

Quantitative Analysis of Placental Mitochondrial DNA in Severe Hypertensive Pregnancies: A Cross-sectional Study

GAYATHRI GANESAN¹, J JOHNSY MERLA², C SELIN SOFIAH³, A SURYA⁴, K SWAMINATHAN⁵

ABSTRACT

Introduction: To address the causes of maternal mortality and morbidity, reproductive health and related disabilities is a motto of World Health Organisation (WHO). Gestational hypertension constitutes as one of the main cause for maternal morbidity and mortality. Aetiology of gestational hypertension- remains a mystery. Mitochondrial dysfunction, particularly in trophoblastic cells, could play an important role in causing gestational hypertension and is postulated to be associated with oxidative stress, impaired differentiation and invasion of trophoblastic tissue. Alterations in peripheral blood mitochondrial Deoxyribonucleic Acid (DNA) copy numbers have been thought as a possible biomarker for mitochondrial dysfunction in disorders induced by oxidative stress.

Aim: To estimate, evaluate and compare the mitochondrial copy numbers between placenta of severe gestational hypertensive pregnancies and normotensive pregnancies.

Materials and Methods: A cross-sectional study was conducted at Tirunelveli Medical College Hospital, Tirunelveli, Tamil Nadu, India, with 25 clinically proven cases of severe gestational hypertensive pregnancies and 25 normotensive pregnancies, for a period of one year (August 2020 to July 2021). The placenta

were collected from the Department of Obstetrics, in containers within two hours of delivery and frozen in liquid nitrogen. Relative quantitative analysis of placental mitochondrial DNA (mtDNA), were done by calculating the copy number value by comparing the levels of two nuclear genes viz., BECN1 and NEB to two mitochondrial genes viz., ND1 and ND6 by using real time quantitative Polymerase Chain Reaction (PCR).

Results: The placental mitochondrial DNA copy number was higher in severe gestational hypertensive patients than in normotensive pregnancies. A total of 21 (84%) cases of the severe hypertensive placenta had higher copy numbers 100-110 vs only 13 (52%) cases had copy numbers 100-105 in the normotensive group. The difference in copy number value between them was statistically significant with p-value of 0.03.

Conclusion: Present study findings suggest significant alterations at the level of the mitochondria in placenta from women with gestational hypertension, which may well contribute to gestational hypertension pathophysiology. Future research should include direct measure of placental and maternal whole body mitochondrial function, and assays that elucidate the potential modifiers of this association.

Keywords: Mitochondrial copy number, Normotensive, Severe gestational hypertension

INTRODUCTION

Many causes are attributed to maternal mortality in India. Gestational hypertension constitutes as one of the main cause for maternal morbidity and mortality [1]. Gestational hypertension is defined as blood pressure more than 140/90 mmHg without proteinuria after 20 weeks in previously normotensive women. Gestational hypertension is considered severe when the systolic level reaches 160 mmHg or the diastolic level reaches 110 mmHg, or both. Aetiology of gestational hypertension-remains a mystery. Hence, it still remains as one of the most intriguing problems to be solved [1,2]. Impaired invasion and differentiation of trophoblastic tissue is considered as one of the main component for the initiation of gestational hypertension [3-5]. Some degree of hypoxia is thought to be essential in the normal placentation process, but defective trophoblastic invasion and remodelling of the uterine spiral arteries seen in pre-eclampsia is thought to result in excessive hypoxia at the placental bed. Pregnancies complicated by pre-eclampsia demonstrate increased indices of systemic oxidative stress when compared to normotensive counterparts [6,7]. For embryogenesis and placentation mitochondrial biogenesis and adequate energy production are crucial. Mitochondria are sources of Reactive Oxygen Species (ROS), lipid peroxides are formed in the placenta due to membrane disruption by ROS and studies have shown significantly higher markers of lipid peroxidation in pre-eclampsia [8].

Mitochondrial dysfunction, particularly in trophoblastic cells of placenta, might play a crucial role in gestational hypertension and is postulated

to be associated with oxidative stress, impaired differentiation and invasion of trophoblastic tissue [8]. Furthermore, mitochondrial DNA is highly susceptible to oxidative stress induced cell damage, due to lack of histone protection [8-10]. Alterations in peripheral blood mitochondrial DNA copy numbers have been indicated as a possible biomarker for mitochondrial dysfunction [8,11,12]. Mitochondrial DNA content has been measured by conventional Southern-blot hybridisation procedure which requires huge amount of sample, and is also strenuous, but still is semiquantitative. More recently quantitative real-time PCR (qPCR) method has been used to measure the content of mitochondrial DNA. Till date most of the studies are done in maternal peripheral blood samples [8,13], however to get closer into the association present study was taken up to analyse this hypothesis in a more direct sample i.e., placental tissue.

Owing to the postulated close association between mitochondrial dysfunction, oxidative stress, impaired trophoblastic invasion leading to gestational hypertension, this study aims to assess any detectable variation in mitochondrial DNA copy number between placenta of normotensive pregnancies and placenta of severe gestational hypertensive pregnancies and comparing the same.

MATERIALS AND METHODS

A cross-sectional study was done at Tirunelveli Medical College Hospital, Tirunelveli, Tamil Nadu, India with study population of 25 clinically proven cases of severe hypertensive pregnancies i.e., when

the systolic blood pressure reaches 160 mmHg or the diastolic level reaches 110 mm Hg, or both [2,3] and 25 cases of normotensive pregnancies i.e., blood pressure less than or equals to 120/80 mmHg, for a period of one year (August 2020-July 2021) after clearance from the Institutional Ethical Committee (IEC). (Ref.NO: 1711/PATHO/2020). Written informed consent was taken from all the participants. Sample size was calculated based on conventional and cost-based approach.

Sample: Placenta extruded during labour vaginalis and placenta removed during caesarean section were collected in containers within two hours of delivery and frozen in liquid nitrogen.

Inclusion criteria: The total number of specimens studied in the present study was 50 placenta (25 severe hypertensive placenta and 25 normotensive placenta).

Exclusion criteria: Gestational hypertension- non severe, chronic hypertension, Diabetes and thyroid complicating pregnancy patients were excluded from the study. The clinical parameters Age, Gravidity, Mode of Delivery, Foetal outcomes were studied in both the groups. The total DNA content, relative quantification of mitochondrial DNA were done by the following steps.

Steps Involved:

1. Collection of placenta- 20 mg of placental tissue excised without placental membrane. Tissue was cut into small pieces and homogenised.
2. DNA extracted from placenta using QIAamp DNA extraction kit. The total amount of DNA present was checked in each sample by Spectrometry with Eppendorf Biospectrometer kinetic.
3. Relative quantification of placental mitochondrial DNA was done by real time PCR. PCR Kit-HELINI was used [14]. The HELINI Human Mitochondrial to Nuclear DNA ratio Realtime PCR Kit is an in-vitro nucleic acid amplification kit to compare the levels of nuclear to mitochondrial DNA Ratio in a human DNA sample. It contains reagents and enzymes for the specific amplification of the conserved region of the Human Chromosomal and Mitochondrial genome, and for the direct detection of the specific amplicon in FAM channel. External positive control is supplied, which can be used as both qualitative and quantitative to determine the copy number. The target sequence (ND1/ND6/BECN1/NEB) is highly conserved and has previously been shown to be a good genetic marker for the determination of copy numbers. This kit includes plates prealiquoted with the primers to compare the levels of two nuclear genes viz., BECN1 and NEB to two mitochondrial genes viz., ND1 and ND6. Mitochondrial ND1 and ND6 encode NADH dehydrogenase 1 or NADH dehydrogenase 6 which forms the core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) which functions in the electron transfer from NADH to the respiratory chain in mitochondria. Nuclear genes BECN1 codes beclin 1, autophagy related and is located in chromosome 17q21, NEB encodes nebulin, a giant protein component of the cytoskeletal matrix which co-exists with the thick and thin filaments of the sarcomeres of skeletal muscle. NEB is found in chromosome 2q22. The quantification was done as per manufacturers instructions [14].
4. Relative copy number method is used to calculate the mitochondrial DNA copy number because this results in the highest sample throughput. Ct values, where, Ct is defined as the cycle number in which fluorescence first crosses the threshold, is obtained for each of the two target genes and two reference genes are used to determine mitochondrial DNA copy numbers. This is done by averaging the copy numbers calculated from the ND1/BECN1 pair and the ND6/NEB pair. To calculate copy number, calculation $N=2^{-\Delta Ct}$, where $\Delta Ct1=CtND1-CtBECN1$ and $\Delta Ct2=CtND6-CtNEB$, is done followed by the average of $2^{-\Delta Ct1}$ and $2^{-\Delta Ct2}$

5. The relative copy numbers of mitochondrial DNA thus derived were categorised into four groups of copy numbers 90.01-95.00, 95.01-100.00, 100.01-105.00, 105.01-110.00 and compared categorically in both the severe hypertensive pregnancies and normotensive pregnancies.

STATISTICAL ANALYSIS

Chi-square test of statistical significance was applied, p-value calculated and analysed for test of significance. A p-value <0.05 was considered to be statistically significant.

RESULTS

The mean age of the study population was 25.04±3.77 years. The minimum age was 19 years and the maximum age was 32 years. Of the 25 cases in hypertensive pregnancies, 23 cases (92%) were in the age group of 21-30 years. Of the 25 cases in normotensive pregnancies, 17 cases (68%) were in the age group of 21-30 years. A total of 52% of the cases studied were primi, 36% cases were IInd gravida and 12% cases were IIIrd gravida. Of the 25 cases in severe gestational hypertensive pregnancies, 12 cases (48%) were Primigravida. Of the 25 cases in normotensive pregnancies, 14 cases (56%) were Primigravida. The difference in gravidity between the two groups was statistically insignificant.

Of the 50 cases, six of them were intrauterine death. Among 25 cases of severe gestational hypertension, the foetal outcome of 5 (20) were intrauterine death and in normotensive pregnancy it was only 1 (4%). This highlights that the adverse foetal outcome (IUD) appears to be more common in gestational hypertension. However, there was no statistical significance between normal and hypertensive pregnancy as the p-value is 0.08- more than 0.05. Of the 25 cases in both normotensive and severe gestational hypertensive pregnancies, 18 (72%) cases were normal delivery in both normotensive and severe gestational hypertensive pregnancies. There is no difference in modes of delivery in both the groups [Table/Fig-1].

Characteristics	Severe gest. Hypertensive (n=25)	Normotensive pregnancies (n=25)	p-value
	N (%)	N (%)	
Age group (years)			
11-20	0	5 (20)	Mean age=25.04 years
21-30	23 (92)	17 (68)	
31-40	2 (8)	3 (12)	
Gravidity			
I	12 (48)	14 (56)	0.66
II	9 (36)	9 (36)	
III	4 (16)	2 (8)	
Foetal outcome			
Alive	20 (80)	24 (96)	0.08
IUD	5 (20)	1 (4)	
Mode of delivery			
LSCS	7 (28)	7 (28)	1
Normal	18 (72)	18 (72)	

[Table/Fig-1]: Maternal and foetal characteristics in severe hypertensive and normotensive pregnancies.

IUD: Intrauterine death; LSCS: Lower segment caesarean section

The results of the Ct numbers for the mitochondrial genes ND1, ND6, Nuclear genes: BECN1, NEB is shown in the [Tables/Fig-2-6]. A higher mitochondrial DNA copy number 17 cases (68%) with copy numbers-100-105, four cases (16%) with copy numbers-105.01-110.00) was found in severe gestational hypertensive patients when compared to normotensive pregnancies (13 cases (52%) with copy numbers- 100.01-105.00, none had copy numbers-105.01-110.00) and this difference in copy number values between normal and severe gestational hypertensive pregnancies was statistically significant as p-value was 0.03.

ND1 (Ct Value)	HT	N	Grand total
10.01-15.00	2	6	8
15.01-20.00	16	15	31
20.01-25.00	5	4	9
25.01-30.00	2	0	2
Grand Total	25	25	50

[Table/Fig-2]: Mitochondrial gene ND1 comparison between severe hypertensive placenta (HT) and normotensive (N) placenta.

Chi-square=4.14337; Degrees of freedom=3; p=0.246393- not significant

ND6 (CT Value)	HT	N	Grand total
10.01-15.00	1	2	3
15.01-20.00	9	21	30
20.01-25.00	10	2	12
25.01-30.00	5	0	5
Grand total	25	25	50

[Table/Fig-3]: Mitochondrial gene ND6 comparison between severe hypertensive placenta (HT) and normotensive (N) placenta.

Chi-square=15.4667; Degrees of freedom=3; p=0.00145831-significant

NEB1	HT	N	Grand Total
10.01-15.00	2	1	3
15.01-20.00	15	19	34
20.01-25.00	7	5	12
25.01-30.00	1	0	1
Grand Total	25	25	50

[Table/Fig-4]: Nuclear gene NEB1 comparison between severe hypertensive placenta (HT) and normotensive (N) placenta.

Chi-square=2.13725; Degrees of freedom=3; p=0.544413-Not significant

BEC1 (CT value)	HT	N	Grand Total
10.01-15.00	5	5	10
15.01-20.00	18	17	35
20.01-25.00	1	1	2
25.01-30.00	1	2	3
Grand Total	25	25	50

[Table/Fig-5]: Nuclear gene BEC1 comparison between severe hypertensive placenta (HT) and normotensive (N) placenta.

Chi-square=0.361905; Degrees of freedom=3; p=0.947995-Not significant

Copy number	HT	N	Grand total
90.01-95.00	1	1	2
95.01-100.00	3	11	14
100.01-105.00	17	13	30
105.01-110.00	4	0	4
Grand total	25	25	50

[Table/Fig-6]: Mitochondrial DNA copy number- comparison between severe hypertensive placenta (HT) and normotensive (N) placenta.

Chi-square=9.10476; Degrees of freedom=3; p=0.02793- significant

DISCUSSION

Placental mitochondria are intracellular structures that provide most of energy production, control a number of processes, like steroid synthesis, fat metabolism, and apoptosis. It also plays a main role in implantation of placenta, development and growth of the foetus [15,16]. Till date, there has been very little research towards the study of maternal mitochondrial function and the subsequent risk of adverse pregnancy outcomes. The defects in mitochondria especially in the trophoblastic cells of placenta might be the crucial step in the initiating the pathophysiological cascade of preeclampsia has been suggested by Widschwendter M et al., [16]. There are many theories why mitochondrial dysfunction are assessed based on mtDNA copy number, may be an helpful risk biomarker marker for adverse placental function including preeclampsia, placental abruption and Intrauterine Growth Reduction (IUGR).

Mitochondrial DNA damage, particularly deletions in the control regions of the circular mitochondrial genome, may alter mitochondrial gene expression and lead to a deficiency in oxidative phosphorylation and enhance generation of ATP by glycolysis. This vulnerability to oxidative damage has been attributed to a number of factors including limited DNA repair mechanisms, lack of histone, DNA-binding proteins protection, resulting in rapid replication without a sufficiently accurate proofreading system [17].

The oxidative stress involving ROS- induces damage to cellular structural elements, like the lipid membranes of mitochondria thereby impairing electron transport chain. Subsequently there is compensatory response to this cellular oxidative stress which causes increase in mitochondrial DNA copy number [17].

This latter hypothesised mechanism is consistent with empirical evidence from experimental animal studies wherein increased mitochondrial damage and subsequent increased in mitochondrial DNA copy numbers were observed with increasing exposure to pro-oxidants [17].

Mitochondrial DNA content has been measured by conventional Southern- blot hybridisation a process which requires enormous amount of sample, but is strenuous and still the results are semiquantitative. More recently quantitative real-time PCR (qPCR) has been used to measure mitochondrial DNA content.

More than 97% of mitochondrial genome is duplicated in nuclear genome. These are also known as pseudogenes/nuclear insertions of mitochondrial origin. They are co-amplified by the primers targeting mitochondrial genome. Therefore, measuring ratio between mitochondrial DNA and nuclear DNA has been method of choice for mitochondrial DNA quantification.

Many studies supported the relationship between mitochondrial DNA and gestational hypertension. Mandò C et al., found increased mitochondrial content in whole placental tissue but decreased content in cytotrophoblast cells in preeclampsia [18].

Studies by Qiu C, et al., Marschalek J et al., found that the median mitochondrial DNA copy number were significantly higher among preeclamptic women than in matched controls [8,13]. All these studies faced a limiting factor that mitochondrial DNA was analysed only using maternal blood which serves as an indirect evidence only.

Hence, to avoid the confounding bias, this study analysed the mitochondrial DNA in a more direct and specific way that is placenta.

In this present study, the median value of placental mitochondrial DNA copy number was higher in severe hypertensive pregnancies than in normotensive pregnancies. The results were statistically significant with a p-value of 0.03 ($p < 0.05$). This was in line with another research article conducted by Pandey D et al., where higher mitochondrial DNA copy number were found in early onset preeclampsia as compared to late onset preeclampsia and control population [19]. However, recently in 2020 while analysing the molecular pathway of mitochondrial abnormalities in preeclamptic women Vangriekie P et al., found lower mitochondrial content in preeclamptic cases due to higher mitophagy [20]. The comparative results of the various studies are summarised in [Table/Fig-7].

Limitation(s)

One limitation in present study was that, whole placental tissue homogenates was used, so it remains to be established precisely in which cell types of the placenta the mitochondrial content is affected. Future research should include direct measure of placental and maternal whole body mitochondrial function, the controllers and the linking pathways behind it and assays that elucidate the potential modifiers of this association. This may pave way for avenues of new treatment options for severe gestational hypertension like mitochondrially targeted antioxidant-based intervention aimed at preventing mitochondrial dysfunction and excessive ROS formation [21].

S. No.	Name of the study	Sample collected	Number of samples	Conclusion
1.	Qiu C et al., [8] 2012, USA	Maternal blood	553	Median mitochondrial DNA copy number was significantly higher among preeclamptic women compared with controls.
2.	Marschalek J et al., [13] Austria, 2018	Maternal serum	40	Mean mitochondrial DNA levels were significantly higher in PE (n=20) than in matched controls (n=20).
3.	Pandey D et al., [19] Manipal, 2020	Placenta	32	Higher mitochondrial DNA copy number in early onset preeclamptic as compared to late onset preeclamptic and control population.
4.	Vangriekie P et al., [20] Netherlands, 2021	Placenta	23	Lower mitochondrial content in preeclampsia due to mitophagy than in matched controls.
5.	Present Study, Tirunelveli, 2023	Placenta	50	Higher mitochondrial DNA copy numbers were significantly higher in severe hypertensive pregnancies than in normotensive pregnancies.

[Table/Fig-7]: Results of comparative studies of mitochondrial DNA in hypertensive pregnancies.

CONCLUSION(S)

Present study findings support an increase in mitochondrial DNA copy number in placenta of severe hypertensive pregnancies. This statistically significant result strengthens the hypothesis that the oxidative stress due to increased mitochondrial dysfunction leads to impaired invasion and differentiation of trophoblastic tissue causing severe gestational hypertension. This opens up more light on placental mitochondria as a probable biomarker and as a therapeutic target in severe gestational hypertension. Further studies can be done to unravel the molecular pathways that control and link mitochondrial content and function in gestational hypertension.

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PARTICULARS OF CONTRIBUTORS:

- Associate Professor, Department of Pathology, Tirunelveli Medical College, Tirunelveli, Tamil Nadu, India.
- Associate Professor, Department of Pathology, Tirunelveli Medical College, Tirunelveli, Tamil Nadu, India.
- Assistant Professor, Department of Pathology, Tirunelveli Medical College, Tirunelveli, Tamil Nadu, India.
- Senior Resident, Department of Pathology, Tirunelveli Medical College, Tirunelveli, Tamil Nadu, India.
- Professor and Head, Department of Pathology, Tirunelveli Medical College, Tirunelveli, Tamil Nadu, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:

Dr. Gayathri Ganesan,
12/38C SBO Colony, Maharaja Nagar, Tirunelveli, Tamil Nadu, India.
E-mail: gg3doc84@gmail.com

PLAGIARISM CHECKING METHODS: [Jan H et al.]

- Plagiarism X-checker: Jan 24, 2023
- Manual Googling: Mar 03, 2023
- iThenticate Software: Mar 22, 2023 (20%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:

- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Jan 18, 2023**
Date of Peer Review: **Feb 17, 2023**
Date of Acceptance: **Mar 23, 2023**
Date of Publishing: **Jul 01, 2023**