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Technical note

Mitochondrial isolation, cryopreservation and preliminary biochemical characterisation from placental cytotrophoblast and syncytiotrophoblast

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1. Introduction

The placenta consists of multiple cell layers, including the underlying cytotrophoblast layer and a multi-nucleated, terminally differentiated syncytiotrophoblast. These two cell types possess mitochondria with unique properties; differing in size, morphology, energy production and steroidogenic capacity [1–3]. Mitochondria are vital to virtually every aspect of cellular function from supplying energy in the form of ATP, processing metabolic intermediates, modulating Ca^{2+} signalling, controlling ROS production and playing a critical anabolic role. Many studies have shown that mitochondrial dysfunction contributes to placental pathology underpinning gestational disorders [4,5]. However, due to the complex cell structure of the placenta and the very different biology of cytotrophoblast and syncytiotrophoblast, mitochondrial populations from both are often studied together so subtle variations are missed. One of the key challenges to date has been the development of methodologies for isolation of functional mitochondria that allow key parameters to be assessed in an efficient and reproducible way [3]. Therefore, the aim of this study was to develop methodologies for the isolation, cryopreservation and biochemical analysis of mitochondria from both cytotrophoblast and the syncytiotrophoblast.

2. Methods

2.1. Placental collection and mitochondrial isolation

Ethical approval was granted by both Queensland Health and Griffith University. Placentae were from healthy term pregnancies, post vaginal birth, and villous tissue was collected as previously described [6]. Isolation of mitochondrial subpopulations was achieved by differential centrifugation adapted from Martinez et al. [3]. Samples were homogenised in a glass dounce homogenizer with 1 mL of chilled isolation media (250 mM sucrose, 0.5 mM Na_2EDTA, 10 mM Tris, pH 7.4). Homogenates were centrifuged at 1,500 g for 10 min at 4 °C and the supernatant collected and centrifuged at 4,000 g for 15 min at 4 °C to produce a pellet of enriched mitochondria (Fraction 1 or Cyto-Mito). The supernatant was collected and centrifuged at 12,000 g for 15 min at 4 °C to produce a second pellet of enriched mitochondria (Fraction 2 or Syncytio-Mito). As the isolation media is designed to allow for cryopreservation of extracted samples, both fractions were analysed fresh or snap frozen and stored at −80 °C. Cryopreserved isolates were revived by warming to 37 °C for 5 min, prior to assessment of mitochondrial respiration. For measurement of mitochondrial membrane potential and ATP production, isolates were revived from storage at −80 °C up to five months later.

2.2. Transmission electron microscopy

Placental tissues were pre-fixed in 3% glutaraldehyde and post-fixed in 1% osmium tetroxide, dehydrated in ethanol, embedded in Epon resin using the Pelco Biowave fixation system and polymerised overnight at 60 °C. Tissue blocks were sectioned (80 nm) using a Leica Ultracut UC6 ultramicrotome and imaged utilising a Joel JSM1011 transmission electron microscope with an Olympus Morada digital camera. Isolated mitochondrial fractions were fixed in 3%

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glutaraldehyde in mitochondrial isolation buffer, centrifuged to create a cell pellet that was processed and imaged as described for placental tissue. The number of mitochondria were counted, and mitochondrial length measured, and mitochondria classified as large (≥0.2μm) or small (≤0.1μm). Ratio of large to small was used to assess % purity.

2.3. Real time respirometry and ATP measurements

Mitochondrial respiration was measured at 37 °C via Oxygraph-2k (OROBOROS, Innsbruck, Austria) using the Substrate-Uncoupler-Inhibitor-Titration (SUIT) protocol. Mitochondrial fractions were added in MiRO5 respiration media (0.5 mM EGTA, 3 mM $MgCl_2{\cdot}6H_2O$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g/L fatty acid free BSA, pH 7.1) for baseline calibration (5mins) prior to the sequential addition of Glutamate (10 mM), Malate (2 mM), Pyruvate (5 mM), ADP (1–5 mM) followed by the addition of cytochrome C and Succinate (10 mM) to activate Complex I + II linked respiration and assess mitochondrial membrane integrity, Carbonyl cyanide m-chlorophenyl hydrazone (CCCP; 1 mM) to measure Maximum respiratory capacity. Complex I and III inhibition with Rotenone (1 μM) and Antimycin A (5 mM) respectively, provided measurement of non-mitochondrial respiration. Non-mitochondrial respiration was subtracted from all obtained values to normalise for solely mitochondrial respiration. Respiratory rates were recorded via OROBOROS DataLab 7.0 software and units of respiration are expressed as pmol O_2/sec per mg protein. The level of ATP was quantified, after removal of contaminating proteins, utilising an ATP Assay Kit (Abcam, Australia) following the manufacturer's instructions. ATP content was is expressed as umole/mg of mitochondrial extract.

2.4. Flow cytometry measurement of membrane potential

Following revival mitochondrial isolates were incubated 100 nM MitoSPY Green (Biolegend, United States) and 50 nM TMRE (tetramethylrhodamine, ethyl ester) for 25 min at 37 °C before centrifugation at appropriate speed and resuspension in PBS. Median fluorescence intensity was measured on a BD SORP LSR II Fortessa flow cytometer (BD Biosciences, United States) with identification a minimum of 50,000 fluorescent positive events. Fluorescence Minus One was utilised to establish appropriate gating controls and the intrinsic autofluorescence of unlabelled mitochondria was taken into account [7]. Data was analysed with FlowJo V10 software (FlowJo LLC).

2.5. Statistical analysis

Data is presented as mean ± SD, with the exception Median Fluorescent Intensity (MFI) for flow cytometry. Analyses were performed using GraphPad PRISM 7.02 (GraphPad, USA), with Student T tests and two-way ANOVA used to determine significance.

3. Results and discussion

As shown in Fig. 1A, mitochondria from cytotrophoblast cells were generally round or ovoid, larger (0.2 μm up to 0.8 μm) with clearly defined cristae (Fig. 1B). Syncytiotrophoblast mitochondria were smaller (0.1 μm or less) with less cristae evident (Fig. 1C). Isolation by differential centrifugation yielded mitochondrial subpopulations of high purity (85–95%), which maintained morphology similar to mitochondria in situ, with no apparent damage induced by centrifugation.

Mitochondrial respiratory capacity was evaluated immediately after isolation and after cryopreservation. Leak state respiration (Fig. 2A) was significantly greater in Cyto-Mito (p = 0.0028) than Syncytio-Mito. Although it appears that isolated mitochondria from both cell lineages have low leak state respiration, it subsequently increased significantly with addition of substrates (17.5 fold for Cyto-Mito and 20 fold for Syncytio-Mito). Complex I + II linked respiration (CI + II; Fig. 2B), and maximum respiratory capacity (Fig. 2C) were significantly higher in Cyto-mito when compared to Syncytio-mito (CI + II: fold change = 2.50, p = 0.0002; Max Res Cap. fold change = 2.36, p = 0.002). The changes in isolated mitochondria observed in this study supports the existing literature from isolated trophoblast cells, which concluded that cytotrophoblast have a higher respiration rate than syncytiotrophoblast [8]. Further, it has been long established that changes in the cristae structure affect bioenergetics [9], and this may explain the lower bioenergetic capacity of Syncytio-Mito. Furthermore, alterations in respirasome complexes and ATP synthase have been associated with altered mitochondrial ultrastructure in the placenta [2]. This study also found that mitochondrial membrane potential was significantly lower in Syncytio-Mito (p = 0.02) compared to Cyto-Mito and this was reinforced by a significant drop in ATP production (p = 0.0016).

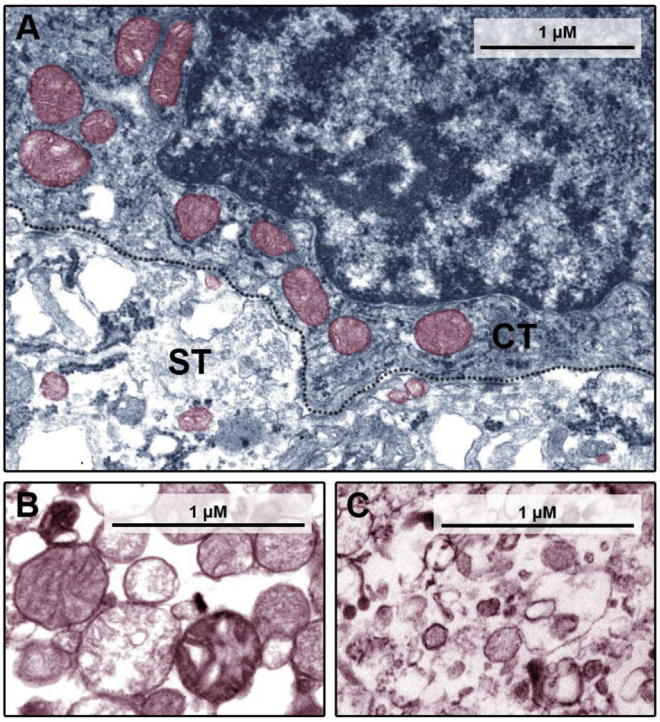

Fig. 1. Placental mitochondria in cytotrophoblasts (CT) and the syncytiotrophoblast (ST). Representative transmission electron microscope images of (**A**) intact villous placental tissue and isolated mitochondria in Fraction 1 (**B**) and Fraction 2 (**C**). Mitochondria were false coloured and a dotted line added to visually separate the cell lineages to aid in identification.

Leak state respiration (without the addition of ADP) was similarly maintained and not significantly affected by the cryopreservation. Respiration and maximum respiratory capacity were not significantly different between cryopreserved mitochondrial fractions and fresh isolates, with recovery of respiratory rates between 83 and 93% of freshly isolated mitochondria. As was the case for fresh isolates, CI + II respiration and maximum respiratory capacity were lower in Syncytio-Mito compared to Cyto-Mito (Fig. 2B; CI + II: fold change 2.36, p = 0.0011; Fig. 2C; Max Res Cap. fold change 2.14, p = 0.0009). Evaluation of the outer mitochondrial membrane via exogenous cytochrome c (Figure D) showed an increase in respiration of only 2% in the cryopreservation isolates compared to fresh isolates, while maintaining the ability to determine significance between Cyto-Mito and Syncytio-Mito (p = 0.0023 fresh and p = 0.0025 frozen). These findings suggest that cryopreservation did not have any significant effect on mitochondrial outer membrane integrity. This result is important as when considered in conjunction with membrane potential and ATP production (Fig. 2, E, F) it confirms that the inner mitochondrial membrane was

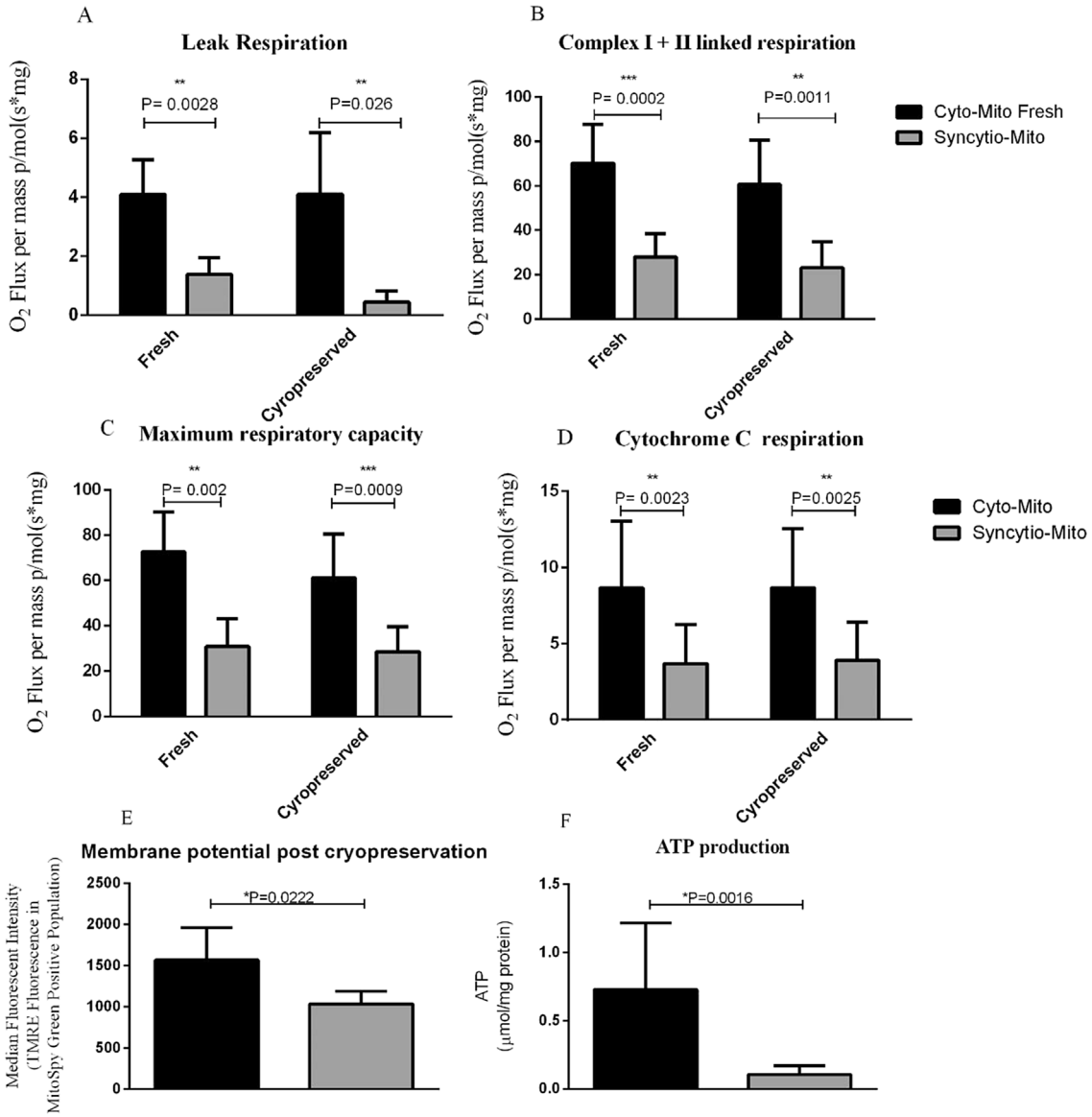

Fig. 2. Mitochondrial respiration, membrane potential and ATP production in fresh and cryopreserved mitochondria from cytotrophoblast (Cyto-Mito (Black bars)) and syncytiotrophoblast mitochondria (Syncytio-Mito (Grey bars)). A) Leak Respiration (n = 7). B) Complex I + II linked respiration (n = 7). C) Maximum respiratory capacity (n = 7). D) Cytochrome C dependent respiration (n = 7). E) Mitochondrial membrane potential post cryopreservation (n = 5). F) ATP production post cryopreservation (n = 9). All data is presented as mean ± SD with exception of E which is presented as median ± SD, and n is the number of individual placentae studied.

protected and functioning. The constituents of the isolation buffer aids in the functional recovery post cryopreservation through the use of sucrose, which preserves physiological osmolarity and prevents the loss of matrix ions, protecting the mitochondria from swelling and lysis [10,11].

When comparing our results to that of other human mitochondrial experiments, the placenta has a lower capacity for oxidative phosphorylation than skeletal muscle and cardiac tissue but may be equivalent to that of the liver [12–14]. These comparisons highlight the need to assess mitochondrial subpopulations when examining bioenergetics as respiration rates can change between tissue or even mitochondrial sub populations with a single tissue such as placenta. This research demonstrates the ability to cryopreserve isolated mitochondria at −80 °C, without changes in key functional parameters. This will facilitate the study of placental mitochondria from normal and abnormal pregnancies.

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