Title

Exploring the role of mitochondrial dysfunction in the pathophysiology of pre-eclampsia.

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Introduction

Pre-eclampsia is a pregnancy disease that complicates 2-5% of pregnancies worldwide (1). It is characterised by the development of hypertension and proteinuria after 20 weeks' gestation (2). Pre-eclampsia is thought to occur secondary to abnormal placentation in early pregnancy (3) resulting from impaired placental trophoblast invasion and subsequent generation of an ischemic environment (4). Placental ischemia is proposed to increase placental oxidative stress leading to the shedding of syncytiotrophoblast debris into the maternal circulation where it initiates a systemic maternal inflammatory response and subsequent endothelial dysfunction (5).

Oxidative stress is a cellular or physiological condition of elevated levels of reactive oxygen species (ROS) which damage cell structure and function. Antioxidant enzymes including superoxide dismutase (SOD) and glutathione peroxidase are components of the body's mechanism for combating oxidative stress. SOD is a powerful antioxidant that catalyses the reaction between two identical molecules of superoxide radical into oxygen or hydrogen peroxide. There is significant evidence that oxidative stress plays a role in the pathophysiology of pre-eclampsia (6, 7). Normal pregnancy is associated with an increase in oxidative stress due to a rise in maternal metabolism and maternal blood flow in the placenta by 10–12 weeks' gestation (8). However, in pregnancies complicated by pre-eclampsia there is an exaggerated oxidative stress phenotype with a correspondent deficient antioxidant response.

Mitochondria are the dominant cellular source of ROS and there is strong evidence that mitochondrial ROS (mROS) play an important role in a variety of physiological processes including the regulation of cell differentiation, apoptosis, redox cell signalling and inflammation (9-11). Furthermore, our research has implicated mitochondrial dysfunction as a potential mediator of oxidative stress in pre-eclampsia (10). Cell-free DNA (cf-DNA) has been investigated as a universal diagnostic biomarker for a number of clinical applications, such as prenatal diagnosis and cancer monitoring (12, 13). Circulating cell free DNA is composed of both nuclear and mitochondrial DNA. Mitochondrial DNA (mtDNA) encodes for 37 genes programmed by the mitochondrial genome (14) and is often used as a biomarker of mitochondrial dysfunction. mtDNA are particularly vulnerable to oxidative damage due to its intimate location in the electron transport chain (ETC) in the mitochondrial matrix and its lack of protective histones (15). More recently, there is emerging evidence suggesting that cell-free mtDNA (cf-mtDNA) is linked to disease progression such as, cardiovascular disease (16). Circulating DNA in maternal plasma is mostly of maternal origin (hematopoietic and

stromal derived) depending on gestational week and maternal bodyweight. Approximately 5-20% of the circulating DNA is derived from fetal/placental cells (17).

Our research has previously shown an increase in mtDNA in plasma samples at time of disease (TOD) in women with pre-eclampsia (18). Hence the aim of this study was to characterise the role of mitochondrial dysfunction in women with pre-eclampsia compared to uncomplicated pregnancies by assessing levels of antioxidant enzyme superoxide dismutase and mtDNA at earlier time-points in pregnancy. We also examined if lifestyle and dietary factors affected mtDNA levels in pregnancy. We hypothesised that mitochondrial dysfunction plays a role in the pathogenesis of pre-eclampsia.

Methods:

Study subjects

Subjects were recruited from the Screening for Pregnancy Endpoints (SCOPE) study Ireland which is an international multicentre prospective cohort study of nulliparous singleton pregnancies aimed to develop a screening test to predict adverse pregnancy outcomes including pre-eclampsia, SGA infants and spontaneous pre-term birth (19, 20). The clinical research ethics committee, University College Cork, approved the collection and use of samples for research purposes. A nested case-control study within SCOPE Ireland was conducted which included all pre-eclampsia cases in SCOPE Ireland and matched controls with a case-to-control ratio of 1:2. Pre-eclampsia cases were defined as women with systolic blood pressure ≥ 140mm Hg and/or diastolic blood pressure ≥ 90 mm Hg on at least two occasions 4 hrs apart after 20 weeks' gestation and with proteinuria (24 hour urinary protein ≥ 300mg or urine dipstick protein ≥ +2). Randomly selected controls were taken from healthy pregnant women who had uncomplicated pregnancies which were defined as pregnancies not affected by pre-eclampsia, preterm birth or growth restriction and delivered at >37 weeks. All

blood pressure readings were <140 and/or <90 mmHg prior to the onset of labour. These were matched with the cases for maternal age, body mass index (BMI) and gestational age. Both 15 and 20 week samples were taken from the SCOPE study from women who subsequently went onto develop pre-eclampsia (n=60) and controls (n=120). Samples were also taken from a subset of women (n=25) at the time of disease (TOD) with pre-eclampsia.

Superoxide Dismutase enzyme activity

Superoxide dismutase activity was quantified in citrate plasma samples using a superoxide dismutase assay kit (Cayman chemical) which was used as per manufacturer's instructions. This assay utilizes tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. This assay measures a combination of activity from all three isoforms.

Sample collection and DNA extractions

Plasma samples were collected in BD Heparin Vacutainer tubes, placed on ice, and centrifuged at 2,400g for 10 minutes at 4˚C according to the standardised protocol. Plasma samples were stored at -80˚C until analysis. Samples were analysed in a blinded manner. Total DNA was extracted from 200µl of plasma from both controls and cases respectively with a QIAamp DNA mini kit (Qiagen). DNA was sonicated at 38 kHz ± 10% for 10 minutes to optimise DNA yield.

mtDNA quantification

Mitochondrial DNA was measured by real-time PCR using StepOne Plus Detection system using Taqman assays for mitochondrial DNA (hMitoF5, hMitoR5) (21). Absolute quantification of the concentration of mitochondrial DNA (mtDNA) was determined by standard curve analysis and presented as copies/ml (21, 22).

Maternal lifestyle factors

Women were asked at recruitment (15 ± 1 weeks of gestation) and at their second visit (20 ± 1 weeks of gestation) how many times each week did they carry out exercise that did not result in heavy breathing, which was the SCOPE definition of moderate-intensity exercise. The response was categorised as never, 1-3 times a week and daily. Similarly, the questionnaire administered at both time points asked women to report the frequency in which the consumed fruit and leafy vegetables. Scoring was similar to exercise, where the response was categorised as never, less than five pieces a week, and daily. Multivitamins were categorised into never, less than daily and daily.

Statistical analysis

Analysis was performed using GraphPad Prism and SPSS version 22 (SPSS Inc. Chicago, Illinois). Data were presented using median (±Interquartile range [IQR]) and comparisons of data between cases and controls were performed using a non-parametric Mann Whitney U test or Wilcoxon signed rank test as appropriate when data was not normally distributed. Data that was normally distributed were represented as mean (±SEM) and comparisons of data between cases and controls were performed using an unpaired t-test. P values <0.05 were considered as statistically significant. Chi-squared test and the odds ratio (OR) was used to compare categorical variables.

Results:

Patient characteristics

There were 1,774 participants in the SCOPE Ireland study. 68 (3.8%) women were diagnosed with pre-eclampsia and 60 were included in the nested case-control study with 120 participants selected as controls. The 60 women with pre-eclampsia were composed of 39

women who developed term pre-eclampsia and 21 preterm pre-eclampsia cases. As all cases and controls were matched nulliparous women, there was no significant differences observed between case-controls studies for maternal age, BMI, and gestational age at delivery. There was a significant difference in mean arterial blood pressure (MAP) in controls versus cases at both 15 and 20 weeks' gestation respectively (median [IQR]; 78.0 mmHg [73.33-83.33], n=120, vs median [IQR]; 82.0 mmHg [75.0- 87.66], n=60, p= 0.0015 and media [IQR] 80.41 mmHg [75.3333- 85.0], n=120, vs median [IQR]; 83.5 mmHg [77.5- 89.83] n=60, p= 0.02). There was a significant difference in birthweight in controls compared to cases (3608.93 g ± 411.90 vs 2990.86 g ± 759.24; n=120, n=60; p<0.0001). (Table 1).

Evidence of altered plasma SOD activity before pre-eclampsia.

There was a statistically significant reduction in antioxidant SOD activity at 15 weeks' gestation between controls and cases (1.94 ng/ml ± 0.06 vs 1.69 ng/ml ± 0.06; n=60, n=119; p< 0.01; 95% CI; 0.04 to 0.45; Figure 1A). There was no significant difference in SOD activity at 20 weeks' gestation between controls and cases, (0.82 ng/ml ± 0.02 vs 0.77 ng/ml ± 0.03; n=119 p=0.21; 95% CI; -0.02 to 0.12; Figure 1B).

Increased ΔmtDNA levels was evident between 15 and 20 weeks' before pre-eclampsia.

There was no significant difference in the amount of total DNA between controls and cases at both 15 and 20 weeks' gestation (15 weeks: 7.06 ng/ml ± 4.08 vs 7.70 ng/ml ± 5.43 , n =60 n=120, p=0.38; 20 weeks 6.71 ± 3.26 vs 7.15 ± 3.44 , n= 60 n=120, p=0.40; Figure 2A). There was no significant difference in mtDNA at 15 weeks' gestation between controls and cases (median [IQR]: 2832.96 copies/ml [1711.17-5002.82] vs 2337.32 copies/ml [1357.11-5328.35], n=58-117; p< 0.3381, Figure 2B). Similarly, there was no significant difference in mtDNA at 20 weeks' gestation between controls and cases and (median [IQR]: 2885.57 copies/ml [1914.54-4834.12] vs 3307.7 copies/ml [1544.49-7396.92], n=58-117; p<0.7873,

Figure 2C). As pregnancy progressed the amount of mtDNA significantly increased in pre-eclampsia and healthy pregnancies (median [IQR]: 2337.32 copies/ml [1357.11-5328.35], 3307.7 copies/ml [1544.49-7396.92] and 6449.8 copies/ml [477.54-11145.9] n=58 and n=22.p< 0.0001, and median [IQR]: 2855.41 copies/ml [1740.19-5322.08], 2900.95 copies/ml [1958.36-5055.16], 5983.88 copies/ml [3209.67-16901.5] n-117 and n=23. P=0.009, Figure 2D) at 15 weeks', 20 weeks' and TOD respectively.

However the mean difference in mtDNA between 15 weeks' and 20 weeks' gestation was significantly higher in cases compared with controls (2236 ± 796.0 copies/ml vs -555.3 ± 599.3 copies/ml mtDNA concentration in plasma, p=0.0065; Figure 2E).

Lifestyle and nutritional factors; effect of these factors on mtDNA

Moderate exercise had no significant impact on the amount of mtDNA in controls vs cases at 15 or 20 weeks' gestation OR 1.00; [CI 1.00-1.00 vs 1.00 CI [1.00-1.00] respectively (Table 2A). When assessing dietary factors such as leafy vegetable intake, fruit intake and multivitamin consumption, similarly there was no effect on the amount of mtDNA at 15 or 20 weeks' gestation in controls vs cases (Table 3A, 3B, 3C).

Discussion

Mitochondrial dysfunction is a pathogenic mediator of oxidative stress in pre-eclampsia with elevated mitochondrial lipid peroxidation and increased vulnerability to oxidation evident in placental mitochondria in pregnancies complicated by pre-eclampsia (23). In this present study we showed a significant reduction in antioxidant SOD activity at 15 weeks' gestation and an increase in the mean difference in mtDNA (between 15 and 20 weeks' gestation) in cases compared to controls.

Oxidative stress results from an imbalance in the production of ROS and the responsive antioxidant levels. There is a vast amount of evidence for antioxidant decline and elevation of ROS in pre-eclampsia (7, 24). SOD is the first barrier and antioxidant defence against ROS and its activity is increased in the placenta of a normal pregnancy (25), while SOD activity in placental tissue from women with pre-eclampsia is decreased (26). In our study, we showed lower levels of SOD activity at 15 weeks' gestation in cases compared to controls. This correlates with previous work which showed lower levels of SOD at both 10-14 and 20-24 weeks' gestation respectively in pre-eclampsia (27). Similarly, the levels of maternal erythrocyte SOD were also lower in the second half of pregnancy in pre-eclampsia when compared with normotensive pregnancies (7).

Mitochondrial DNA is correlated with the number and size of the mitochondria (28), furthermore mtDNA are particularly susceptible to oxidative damage. While the origin of cf-mtDNA is difficult to phenotype, the quantitative assessment of cf-mtDNA may permit the evaluation of mitochondrial dysfunction in pre-eclampsia. There have been a number of studies that suggest mitochondrial abundance may be associated with placental insufficiency and pre-eclampsia (29-32). Our research previously showed evidence of increased mtDNA at time of disease in women with pre-eclampsia, furthermore, we provided additional evidence of mitochondrial dysfunction by demonstrating increased mitochondrial-specific ROS and reduced oxygen consumption (18). In this current study, while there was no significant difference in mtDNA copy number at both 15 and 20 weeks' gestation respectively in cases compared with controls, we reported a significant increase in the mean difference in mtDNA copy number between 15 and 20 weeks' gestation in cases compared with controls.

Given the critical role of SOD antioxidants in mediating oxidative damage provoked by exaggerated superoxide generation, the compromised antioxidant defence evident at 15 weeks' gestation in cases in our study group may be partly responsible for increased

vulnerability of mtDNA damage as evident by the increase in mean difference in mtDNA between 15-20 weeks' gestation. Previous work in retinal endothelial cells overexpressing SOD2 (33) and in SOD2-depleted chrondrocytes (34) has established an essential protective role for this enzyme in preventing mtDNA damage. Furthermore, we showed that mtDNA copy number increases as pregnancy progresses in women with pre-eclampsia and we hypothesise that the initial insult to mitochondrial antioxidant function seen early in pregnancy (15 weeks) could be exacerbated later in pregnancy resulting in a more significant increase in mtDNA copy number in pre-eclampsia as we previously described.

The strength of our data compared to previous work in this area is attributable to the longitudinal examination of mtDNA through gestation, whereas previous studies have focused on mtDNA quantitation in the third trimester of pregnancy. This work correlates with previous studies, where mtDNA copy number was assessed in a case-control study and reported that the odds of pre-eclampsia were positively associated with increased maternal blood mtDNA copy number (31). These findings strongly suggest that altered mitochondrial function is evident very early in the development of pre-eclampsia. This study was performed in the Irish SCOPE cohort and while we provide evidence of mitochondrial dysfunction in pre-eclampsia, further larger studies in different populations are warranted.

Lifestyle interventions such as healthy diet (fruit and vegetable consumption) and exercise have been examined in mitochondrial diseases (35, 36). In recent years, exercise has been intensively researched in relation to reducing risk of pre-eclampsia (37, 38). The next step in this study was to assess whether lifestyle and nutritional factors had an effect on the amount of mtDNA in controls and cases. However, we showed that exercise had no significant difference on mtDNA copy number during pregnancy in controls or cases. Similarly, a previous study assessed exercise and mtDNA copy number in controls and pre-eclampsia cases and showed no association between exercise and mtDNA copy number (31).

Diet has been suggested to play a potential role in the management of pre-eclampsia (39). In our study we found no association between fruit and vegetable intake on mtDNA copy number in control and cases. Similarly, Clausen et al, showed no association between pregnancies affected by pre-eclampsia and healthy pregnancies when investigating meat, fish, vegetables and fruit intake (40). Finally we investigated multivitamin intake and its association with mtDNA copy number in control and cases and found no association. Both Vitamin C and E have been extensively studied as antioxidant therapeutic options in pre-eclampsia (41, 42), however the results were largely disappointing. This may have occurred as these exogenous antioxidant vitamins do not penetrate the intracellular source of ROS, the mitochondria, and are sequestered in the cytosol. Therefore, we propose a mitochondrial-targeted antioxidant may represent a more promising clinically effective treatment strategy for pre-eclampsia.

Conclusion

In this study, we provide evidence that in early gestation there is a significant reduction in mitochondrial antioxidant SOD activity in women who developed pre-eclampsia. Furthermore, there is a significant increase in ΔmtDNA levels between 15 and 20 weeks' in women who subsequently went on to develop pre-eclampsia. Our findings support a pathogenic role for mitochondrial dysfunction in the pathophysiology of pre-eclampsia. Finally, we found no effect of either lifestyle or dietary factors in mediating mitochondrial dysfunction in this study cohort, highlighting the potential need for the development for mitochondrial targeted antioxidants as potential therapeutic targets to treat pre-eclampsia.

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Table 1: Patient Characteristics in the study cohort

	Preterm pre-eclampsia (n=21)	**Term pre-eclampsia (n=39)**	**No pre-eclampsia (n=120)**
Mean Maternal age, years	31	29	29
Mean BMI	25	26	25
Maternal			
Mean Arterial Blood Pressure at 15 weeks	82.33 [74.16-86.33]	81.5 [74.58-88.0]	78 [73.33-83.33]
Mean Arterial Blood Pressure at 20 weeks	81.33 [77.66-89.66]	83 [76.08-87.0]	80.41 [75.33-85.0]
Fetal			
Mean Birth weight, g	2104	3300	3608
Mean gestational age at delivery	34	38	40
Perinatal death	1	1	0

Data are presented as mean or Median [IQR]. Mean Arterial blood pressure was calculated as MAP = (2 x diastolic) + systolic/3 and

Table 2: The effect of lifestyle factors (Exercise) on mtDNA in cases compared to controls at 15 and 20 weeks' gestation

Moderate exercise activity	**N=58**	**Case (mtDNA copy number/ml)**	**N=117**	**Control (mtDNA (copy number/ml**	**OR**	**(95% CI)**
Never exercised	19	1631.02 (815.92-3445.15)	23	2817.51 (1856.43-5602.91)	1.00	1.00-1.00
More than Once a week	31	2742.43 (1510.48-5328.35)	60	2676.89 (1669.87-3734.28)		
Daily	8	3928.08 (934.06-11509.39)	34	3070.21 (1912.75-5322.08)		
Never exercised	12	3242 (1367.75-9276.86)	20	2649.15 (1849.56-4454.55)	1.00	1.00-1.00
More than once a week	37	3249.79 (1493.10-7562.22)	67	2847.66 (1758.46-4248.59)		
Daily	9	4198.29 (1632.68-5398.94)	30	2790.07 (1998.00-5385.22)		

Table 3 (A): The effect of dietary factors (Fruit intake) on mtDNA in cases compared to controls at 15 and 20 weeks' gestation

High fruit intake	**N=58**	**Case (mtDNA copy number/ml)**	**N=117**	**Control (mtDNA copy number/ml)**	**OR**	**(95% CI)**
Never	7	2158.15 (1357.11-3119.63)	9	1856.43 (617.47-9759.18)	1.00	1.00-1.00
<6 times a week	11	3445.15 (1140.94-6661.19)	25	2617.49 (1683.27-4005.63)		
>5 a day	40	2466.71 (1339.17-4526.35)	83	2829.32 (1868.68-4786.10)		
Never	0		1	6802.61	1.00	1.00-1.00
<6 times a week	6	2650.57 (1063.15-6136.60)	16	2384.71 (1471.25-5954.11)		
>5 a day	52	3344.25 (1548.32-7395.89)	108	2856.83 (1980.26-4711.28)		

Table 3 (B): The effect of dietary factors (leafy vegetable intake) on mtDNA in cases compared to controls at 15 and 20 weeks' gestation

Leafy vegetable intake	**N=58**	**Case (mtDNA copy number/ml)**	**N=117**	**Control (mtDNA (copy number/ml**	**OR**	**(95% CI)**
Never	22	2064.62 (1443.17-5873.68)	49	2862.43 (1964.75-5110.42)	1.00	1.00-1.00
<6 times a week	27	2133.39 (1140.94-4068.91)	42	2547.10 (1644.76-4838.29)		
>5 a day	9	3119.63 (1677.58-5812.97)	26	2676.89 (1184.76-3950.15)		
Never	1	4198.29 (4198.29-4198.29)	3	1846.20 (1108.22-)	1.00	1.00-1.00
<6 times a week	23	3104.99 (1366.01-7396.91)	46	2596.07 (1636.75-4126.70)		
>5 a day	34	3315.32 (1518.79-6499.72)	68	3067.12 (2151.85-5347.62)		

Table 3 (C): The effect of dietary factors (Multivitamin intake) on mtDNA in cases compared to controls at 15 and 20 weeks' gestation

Multivitamin intake	**N=58**	**Case (mtDNA copy number/ml)**	**N=117**	**Control (mtDNA (copy number/ml**	**OR**	**(95% CI)**
No	44	2206.55 (1186.20-4709.44)	71	2617.49 (1856.43-4778.04)	1.00	1-1.00
Daily	14	2745.61 (1504.21-6598.51)	39	2850.34 (1626.85-5111.71)		
Less than daily	0		7	3518.35 (1856.43-4315.26)		
No	38	4032.60 (1744.82-7789.96)	57	2777.50 (1769.47-5184.31)	1.00	1.00-1.00
Less than daily	4	1374.57 (332.18-5948.15)	15	3647.68 (2278.63-4711.28)		
Daily	16	2384.71 (1471.25-5954.11)	45	2754.95 (1822.09-4421.01)		

Figure Legends

Figure 1: A) SOD activity (U/ml) was significantly reduced at 15 weeks in cases compared to healthy controls (P<0.01). B) SOD activity (U/ml) at 20 weeks was reduced in cases compared to healthy controls. Data are expressed as mean ± SEM.

Figure 2: A) Total DNA in maternal plasma in controls and cases showed no significant difference. B) mtDNA at 15 weeks showed no significant difference in controls when compared to cases C) mtDNA at 20 weeks was higher in controls compared to cases but not statistically significant D) Mitochondrial dysfunction is significantly increased as gestation progresses in pre-eclampsia and uncomplicated pregnancies ($p<0.001$ and $p=0.0009$) respectively.●=cases, ■=control. Data represented as the median; [IQR]. E) Significant increase in the mean difference in mtDNA concentration between 15 and 20 weeks gestation in cases compared to controls (P<0.01). Data represented as the mean; [SEM].