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Study of mitochondrial function in placental insufficiency

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ABSTRACT

Introduction: It has been suggested that mitochondria play a crucial role in sustaining pregnancy and foetal growth. The aim of the study was to assess the influence of mitochondrial functions and genetics on placental insufficiency diseases.

Methods: A total of 115 patients were recruited, subdivided into 74 placenta samples and 41 maternal blood samples: placental insufficiency diseases including intra uterine growth restriction (IUGR) (n = 35), pre-eclampsia (PE) (n = 13), IUGR associated to PE (PER) (n = 25); and controls (n = 42). Haplogroups were determined for all patients. Eighty-six placenta samples were studied for quantitative and qualitative analyses of mtDNA: IUGR (n = 25), PE (n = 1), PER (n = 18) and controls (n = 42). Sixteen placenta samples were selected for functional analysis: IUGR (n = 6), PER (n = 2) and controls (n = 8).

Results: Mitochondrial DNA copy numbers and rearrangements and haplogroup distribution were not significantly altered in the patient group. Enzyme activity and expression of respiratory chain complexes were also comparable between both groups.

Discussion: Our results do not argue in favour of a mitochondrial involvement in placental insufficiency, suggesting that the glycolytic pathway may represent a key energetic source in placental insufficiency diseases.

1. Introduction

Foetal growth is a complex process that depends on genetic, nutritional and hormonal factors. Foetal growth is inseparable from placental growth and requires a nutrient supply both continuous and adapted to each pregnancy period. Intra-uterine growth restriction (IUGR) is defined by a failure of the foetus to reach normal (or genetically defined) growth potential and can lead to a small for gestational age (SGA) new born. Vascular IUGR is the most frequent cause of IUGR, due to placental insufficiency secondary to a disturbed remodelling of the uteroplacental arteries [1].

Energy-generating metabolic pathways play a key role in sustaining pregnancy and foetus growth [2]. In aerobic conditions, mitochondria provide most of the cell energy supply. An electron flow across the respiratory chain, generated by nutrients oxidation, feeds a proton gradient across the inner membrane subsequently used by ATP synthase to provide adenosine triphosphate (ATP). Components of the oxidative phosphorylation (OXPHOS) machinery are encoded by both nuclear and mitochondrial genomes. Transmitted exclusively from maternal DNA, Mitochondrial DNA (mtDNA) is a circular double strand DNA of 16569 base pairs. mtDNA is highly polymorphic and is commonly used as a genetic marker for association studies. Indeed, mtDNA haplogroups, determined by the combination of variants, reflect geographical origin, human phylogeny and migratory flows of populations. Selected haplogroups have been shown to play an important role in the protection or predisposition to various diseases, especially

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neurodegenerative disorders (Parkinson's and Alzheimer's diseases) [3]. Some haplogroups, such as U, J and T, affect mitochondrial respiration and reactive oxygen species (ROS) production [4]. The mtDNA content is also considered as a reliable marker of mitochondrial biogenesis, since it varies according to the cell type, the development stage and the need for energy [5].

As the placenta consumes large volumes of oxygen, it has been suggested that placenta physiology is strongly dependent on mitochondrial activity [6,7]. An imbalance of the placental mitochondrial function combined with an increase in oxidative stress in placentas with vascular diseases may be responsible for IUGR. Recently, using transcriptomic analysis, we proved that gene expression levels of 3 out of 5 OXPHOS complexes were significantly reduced in IUGR placentas [8]. Nevertheless, not much is known about the role and consequences of this mitochondrial imbalance on the well-being of the foetus and the future of the child. The aim of our study was to investigate whether mitochondrial factors such as the OXPHOS complexes and mtDNA genetics may be involved in the pathogenesis of placental insufficiency.

2. Materials and methods

2.1. Ethical issues and patients

Placentas and blood samples were collected at Angers University Hospital. This study was approved by the Ethics Committee of Angers University and written consent was obtained for all patients. Clinical data related to the mother and foetus, as well as neonatal data, were collected from the patients' obstetrical files. The cohort was registered at the French National Commission on Informatics and Liberty (*Commission Nationale Informatique et Libertés* no. pWP03752UL, ethics committee for the collection of the clinical data).

Placentas were obtained from caesarean sections before or during the onset of labour or from vaginal delivery. For the analysis, patients were classified in two groups: placental insufficiency and control groups. Placental insufficiency diseases consisted of the following three sub-groups: Intra-Uterine Growth Restriction (IUGR), defined by a reduction of foetal growth during gestation, with a notch observed by Echo-Doppler in at least one uterine artery and with Doppler abnormalities on umbilical Doppler and/or cerebral Doppler and/or *ductus venosus*, and with a birth weight below the 10th percentile according to Audipog growth curves [9] and confirmed by the anatomopathological analysis of the placenta after birth. The second subgroup (PE) included pre-eclamptic pregnancies, defined by systolic pressure above 140 mmHg, diastolic pressure above 90 mmHg and proteinuria above 0.3 g per day. The third subgroup consisted of patients with a combination of vascular IUGR and PE (PER). The control group was defined by women with normal pregnancies, although most of them underwent a planned Caesarean section. All obstetrical and neonatal data were collected prospectively from medical records. All patients recruited were European.

2.2. Placental and blood samples

To avoid degradation, only placental tissues dissected within a timeframe of 30 min after delivery were included. After removal of maternal deciduas and amniotic membranes, sections of 1 cm^3 of placental villi were dissected from four different cotyledons between the basal and chorionic plates, as previously described [10,11]. After vigorous washing with PBS to remove maternal blood, tissues were immediately frozen at −80 °C, to further extract DNA, or frozen at −80 °C in Tris-HCl and protease inhibitor buffer, to further extract proteins. Placentas were then sent for anatomopathological analysis or stored at the biological core facility at Angers University Hospital.

Maternal blood samples were taken at the time of intravenous laying device before delivery and stored at the biological core facility for further analysis of the mitochondrial genome.

3. Analysis of the mitochondrial genome

3.1. DNA extraction from placenta and blood

DNA was extracted from chorionic villus samples or mother blood samples using the *QIAamp DNA mini QIAcube Kit* (Qiagen®) following the manufacturer recommendations.

3.2. Mitochondrial haplogroup analysis

Firstly, haplogroup H was assessed as previously described [12]. Other non H-haplogroups were determined using Next Generation Sequencing (NGS). Library preparation was performed using the Ion Xpress Plus Fragment Library Kit. Sample emulsion PCR, emulsion breaking, and enrichment were performed using the Ion 540 Kit–Chef and sequenced on the Ion S5 Sequencer.

3.3. Quantitative variations of mtDNA

MtDNA was quantified by quantitative PCR using the *Bio-Rad iTaq SYBR Green* kit (Bio-Rad, France) on the device *CFX Touch* (Bio-Rad, France). The mtDNA/nucDNA ratio was measured by PCR using the following primers: *MT-ND4* (NADH dehydrogenase 4) (Forward: 5′-ACTCTCACTGCCCAAGAACT-3′ and Reverse: 5′-GTGTGAGGCGTAT TATACCA-3′) and *MT-COX1* (cytochrome c oxidase I) (Forward: 5′-TACGTTGTAGCCCACTTCCACT-3′ and Reverse: 5′-AGTAACGTCGG GGCATTCCG-3′) and *B2M* (beta-2 microglobulin) (Forward: 5′-CAGC CTATTCTGCCAGCC-3′ and Reverse: 5′-CAATGTTCTCCACATAGTGA GGG-3′) and *GAPDH* (glyceraldehyde 3 phosphate dehydrogenase) (Forward: 5′-CCCTGTCCAGTTAATTTC-3′ and Reverse 5′-CACCCTTTA GGGAGAAAAA). Reactions were performed as follows: initial denaturation at 95 °C for 10 min, and 35 cycles at 95 °C for 30 s, 60 °C for 1 min (ND4/B2M), 57 °C for 1 min (COXI/GAPDH).

Each sample was analysed in triplicate. DNA extracted from cells deprived of mtDNA (Rho0) was used to check the primer specificity. The relative number of mtDNA copies was then calculated by the mtDNA/nucDNA ratio (*ND4/B2M* and *COXI/GAPDH*) using the reference method “delta delta Ct (ΔΔCT)” [13].

3.4. Analysis of mtDNA deletions by long-range PCR

The presence of mtDNA rearrangements was assessed by measuring the entire mtDNA through long-range PCR. Two semi-long PCRs (around 8000 base pairs) and one long PCR (16300 bp) were carried out on the 86 DNA samples. The PCR reaction mixture was performed in a 20 µl reaction volume containing a final concentration of 0.5 mM of each gene-specific primer (primer sequences upon request). A 1% agarose gel electrophoresis was then carried out in order to separate the PCR products. The fluorescence reading was carried out using the *Odyssey Li-Cor* imaging system (*Odyssey Fc imager*, Li-Cor).

4. Functional analysis of mitochondria

4.1. Placental protein analysis

Protein concentrations extracted from chorionic villus samples were measured according to a colorimetric method using bicinchoninic acid following the manufacturer instructions (*BC Assay kit*, Interchim).

4.2. Analysis of the mitochondrial enzymatic activities

The enzymatic activities of cytochrome c oxidase (COX or complex IV), NADH ubiquinone reductase (NUR or complex I), ubiquinol-cytochrome c reductase (UCCR or complex III), succinate ubiquinone reductase (SUR or complex II), lactate dehydrogenase (LDH) and citrate synthase (CS) were measured by spectrophotometry (*UVmc2*, SAFAS),