ATA CCC ATG GCC AAC CTC CT-3'
3. reverse 5'-GGG CCT TTG CGT AGT TGT AT-3'⁽¹⁴⁾.
4. Bacterial 16S ribosomal RNA:
5. forward 5'-CGT CAG CTC GTG TTG TGA AA-3'
6. reverse 5'-GGC AGT CTC CTT GAG TTC C-3'⁽¹⁵⁾.

DNA solutions were diluted in 10-fold serial dilutions and used as standards. Thermal profile for detecting mtDNA was performed as following: initially for 2 min at 50°C, 10 min at 95°C and a further step consisting of 40 cycles for 15 s at 95°C and for 1 min at 60°C⁽¹⁶⁾.

Statistical analysis:-

Results were analyzed using One-way ANOVA Test and Chi-square test (X² test). Possible relationships were investigated using Spearman's non-parametric method. Predictability of estimated parameters was evaluated using the receiver operating characteristic (ROC) curve analysis. Evaluation of the single predictor was performed using Regression analysis (Stepwise Method). Statistical analysis was conducted using the IBM SPSS (Version 23, 2015) for Windows statistical package. P value <0.05 was considered statistically significant.

Results:-

One hundred and seventy-four primigravida were eligible for evaluation, 22 women were excluded, 44 women developed PE during their course of pregnancy (PE group) and the remaining 108 women had completed their pregnancy free of PE and 10 of them were chosen as control group (Fig. 1). There was non-significant (p>0.05) difference between both groups as regards enrolment data determined at 12th week GA as shown in table 1.

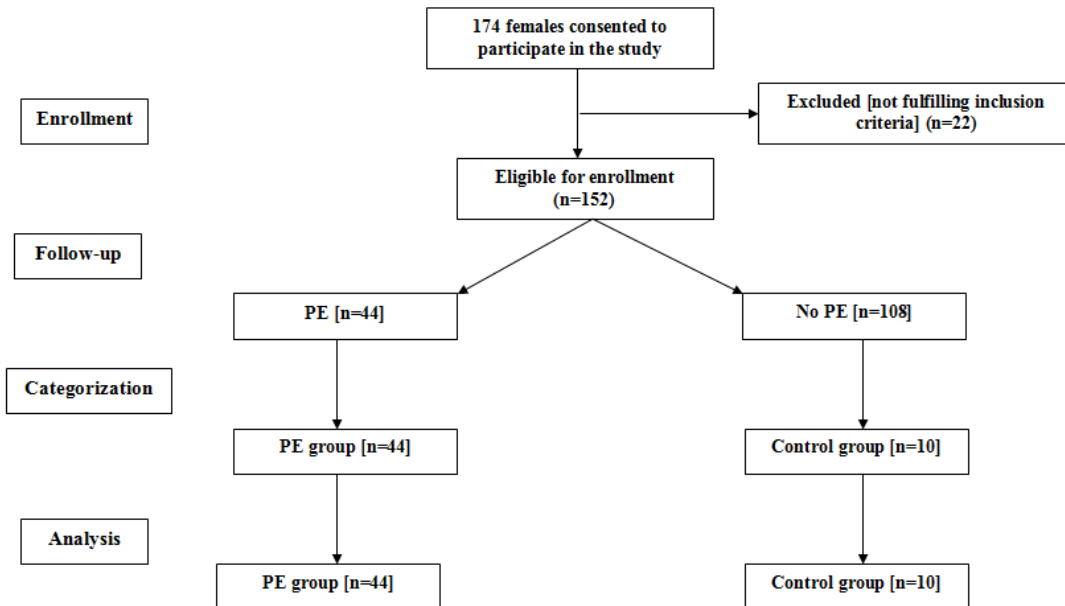


Figure 1: Consort Flow sheet

Table 1:-Baseline data of enrolled primigravida women determined at the 12th week GA

Data		Control (n=10)	PE (n=44)	P value
Age (years)		29.1±6.7	26.7±6.5	0.298
BMI data	Weight (kg)	74±4.1	75.8±6.3	0.376
	Height (cm)	169.6±2	169.7±2.8	0.767
	BMI (kg/m ²)	25.6±1.5	26.3±2.1	0.263
Blood pressure measures (mmHg)	Systolic	118.8±6.3	117.3±4.1	0.597
	Diastolic	79.9±4.3	83±4.4	0.053
Proteinuria‡	No	7 (70%)	34 (77.3%)	0.627
	Present (+)	3 (30%)	10 (22.7%)	
Fasting blood glucose (mg/dl)		82.7±8.4	84.1±6	0.078

Data are presented as mean±SD, numbers, percentages; PE: Pre-eclampsia; BMI: Body mass index; $p > 0.05$: indicates non-significant difference; ‡: Level of protein in urine as judged by dipstick measurement and expressed as number of + marks

Among PE women, 17 women developed early PE; 12 had mild and 5 had severe PE. The other 27 women developed late PE; 23 had mild and 4 had severe PE. Systolic and diastolic BP measures of women who developed early PE were significantly ($p=0.0015$) and non-significantly ($p=0.053$) higher, respectively, compared to measures recorded with late PE at time of development of PE. Differentially, SBP and DBP of women who developed mild early PE were significantly higher ($p=0.0005$ & 0.033 , respectively) than women who developed mild late PE. SBP measures were significantly ($p=0.001$) higher, while DBP measures were non-significantly higher ($p=0.153$) with early versus late severe PE. Regarding proteinuria, women who developed severe PE; early versus late-onset, showed non-significant ($p=0.872$) variance, while women had mild late PE had significantly ($p=0.045$) lower level of proteinuria compared to those had developed mild early PE (Table 2).

Table 2:-Clinical data of PE women determined at time of development of PE

Data		Mild PE (n=35)	Severe PE (n=9)	Total
SBP (mmHg)	Early PE (n=17)	152.1±4.7	172.5±2.7	158.1±10.5
	Late PE (n=27)	145.7±4.5	164.8±2.2	148.5±8.1
	P=	0.0005	0.001	0.0015
DBP (mmHg)	Early PE (n=17)	95.6±3.6	115±3.5	101.3±9.7

	Late PE (n=27)	93.3±2.5	112.5±2.9	96.1±7.4
	P=	0.033	0.153	0.053
Proteinuria ‡	Early PE (n=17)	3±0.9	3.5±0.6	3.1±0.8
	Late PE (n=27)	2.5±0.5	3.4±1.1	2.7±0.7
	P=	0.045	0.872	0.059

Data are presented as mean±SD & numbers; percentages are in parenthesis; PE: Pre-eclampsia; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; P: significance versus women developed late PE; p<0.05: indicates significant difference; p>0.05: indicates non-significant difference; ‡: Level of protein in urine as judged by dipstick measurement and expressed as number of + marks

Mean plasma levels of mtDNA estimated at time of enrollment were significantly (p=0.022) higher in PE women compared to controls. Mean plasma mtDNA levels estimated in women developed early-onset PE was significantly higher compared to controls (p=0.0022) and women developed late-onset PE (p=0.011), with non-significantly (p=0.091) higher levels in women developed late-onset PE compared to controls (Fig. 2). Concerning severity, plasma mtDNA levels estimated in women developed severe PE were significantly higher compared to controls (p=0.0002) and women developed mild PE (p=0.0016) with non-significantly (p=0.097) higher levels in women developed mild PE compared to controls (Table 3, Fig. 3).

Table 3:-Mean (±SD) levels of plasma mtDNA estimated at time of development of PE

Group		Plasma level (copies/μl)	P value
Control group		161.5±66.9	
PE group	Total	253.6±118.5	P1=0.022
	Early-onset	328.2±140.7	P1=0.0022
	Late-onset	206.6±64.8	P1=0.091 P2=0.011
	Mild PE	225.5±102.7	P1=0.0002
	Severe PE	362.9±105.3	P1=0.097 P3=0.0016

PE: Pre-eclampsia; mtDNA: mitochondrial DNA; P1: significance versus control women; P2: significance versus women developed late PE; P3: significance versus women developed mild PE; p<0.05: indicates significant difference; p>0.05: indicates non-significant difference

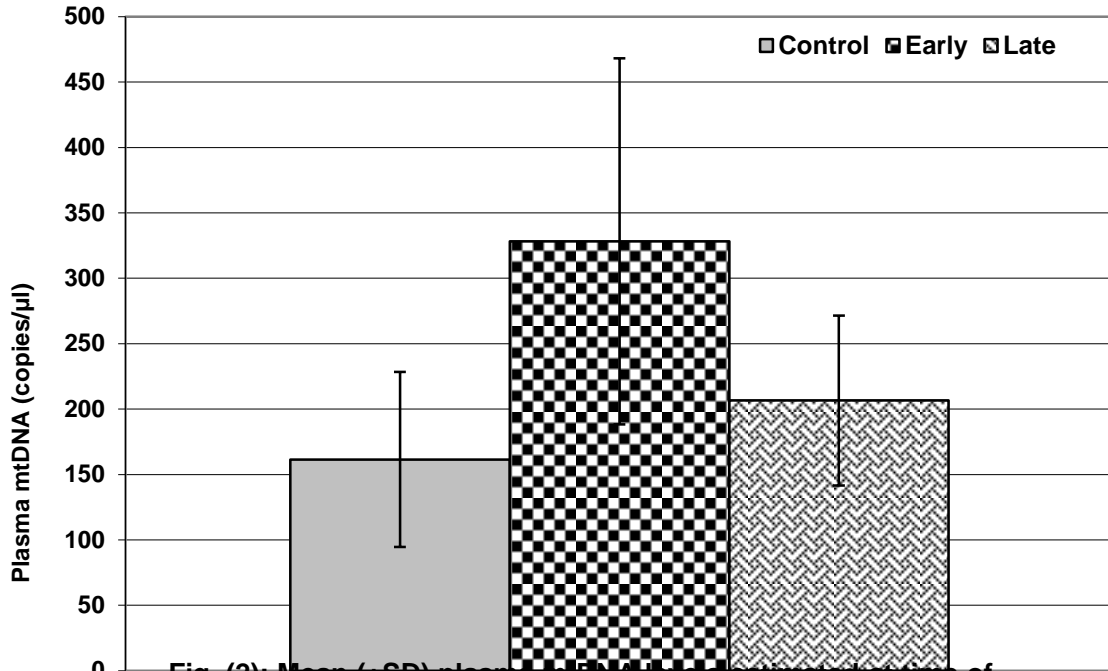


Fig. (2): Mean (\pm SD) plasma mtDNA levels estimated at time of enrolment of studied women categorized according time of onset

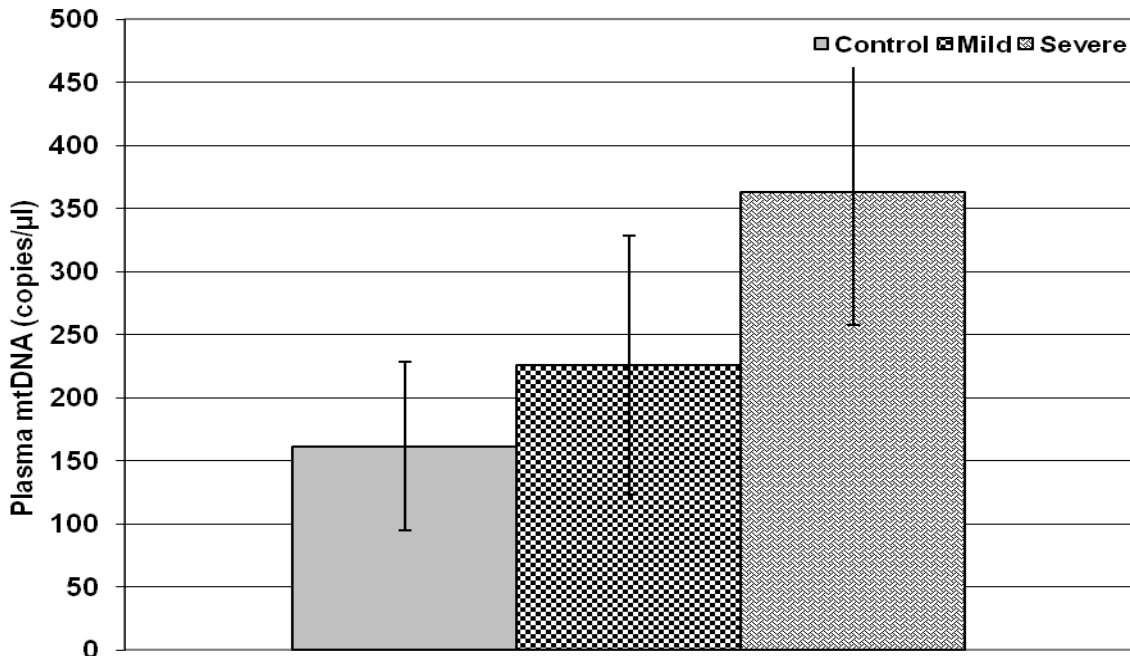


Fig. (3): Mean (\pm SD) plasma mtDNA levels estimated at time of enrolment of studied women categorized according to PE severity

Development of PE especially severe PE showed positive significant correlation with at enrolment blood pressure measures and plasma mtDNA level, while development of early-onset PE showed positive significant correlation with at enrolment plasma mtDNA levels, while showed negative significant correlation with age and at admission FBG. ROC curve analysis assured the detected correlations and arranged the variables in decreasing order of significance as early predictor for possibility for development of PE (Fig. 4) and severe PE (Fig. 5) as follows: high at enrolment plasma mtDNA, DBP and SBP. Also, ROC curve analysis arranged variables for early detection of

early-onset PE as follows: high at enrolment plasma mtDNA, younger maternal age and high at enrolment FBG level, in decreasing order of significance (Fig. 6). Regression analysis, Stepwise method, defined high at enrolment plasma mtDNA as the only significant early predictor for development of PE and defined high at enrolment plasma mtDNA combined with high at enrolment SBP as the significant early predictor for development of severe PE. However, high at enrolment plasma mtDNA combined with young maternal age as the significant early predictor for development of early-onset PE (Table 4).

Table 4:-Statistical analyses for early predictors for PE development

Data			Age	Weight	SBP	DBP	FBG	mtDNA
Spearman's correlation	PE	Rho	-0.238	0.069	0.292	0.359	0.088	0.342
		p	0.083	0.620	0.032	0.008	0.528	0.011
	Early-onset	Rho	-0.371	-0.087	0.156	-0.126	-0.304	0.394
		p	0.013	0.576	0.313	0.414	0.045	0.008
	Severe PE	Rho	-0.106	0.218	0.309	0.328	0.074	0.482
		p	0.492	0.155	0.042	0.030	0.634	0.001
ROC curve	PE	AUC			0.284	0.763		0.753
		p			0.034	0.010		0.013
	Early-onset	AUC	0.284				0.321	0.733
		p	0.017				0.048	0.010
	Severe PE	AUC			0.719	0.271		0.156
		p			0.045	0.036		0.002
Regression analysis	PE	β						0.310
		p						0.022
	Early-onset	β	-0.308					0.483
		p	0.019					<0.001
	Severe PE	β			0.286			0.492
		p			0.034			0.001

Rho: Spearman's correlation coefficient; PE: Preeclampsia; ROC: Receiver operating characteristic curve; AUC: Area under curve; β : Standardized regression coefficient

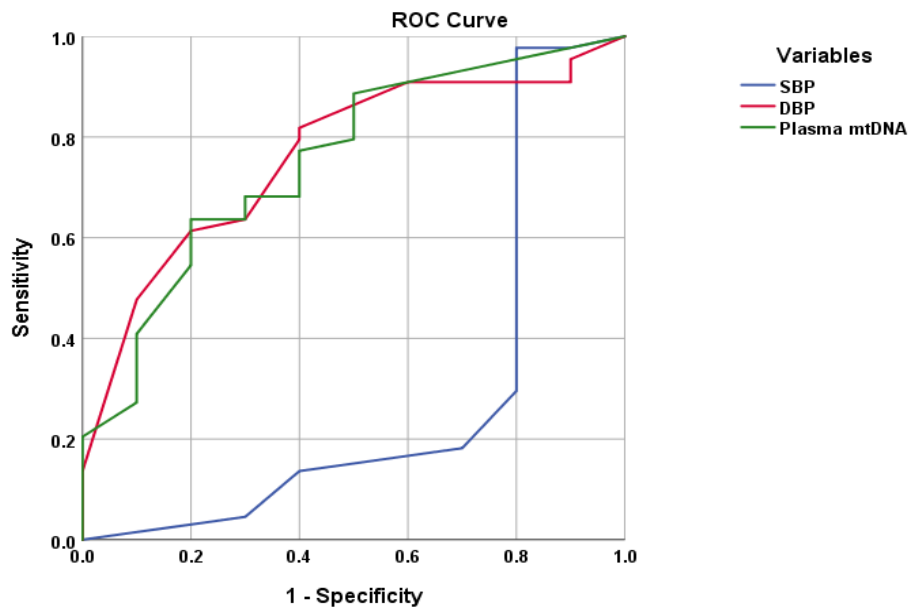


Fig 4:-ROC curve analysis for significant early predictors for PE

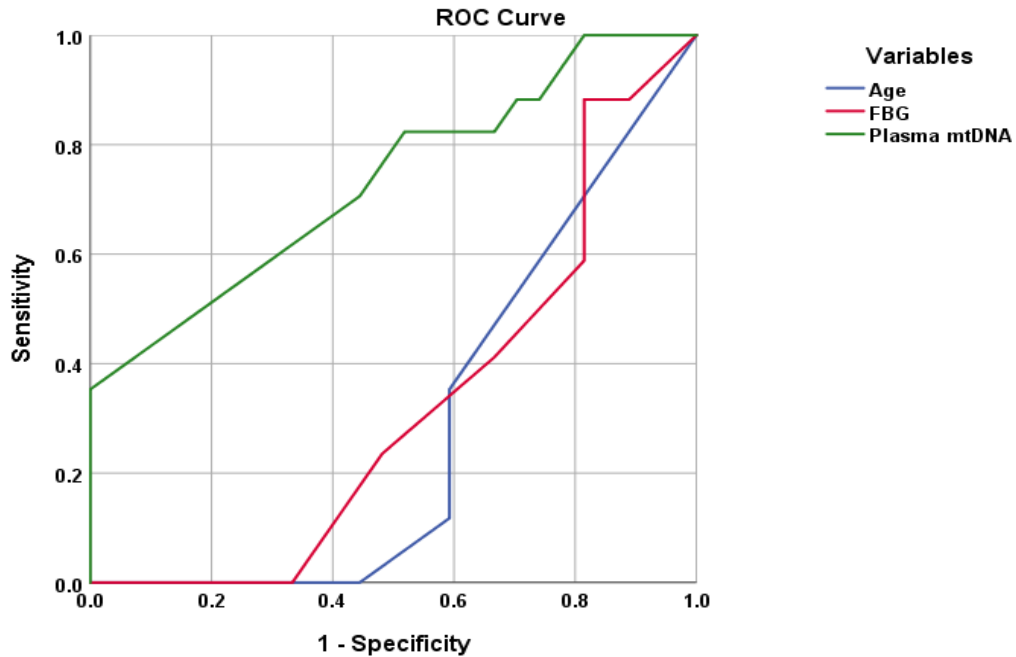


Fig 5:-ROC curve analysis for significant early predictors for early-onset PE

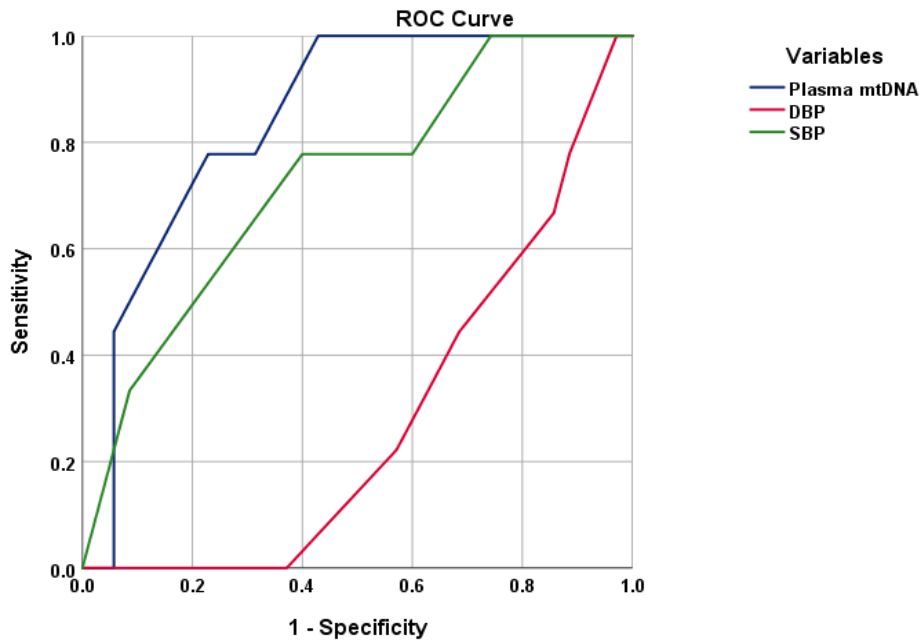


Fig 6:-ROC curve analysis for significant early predictors for severe PE

Discussion:-

The current study detected significantly higher plasma mitochondrial DNA (mtDNA) in preeclamptic women than women who continued their pregnancy free of hypertensive manifestations. This finding indicated an association between mitochondrial injury early in the pregnancy course and subsequent development of PE. In support of this assumption, there was positive significant correlation between development of PE and estimated levels of plasma mtDNA and statistical analyses defined high plasma mtDNA as a significant early predictor for development of PE.

Moreover, at enrolment plasma mtDNA levels were found to be the single significant early predictor for discrimination between mild and severe PE and its level could identify women liable to develop severe PE. The possibility for development of early-onset PE showed positive significant correlation with at enrolment plasma mtDNA and fasting blood glucose levels, and with young maternal age. Statistical analyses defined both high plasma mtDNA level and young maternal age as the significant predictors for upcoming early-onset PE. These data indicate a relation between mitochondrial dysfunction on one side and development early-onset PE and disturbed glucose homeostasis on the other side with an interrelation between development of PE and disturbed glucose homeostasis.

In line with these findings, **Qiu et al.**⁽¹⁷⁾ detected an association of placental mtDNA copy number with placental DNA oxidation secondary to oxidative stress and suggested that placental oxidative stress and mitochondrial dysfunction deleteriously affect course and outcomes of pregnancy. Thereafter, **Qiu et al.**⁽¹⁸⁾ found mtDNA copy number, a marker for mitochondrial dysfunction, was positively associated with telomere length which is associated with pregnancy complications, such as PE and IUGR. Also, **Salvianti et al.**⁽¹⁹⁾ documented a good diagnostic performance for cell free DNA in distinguishing PE women from healthy controls and considered it as a marker that could allow accurate monitoring and prevention of PE development.

Furthermore, **Muñoz-Hernández et al.**⁽²⁰⁾ detected significantly higher serum levels of total and fetal circulating cell-free DNA in PE women than in healthy control pregnant women with strong relationship to the range of PE severity. **Jiang et al.**⁽²¹⁾ reported that advanced glycation end products may be involved in PE pathogenesis through the enhancement of mitochondrial oxidative stress damage that was reflected as number of mtDNA copies in plasma. **Novielli et al.**⁽²²⁾ reported increased mtDNA levels in cord blood of IUGR, PE/IUGR and PE newborns in comparison to its levels in cord blood of control newborns and attributed this increase to a fetal response to placental insufficiency. Recently, **Marschalek et al.**⁽²³⁾ reported significantly higher mean mtDNA levels in PE than in matched control pregnant women.

Multiple studies tried to evaluate the role played by mitochondrial dysfunction and development of PE, where **Kalkanli et al.**⁽²⁴⁾ studied the ultrastructure of placentae of PE women and detected dilatation of endoplasmic reticulum, degeneration of mitochondria in endothelial cells with capillary vessel edema. Also, **Meng et al.**⁽²⁵⁾ using transition electron microscopy found vasculosyncytial membrane had significantly fewer or even absent syncytiotrophoblastic microvilli, swollen or completely destroyed mitochondria and endoplasmic reticulum in placentae of women developed gestational complications

D'Souza et al.⁽²⁶⁾ on examination of placental tissue extracts from PE women detected down-regulation of superoxide dismutase and glutathione peroxidase and up-rise of reactive oxygen species (ROS), mainly produced by mitochondria. **Haram et al.**⁽²⁷⁾ found decreased glutathione peroxidase activity is associated with upregulation of synthesis of vasoconstrictive eicosanoids which participate in PE pathophysiology through contributing to reduced trophoblastic invasion with increased vascular endothelial damage. Moreover, ROS may trigger platelet adhesion and aggregation causing intravascular coagulopathy which lead to placental infarction and impairs the uteroplacental blood flow⁽²⁸⁾.

Conclusion:-

Preeclampsia could be considered as a syndrome affecting pregnant women with varied etiologies and pathophysiology. Placental oxidative stress secondary to mitochondrial dysfunction may underlie PE development and the earlier the mitochondrial affection the earlier the presentation of PE. Moreover, the severer the mitochondrial injury, the severer the PE manifestations. Mitochondrial destruction is associated with release of mitochondrial DNA in maternal general circulation, so high level of mtDNA in plasma could be considered as a measure for mitochondrial dysfunction with subsequent liability to develop PE especially the early-onset type and severe manifestations. Wider scale studies are mandatory to evaluate the levels of oxidative stress markers and its relation to levels of mtDNA so as to find a marker for screening pregnant women for liability for PE development.

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