**Mitochondrial isolation, cryopreservation and biochemical characterisation
from placental cytotrophoblast and syncytiotrophoblast.**

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Abstract

Placental function and homeostasis is very much dependent upon trophoblast mitochondria. Mitochondria are responsible for a myriad of functions including energy production, endocrine functions, nutrient and oxygen transfer as well as protecting the placenta from oxidative insult and cell stress. The materno-fetal interface is comprised of 2 related cell lineages, cytotrophoblast cells which fuse to form the overlaying syncytium. Mitochondrial morphology and function in these 2 lineages is different and, in this study, we describe methods for mitochondrial isolation, cryopreservation and preliminary biochemical characterisation of these discrete forms of mitochondria.

Villous tissue was collected from normal term placentae following vaginal delivery (n=7). Differential centrifugation was used to isolate 2 fractions, larger mitochondria predominantly from the cytotrophoblast cells and smaller mitochondria from the syncytiotrophoblast. Freshly isolated mitochondria were assessed for Complex I and Complex II respiration and maximal respiratory capacity and these were compared to equivalent isolates that had been frozen, stored at -80°C and revived. Other important functional parameters of mitochondrial activity were also assessed post cryopreservation including membrane potential, ATP production, progesterone biosynthesis and anti-oxidant expression.

The methods described resulted in 2 mitochondrial populations which match the types of mitochondria observed in situ with electron microscopy. Both forms of mitochondria were metabolically active and recovered well from cryopreservation. There were important functional differences between cyto-mitochondria and syncytio-mitochondria including decreased respiration, decreased oxidative phosphorylation, decreased anti-oxidant expression but increased expression of the enzyme CYP11A1 and increased progesterone production. This study highlights the need to consider different types of trophoblast mitochondria in placental studies and provides methods to facilitate this.

Introduction

The placenta is a highly specialised organ that forms the interface between maternal uterine tissue and the developing fetus. The placenta is a unique organ, consisting of multiple cell layers, including, most importantly to this study, the underlying cytotrophoblast layer and a multi-nucleated, terminally differentiated syncytiotrophoblast. These cell lineages are constituents of the villous network, responsible for the maternal fetal exchange of nutrients and oxygen, as well as important metabolic and endocrine functions. The syncytiotrophoblast is in a constant state of renewal with cytotrophoblast fusion from under the basal membrane and shedding of cell debris into the maternal circulation from the outer surface. The differentiation and fusion of cytotrophoblasts into the syncytium radically changes the normal organelle and cytoskeletal organisation of these mononuclear cells as they fuse into the multinuclear and disorganised environment of the syncytium. This differentiation involves more than just morphological changes, and is reflected in the mitochondria of the cell types, with each cell type possessing mitochondria with unique properties [1].

Mitochondria are organelles responsible for a myriad of cell functions including energy production in the form of adenosine triphosphate (ATP), metabolism of lipids and proteins, hormone production, and the generation of important signals that determine cell fate. Mitochondrial dysfunction has been associated with a wide array of disorders, encompassing; cardiovascular disease, diabetes, neurological disorders and more recently gestational disorders [2]. Many of these disorders centre around oxidative stress, an imbalance between the mitochondrial generation of reactive oxygen species (ROS) and antioxidant function [3]. Many studies have shown that mitochondrial dysfunction in the placenta contributes to placental distress and pregnancy pathologies including fetal growth restriction [4], preeclampsia (PE) [5], and gestational diabetes mellitus (GDM) [6]. However, due to the complex cell structure of the placenta and the two vastly different cell lineages of the chorionic villi, questions regarding mitochondrial function and indeed dysfunction remain unresolved as placental mitochondrial sub-types are often considered as one. Our recent research has focused on better understanding mitochondrial function within an organ which possess

different populations of mitochondria, and hopefully this will ultimately allow for a better understanding of mitochondrial dysfunction in placental disorders [7].

One of the key challenges to date has been the development of methodologies for the isolation of functional mitochondria that allow key parameters to be assessed in an efficient and reproducible way. While methodologies surrounding isolation of ultrapure samples exist, once isolated, the mitochondria lose many of their functional capabilities and physiological attributes over short periods of time [8]. Therefore, the aim of this study was to develop methodologies for the isolation and cryopreservation of enriched mitochondrial fractions from both cytotrophoblast and the syncytiotrophoblast.

Methods

Placental collection

Ethical approval was granted by the Queensland Health Human Research Ethics Committee, Australia; HREC/14/QPCH/246 and Griffith University Human Research Ethics Committee; MSC/05/15/HREC. Term placentae, normal vaginal birth, were collected after written informed consent from healthy control pregnancies.

Mitochondrial isolation

Villous tissue samples were collected as previously described [9]. Isolation of mitochondrial subpopulations was achieved by differential centrifugation adapted from protocols published by Martinez et al. [8]. Villous tissue samples were homogenised in a glass dounce homogenizer with 1 mL of chilled isolation media (250 mM sucrose, 0.5 mM Na₂EDTA, 10 mM Tris, pH 7.4) on ice. The homogenate was centrifuged at 1,500 g for 10 minutes at 4 °C to pellet the cell debris. The supernatant was collected and centrifuged at 4,000 g for 15 minutes at 4 °C to produce enriched mitochondrial isolate, Fraction 1 (Cyto-Mito). The supernatant was again collected and centrifuged at 12,000 g for 15 minutes at 4 °C to produce enriched mitochondrial Fraction 2 (Syncytio-Mito). As the isolation media is designed to allow for cryopreservation of extracted samples both mitochondrial-enriched isolates were analysed fresh or snap frozen in liquid nitrogen and stored at -80 °C for subsequent analysis.

Cryopreserved isolates were revived by warming to 37°C in a water bath for approximately five minutes, prior to assessment of mitochondrial respiration. Isolates used for biochemical assays were thawed slowly on ice (between 10 and 30 minutes). For measurement of mitochondrial membrane potential, ATP production, and antioxidant capacity, mitochondria were revived from storage at -80°C up to five months later.

Electron microscopy

Placental tissues were resuspended in 3% glutaraldehyde prior to fixing, samples were post-fixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol, embedded in Epon resin using the Pelco Biowave fixation system and polymerised overnight at 60°C. Tissue blocks were sectioned using a Leica Ultracut UC6 ultramicrotome at a thickness of 80nm and imaged utilising a Joel JSM1011 transmission electron microscope fitted with an Olympus Morada digital camera. Similarly, isolated mitochondrial fractions were fixed in 3% glutaraldehyde in mitochondrial isolation buffer prior to centrifugation to create a pellet for processing. Each pellet was processed, sectioned and imaged as per the fixed intact placental tissue above. To assess mitochondrial enrichment during isolation, the total number of mitochondria were counted, and mitochondrial length measured. Mitochondria were classified as large (0.2µm or greater) or small (0.1µm or below). Mitochondria in Images have been colour enhanced to aid in identification.

Real time respirometry

Mitochondrial respiration was measured at 37°C via polarographic oxygen sensors within the Oxygraph-2k instrument (OROBOROS Instruments, Innsbruck, Austria). Mitochondrial respiration was observed by performing a SUIIT (substrate-uncoupler-inhibitor-titration) protocol examining oxygen flux and recorded via DataLab 7.0 software (OROBOROS Instruments, Innsbruck, Austria). The SUIIT protocol was as follows; isolated mitochondrial fractions were added to the Oxygraph-2k chamber in MiRO5 respiration media (0.5 mM EGTA, 3 mM MgCl₂·6H₂O, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM sucrose, 1 g/L BSA essentially fatty acid free, pH 7.1) with time allowed for baseline calibration (5mins) prior to the sequential addition of Glutamate (10mM), Malate

(2mM), Pyruvate (5mM) and ADP (1–5mM) to activate Complex I linked respiration, Succinate (10mM) to activate Complex I + II linked respiration, Carbonyl cyanide m-chlorophenyl hydrazone (CCCP; 1mM) to produce an uncoupled or Maximum respiratory capacity. Rotenone (1µM) to inhibit complex I linked respiration, Antimycin A (5mM) to inhibit complex III respiration providing a measurement of non-mitochondrial respiration. Units of respiration are expressed as p/mol O₂/sec per mg protein

ATP production

The level of ATP was quantified utilising an ATP Assay Kit (Abcam, Australia) following the manufacturer's instructions. To remove enzymes that may interfere with ATP quantification, protein was removed from the mitochondrial extracts using the Deproteinizing Sample Preparation Kit (Abcam) following the manufacturer's instructions. ATP content was is expressed as umole/mg of mitochondrial extract.

Flow cytometry measurement of membrane potential

Mitochondrial membrane potential was examined using established fluorogenic flow cytometric principles [10]. Mitochondrial isolates were incubated with 100nM MitoSPY Green (Biolegend, United States) and 50nM TMRE (tetramethylrhodamine, ethyl ester) for 25 minutes at 37°C [11]. Following incubation, samples were pelleted (12,000g, 15min), supernatant discarded, and the pellet resuspended in ice cold PBS. Samples were stored on ice and shielded from light prior to assessment of fluorescence by a BD SORP LSR II Fortessa flow cytometer (BD Biosciences, United States). Mitochondrial fractions were identified by characteristic forward and side scatter parameters with a minimum of 50,000 MitoSPY Green positive events captured. TMRE and MitoSPY Green fluorescence emission was captured by 588/12BP and 530/30BP filter sets following 563nm and 488nm excitation respectively.

Compensation by way of single stain samples at double the analytical concentration of relevant fluorogenic dye mixed with unstained sample immediately before capture [12].

Fluorescence Minus One samples were utilised to establish appropriate gating controls [10, 13]. Unstained mitochondrial fractions had an intrinsic autofluorescence of 75.53 ± 29.04 in

cytotrophoblast isolates and 62.58 ± 28.16 for syncytiotrophoblast which was taken into consideration. Data was analysed with FlowJo V10 (FlowJo LLC).

Superoxide dismutase activity

Superoxide dismutase (SOD) activity was quantified by fluorometric analysis performed utilising a Superoxide Dismutase Assay Kit (Cayman Chemicals) in accordance to the supplier's instructions. Enzyme activity was expressed a Units.mg of protein.

Progesterone production

Progesterone production was assessed by Human Total Progesterone ELISA (DuoSet, Australia) in accordance to manufacturer's guidelines. Progesterone activity is expressed as pg/mg of protein.

Western blotting

Protein concentrations of mitochondrial isolates were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Australia) following the manufacturer's instructions. 20 µg of protein was loaded onto 12% Polyacrylamide gels then separated electrophoretically. Upon completion, proteins were wet-transferred to polyvinylidene fluoride (PVDF) membrane which was then blocked with Odyssey® blocking buffer. Membranes were incubated with the primary antibody overnight with agitation at 4°C. Primary antibodies utilised in these experiments consisted of β-actin (ab8227) at 1:1000 dilution and CYP11A1 (ab175408) at 1:1000 dilution. The membranes were washed extensively with Tris Buffered Saline (TBS/Tween) and TBS followed by a subsequent incubation with secondary antibody anti-rabbit (IRDye 680 goat, Licor, Lincoln, NE,USA) at 1:1000 dilution. The developed blot was then imaged by Licor Odyssey and quantified by Image studio v 5.2.

Statistical analysis

All values are mean $\pm$ SD, with exception of median used to calculate Median Fluorescent Intensity (MFI) for flow cytometry. Students T test was used to determine significance between groups. Significance level was set at 0.05. Analyses were performed using GraphPad PRISM 7.02 (GraphPad, USA).

Results

Mitochondrial isolation.

Mitochondrial morphology in placental villous tissue sections and in mitochondrial isolates was assessed using transmission electron microscopy. Significant morphological differences were observed between cytotrophoblast and syncytiotrophoblast mitochondria (Figure 1A). Mitochondria from cytotrophoblasts were generally larger (0.2 μm up to 0.8 μm), displayed various shapes including circular, ovoid and rod like morphology, and had well defined cristae structure. Syncytiotrophoblast mitochondrial were smaller (0.1 μm or less), predominantly spherical and possessing sparse cristae. Mitochondrial enriched Fraction 1 contained 85–95% mitochondria of 0.2 μm and above, with an average size of 0.5 μm , and possessing well-defined cristae (Figure 1B). Mitochondrial enriched Fraction 2 contained 90–95% mitochondria less than or equal to 0.1 μm , spherical or punctate shape, with less well-defined cristae (Figure 1C).

Figure 1: Placental mitochondria in cytotrophoblasts (CT) and the syncytiotrophoblast (ST). Representative transmission electron microscope images of (A) intact villous placental tissue and following isolation protocol to generate Fraction 1 (B) and Fraction 2 (C). Mitochondria were false coloured and a dotted line added to visually separate the cell lineages.

Mitochondrial respiration is maintained in cryopreserved placental mitochondria

To determine if differences in mitochondrial respiration occur between the mitochondrial enriched fractions in freshly isolated samples, mitochondria respiratory capacity was evaluated immediately after isolation. Complex I + II linked respiration (CI+II; Figure 2A), and maximum respiratory capacity (ETS; Figure 2B) were significantly higher in Fraction 1 when compared to Fraction 2 (CI+II: fold change=2.50, $P=0.0002$; ETS fold change=2.36, $P=0.0002$).

Following isolation, cryopreservation and revival, mitochondrial respiratory states were assessed in matched samples to assess if the functional differences observed between the mitochondrial fractions (Figure 2A and B) were maintained. All revived isolates respired successfully ($n=7$ individual placentae). Mitochondrial respiration and maximum respiratory capacity of the ETS were not significantly different in cryopreserved mitochondrial fractions when compared to fresh isolates, with recovery of respiratory levels between 83–93% of freshly isolated mitochondria (Table 1). In all isolates studied the CI+II respiration (Figure 2C) and maximum respiratory capacity (Figure 2D) were higher in Fraction 1 (Cyto-Mito) compared to Fraction 2 (Syncytio-Mito), with similar fold changes observed between the cryopreserved mitochondria and fresh isolates (Figure 2C; CI+II: fold change 2.36, $P=0.0011$; Figure 2D; ETS fold change 2.14, $P=0.0009$).

Figure 2: Mitochondrial respiration differed between distinct placental mitochondrial isolates. A) Complex I+II linked respiration with fresh mitochondrial isolates. B) Maximum respiratory capacity of fresh mitochondrial isolates. C) Complex I+II linked mitochondrial respiration in cryopreserved mitochondrial isolates. D) Maximum respiratory capacity of cryopreserved isolates. Cyto-Mito (Black), Syncytio-Mito (Grey). $n=7$. *, $P<0.05$; **, $P<0.01$, ***, $P<0.001$.

Table 1. Recovery of mitochondrial respiration in cryopreserved placental mitochondria compared to fresh mitochondria.

	Complex I+II respiration				Maximum respiratory capacity			
	Fraction 1		Fraction 2		Fraction 1		Fraction 2	
	Respiration	% Recovery	Respiration	% Recovery	Respiration	% Recovery	Respiration	% Recovery
Fresh	70.09±17.6		28.00±10.5		72.66±17.4		30.79±12.4	
Frozen	60.60±20.0	86%	23.13±11.7	83%	61.20±19.	84%	28.61±10.9	93%

Data shown as mean±SD ($n=7$). Respiration units p/mol O_2 /sec per mg protein.

Membrane Integrity of cryopreserved placental mitochondria

Mitochondrial respiration is an assessment of oxidative phosphorylation and the production of ATP and requires mitochondria to maintain membrane potential. ATP production (Figure 3A) and mitochondrial membrane potential (Figure 3B) were maintained in all isolated, cryopreserved and revived mitochondria. ATP production and membrane potential were significantly higher in Fraction 1 compared to Fraction 2 (ATP production $P=0.0016$; mitochondrial membrane potential: $P=0.0022$).

Figure 3: A) ATP production in cryopreserved mitochondrial isolates Cyto-Mito (Black), Syncytio-Mito (Grey) expressed as $\mu\text{mol/mg protein}$ ($n=9$). **B)** Mitochondrial membrane potential in cryopreserved mitochondrial isolates, measured via MFI (Median Fluorescent Intensity of TMRE in a MitoSPY green positive population obtained by flow cytometry). Cyto-Mito (Black), Syncytio-Mito (Grey) Mean $\pm$ SD ($n=5$).

Biochemical potential of cryopreserved placental mitochondria.

To investigate the steroidogenic capacity of cryopreserved mitochondria, progesterone synthesis was examined. Protein levels of the cholesterol side-chain cleavage enzyme (CYP11A1) was significantly higher in Fraction 2 compared to Fraction 1 ($P=0.0389$, Figure 4A), showing an increase in CYP11A1 expression post cytotrophoblast differentiation. This was supported by higher progesterone production, which was significantly increased in Fraction 2 (Syncytio-Mito) when compared to Fraction 1 (Cyto-Mito) ($P<0.0001$; Figure 4B).

Figure 4: Mitochondrial endocrine function is maintained following cryopreservation.

A) Western blots of Cyto-Mito and Syncyto-Mito for CYP11A1 expression and densitometry relative to β -actin (N=7). **B)** Progesterone production (pg/mg of protein) Cyto-Mito (Black), Syncyto-Mito (Grey) Mean $\pm$ SD (n=9).

To investigate antioxidant capacity of mitochondria from separate cellular compartments in the placenta, superoxide dismutase activity (SOD) was measured in Fraction 1 and Fraction 2 cryopreserved mitochondria. SOD activity was significantly decreased in Fraction 2 compared to Fraction 1 (P=0.0485; Figure 5).

Figure 5: Antioxidant capacity of mitochondria following cryopreservation in enrich mitochondrial fractions. Super oxide dismutase (SOD) activity compared between Cyto-Mito (Black), Syncytio-Mito (Grey). Mean values $\pm$ standard deviation (n=9).

Discussion

Mitochondrial function in the placenta is central to pregnancy health, with a range of pregnancy complications associated with placental mitochondrial changes [1, 7]. Assessment of placental mitochondrial function is critical to investigating placental physiology as well as developing a better understanding of important pathologies such as fetal growth restriction, PE and GDM [1, 7]. However, mitochondria are highly responsive to environmental changes and many functions need to be assessed in functioning tissue. Further, mitochondria within two of the functional cell lineages of the placenta — cytotrophoblasts and the syncytiotrophoblast — are morphologically and functionally distinct, and may be affected in different ways by a range of factors [14]. Investigations often consider all placental mitochondria together, which can fail to detect changes that occur in only one mitochondrial subpopulation. Here we describe a simple and effective method to isolate specific mitochondrial subpopulations from the human placenta, store these isolates long term, and

revive mitochondria that maintain functional capacity. The cryopreservation and revival of mitochondrial subpopulations allows for the specific assessment of mitochondrial function in placental tissue and will be an important tool in determining placental function in health and disease. This methodology will also facilitate biobanking of placental mitochondria and allow sharing of valuable resources.

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 isolation. Isolates were able to be revived with minimal changes in respiration, respiring at 83–93% of respiratory levels of the same mitochondria isolated fresh. Further, the characteristic differences in respiration between the isolates were maintained in cryopreserved mitochondria. Following cryopreservation, mitochondria from the cytotrophoblast maintained 86% of complex I+II respiration and 84% of maximal respiration. Notably, the standard deviations of the paired samples pre and post cryopreservation are comparable, giving confidence in the ability to detect significant changes between populations regardless of the isolates being subjected to cryopreservation. With respiration of syncytiotrophoblast mitochondria through complex I+II and maximum capacity was maintained 82% and 92% of functionality, respectively.

A method to isolate mitochondria from the various cell lineages of the placenta is important as it will better facilitate the characterisation of adaptations mitochondria undergo throughout gestation, and their role in healthy and complicated pregnancies. Previous publications have presented extensive evidence of mitochondrial alterations in gestational diabetes mellitus through mitochondrial content [1], mitochondrial linked apoptosis [15], morphological alterations specific to syncytiotrophoblast [16], and oxidative stress linked to excessive ROS production [17, 18]. Additionally, mitochondrial changes have been indirectly linked to insulin resistance [19] and hyperglycemia [20]. These observations have only been considered with mitochondria as a uniform, homogenous population, yet there may be vastly different effects in the cytotrophoblast mitochondria versus the syncytiotrophoblast. The

methods presented in this study produces enriched populations of mitochondria enabling a more in depth analysis of mitochondrial function within the placenta.

Our results confirm observations made by others that morphologically and functionally mitochondria from cytotrophoblasts and the syncytiotrophoblast are different. Mitochondria from the cytotrophoblast appear as larger organelles with a variety of shapes, suggesting an active fission/fusion process. The small circular morphology of syncytial mitochondria suggest that normal processes of mitochondrial fusion may be compromised, and this would limit the ability of these organelles to deal with stress and may make them more vulnerable to mitophagy and release of mitochondrial debris. This may have implications in placenta experiencing various gestational complications that would need to adapt to cellular stress. Cytotrophoblast mitochondria are generally uniformly dispersed throughout the cytoplasm, suggesting normal movement via the cytoskeleton network [21]. In the syncytiotrophoblast, many elements of the cytoskeleton are disrupted, resulting in organelle disorganisation as is often observed with nuclear clumping near the syncytial surface [22]. This may impact on mitochondria mobility and positioning prior to fusion.

In the current study, cytotrophoblast mitochondria had well-defined cristae, indicative of actively respiring organelles producing ATP. On the other hand, syncytiotrophoblast mitochondria were mostly smaller, predominantly circular and punctate in appearance with poorly defined cristae structure. This suggests that syncytial mitochondria have shifted their metabolism away from being highly aerobic ATP producers to a more steroidogenic phenotype and this is supported by the evidence presented in this study. The rate of oxygen consumption was 2.50 and 2.36 fold less in syncytiotrophoblast mitochondria compared cytotrophoblast mitochondria. There is evidence that lower respiratory levels in syncytiotrophoblast mitochondria may be due to their atypical cristae structure, with alterations in levels of electron transfer chain complexes and function [23-25]. Furthermore, this may be a result of the inability to form respirasome complexes with a decrease in oxidative phosphorylation [23, 26]. In the syncytiotrophoblast it has been shown that ATP synthase structure is altered, with a monomeric state favoured in syncytiotrophoblast and a larger concentration of F_0F_1 -ATP

synthase dimers observed in cytotrophoblasts [27]. This potentially would have a significant effect on both mitochondrial cristae structure and respirasome formation as well as ATP production as shown in this study.

Measurement of ATP generation and membrane potential are critical components of mitochondrial physiology and indicate a population of functional mitochondria. Assessment of these parameters in cryopreserved samples illustrated an effective and reproducible methodology for observing changes in mitochondrial physiology between pathologies or subpopulations of mitochondria. Our results show ATP generation was decreased by 86% in syncytiotrophoblast compared to cytotrophoblast mitochondria, and mitochondrial membrane potential decreased by 34%. The lower ATP production may correspond to the aforementioned decreases in respiration, with less electrons transitioning through the ETC resulting in a lack of protons in the intramembranous space to activate ATP synthase [28]. In addition, the decrease in ATP production may also be due to the lower membrane potential observed in syncytiotrophoblast mitochondria [29, 30]. The increased mean fluorescent intensity associated greater membrane potential in cytotrophoblast mitochondria suggests a mitochondrial subpopulation more involved in ATP production, which is supported by the ATP results obtained in this study. The physiological ramification of this result reflects what has been observed in other studies that ATP production is not crucial for progesterone synthesis in syncytiotrophoblasts [31].

An important facet of syncytial function is to produce the large amount of progesterone needed to sustain a viable pregnancy. The first enzymatic step is the mitochondrial conversion of cholesterol to pregnanolone by the enzyme CYP11A1. Our results indicate that cell differentiation from cytotrophoblast to syncytiotrophoblast leads to enhanced expression of CYP11A1 and produces more progesterone per mg of extracted mitochondrial protein. This is consistent with the literature and suggests that the primary role of syncytiotrophoblast mitochondria is shifted towards steroidogenesis and particularly the production of progesterone, rather than the production of energy through ATP generation [32]. Morphological alterations in size and cristae structure observed between the two mitochondrial

populations have also been speculated to improve the transfer of cholesterol into syncytiotrophoblast mitochondria, as increasing the surface to volume ratio may increase the rate of cholesterol translocation into the inner membrane [8, 32]. This would also facilitate enhanced progesterone production.

The cryopreservation and revival of mitochondria allowed detailed assessment of important mitochondrial-linked processes in samples matched for potentially confounding features (e.g. gestation, method of delivery). This is an important method to consider as it provides the ability to isolate and assess mitochondrial characteristics and interactions. Due to the highly dynamic mitochondrial reticulum that constantly undergoes cycles of fission and fusion, it may prove difficult to continue to determine specific differences between mitochondrial subpopulations in a traditional in-vitro cell models or in primary tissue explants, as mitochondria will selectively degrade poorly functioning mitochondria, and quickly adapt to the new environment [33-36]. Cryopreserved mitochondria of the two isolated subpopulations, cytotrophoblasts (Fraction 1) and the syncytiotrophoblast (Fraction 2), allow for the examination of mitochondrial physiology of subpopulations after cryopreservation of up to five months, and showed that these mitochondria maintained critical functions including the ability to generate ATP, maintain mitochondrial membrane potential, generate progesterone, and exhibit an efficient antioxidant response. We believe that the selection of isolation buffer aids in this recovery post cryopreservation through the use of sucrose as a non-electrolyte, which when combined in the buffer to achieve physiological osmolarity prevents the loss of matrix ions and protects the mitochondria losing osmotic support throughout storage [37, 38]. Furthermore, cryopreserved mitochondria exhibited distinct functional differences similar to those reported for freshly isolated mitochondria isolated by similar methodologies [8].

Mitochondria produce the majority of ROS, and are also susceptible to ROS-mediated damage. ROS are countered by antioxidants, and the disruption of this ROS/antioxidant balance is associated with a range of pathologies [1]. Antioxidant activity of SOD was significantly higher in cytotrophoblast compared to the syncytiotrophoblast mitochondrial. Cytotrophoblasts may have a higher inert level of antioxidants to counter their relatively greater

mitochondrial respiration and therefore a greater level of ROS (due to the inert electron leakage from the ETC). This result may also suggest a reduced ability to scavenge ROS in syncytiotrophoblast mitochondria, which has been previously observed in the syncytium [39]. Examination into the mechanisms controlling antioxidant expression between these mitochondrial subpopulations is required.

This study demonstrates the importance of assessing mitochondria from varying cell lineages of the placenta as separate subpopulations, as their different respiratory and hormonal functional capacity will likely be altered due to cell differentiation and fusion. However, before the field advances further into pregnancy pathologies a better understanding of mitochondrial mechanics in healthy pregnancies is required. Further, this research demonstrates the ability to cryopreserve mitochondria at -80°C following an enriched isolation procedure without significant changes in functional parameters between fresh and frozen isolates from the same placentas, all the while, maintaining the ability to determine significant alterations between subpopulations from the cytotrophoblast and syncytiotrophoblast. This research has confirmed that syncytiotrophoblast mitochondria have significantly repressed bioenergetic capacity compared to cytotrophoblast mitochondria, and a general shift in physiological function towards steroidogenesis and the production of progesterone. This methodology provides the ability to perform more in depth analysis and characterisation in mitochondria and mitochondrial associated mechanisms from the placenta across healthy and complicated pregnancies, removing the often critical time dependant and limiting factor of mitochondrial degradation and availability.

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References

- [1] O. Holland, M. Dekker Nitert, L.A. Gallo, M. Vejzovic, J.J. Fisher, A.V. Perkins, Review: Placental mitochondrial function and structure in gestational disorders, *Placenta* 54 (2017) 2-9.
- [2] M. Gomez-Serrano, E. Camafeita, M. Loureiro, B. Peral, Mitoproteomics: Tackling Mitochondrial Dysfunction in Human Disease, *Oxidative medicine and cellular longevity* 2018 (2018) 1435934.
- [3] E. Georgieva, D. Ivanova, Z. Zhelev, R. Bakalova, M. Gulubova, I. Aoki, Mitochondrial Dysfunction and Redox Imbalance as a Diagnostic Marker of "Free Radical Diseases", *Anticancer research* 37(10) (2017) 5373-5381.
- [4] G.J. Burton, E. Jauniaux, Pathophysiology of placental-derived fetal growth restriction, *American journal of obstetrics and gynecology* 218(2s) (2018) S745-s761.
- [5] E. Teran, I. Hernández, L. Tana, S. Teran, C. Galaviz-Hernandez, M. Sosa-Macías, G. Molina, A. Calle, Mitochondria and Coenzyme Q10 in the Pathogenesis of Preeclampsia, *Frontiers in physiology* 9 (2018) 1561-1561.
- [6] C. Mandò, G.M. Anelli, C. Novielli, P. Panina-Bordignon, M. Massari, M.I. Mazzocco, I. Cetin, Impact of Obesity and Hyperglycemia on Placental Mitochondria, *Oxidative medicine and cellular longevity* 2018 (2018) 2378189-2378189.
- [7] O.J. Holland, J.S.M. Cuffe, M. Dekker Nitert, L. Callaway, K.A. Kwan Cheung, F. Radenkovic, A.V. Perkins, Placental mitochondrial adaptations in preeclampsia associated with progression to term delivery, *Cell death & disease* 9(12) (2018) 1150.
- [8] F. Martinez, M. Kiriakidou, J.F. Strauss, 3rd, Structural and functional changes in mitochondria associated with trophoblast differentiation: methods to isolate enriched preparations of syncytiotrophoblast mitochondria, *Endocrinology* 138(5) (1997) 2172-83.
- [9] G.J. Burton, N.J. Sebire, L. Myatt, D. Tannetta, Y.L. Wang, Y. Sadovsky, A.C. Staff, C.W. Redman, Optimising sample collection for placental research, *Placenta* 35(1) (2014) 9-22.
- [10] H.T. Maecker, J. Trotter, Flow cytometry controls, instrument setup, and the determination of positivity, *Cytometry. Part A : the journal of the International Society for Analytical Cytology* 69(9) (2006) 1037-42.
- [11] S. Dingley, K.A. Chapman, M.J. Falk, Fluorescence-activated cell sorting analysis of mitochondrial content, membrane potential, and matrix oxidant burden in human lymphoblastoid cell lines, *Methods in molecular biology (Clifton, N.J.)* 837 (2012) 231-9.
- [12] W.G. Telford, S.A. Babin, S.V. Khorev, S.H. Rowe, Green fiber lasers: an alternative to traditional DPSS green lasers for flow cytometry, *Cytometry. Part A : the journal of the International Society for Analytical Cytology* 75(12) (2009) 1031-9.
- [13] M. Roederer, Spectral compensation for flow cytometry: Visualization artifacts, limitations, and caveats, *Cytometry* 45(3) (2001) 194-205.
- [14] O.J. Holland, A.J.R. Hickey, A. Alvsaker, S. Moran, C. Hedges, L.W. Chamley, A.V. Perkins, Changes in mitochondrial respiration in the human placenta over gestation, *Placenta* 57 (2017) 102-112.
- [15] T.R. Magee, M.G. Ross, L. Wedekind, M. Desai, S. Kjos, L. Belkacemi, Gestational diabetes mellitus alters apoptotic and inflammatory gene expression of trophoblasts from human term placenta, *Journal of diabetes and its complications* 28(4) (2014) 448-59.
- [16] Q. Meng, L. Shao, X. Luo, Y. Mu, W. Xu, C. Gao, L. Gao, J. Liu, Y. Cui, Ultrastructure of Placenta of Gravidas with Gestational Diabetes Mellitus, *Obstetrics and gynecology international* 2015 (2015) 283124-283124.
- [17] M. Lappas, U. Hiden, G. Desoye, J. Froehlich, S.H.-d. Mouzon, A. Jawerbaum, The Role of Oxidative Stress in the Pathophysiology of Gestational Diabetes Mellitus, *Antioxidants & Redox Signaling* 15(12) (2011) 3061-3100.
- [18] L. Chaudhari, O.P. Tandon, N. Vaney, N. Agarwal, Lipid peroxidation and antioxidant enzymes in gestational diabetics, *Indian journal of physiology and pharmacology* 47(4) (2003) 441-6.
- [19] J.A. Kim, Y. Wei, J.R. Sowers, Role of mitochondrial dysfunction in insulin resistance, *Circulation research* 102(4) (2008) 401-14.

- [20] T. Yu, J.L. Robotham, Y. Yoon, Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology, *Proceedings of the National Academy of Sciences of the United States of America* 103(8) (2006) 2653-8.
- [21] C.J. Jones, H. Fox, Ultrastructure of the normal human placenta, *Electron microscopy reviews* 4(1) (1991) 129-78.
- [22] S.J. Calvert, M.S. Longtine, S. Cotter, C.J. Jones, C.P. Sibley, J.D. Aplin, D.M. Nelson, A.E. Heazell, Studies of the dynamics of nuclear clustering in human syncytiotrophoblast, *Reproduction* (Cambridge, England) 151(6) (2016) 657-71.
- [23] S. Cogliati, C. Frezza, Maria E. Soriano, T. Varanita, R. Quintana-Cabrera, M. Corrado, S. Cipolat, V. Costa, A. Casarin, Ligia C. Gomes, E. Perales-Clemente, L. Salviati, P. Fernandez-Silva, Jose A. Enriquez, L. Scorrano, Mitochondrial Cristae Shape Determines Respiratory Chain Supercomplexes Assembly and Respiratory Efficiency, *Cell* 155(1) (2013) 160-171.
- [24] K.M. Davies, M. Strauss, B. Daum, J.H. Kief, H.D. Osiewacz, A. Rycovska, V. Zickermann, W. Kühlbrandt, Macromolecular organization of ATP synthase and complex I in whole mitochondria, *Proceedings of the National Academy of Sciences* 108(34) (2011) 14121.
- [25] R. Guo, J. Gu, S. Zong, M. Wu, M. Yang, Structure and mechanism of mitochondrial electron transport chain, *Biomedical Journal* 41(1) (2018) 9-20.
- [26] P. Paumard, J. Vaillier, B. Coulary, J. Schaeffer, V. Soubannier, D.M. Mueller, D. Brethes, J.P. di Rago, J. Velours, The ATP synthase is involved in generating mitochondrial cristae morphology, *The EMBO journal* 21(3) (2002) 221-30.
- [27] D. De los Rios Castillo, M. Zarco-Zavala, S. Olvera-Sanchez, J.P. Pardo, O. Juarez, F. Martinez, G. Mendoza-Hernandez, J.J. García-Trejo, O. Flores-Herrera, Atypical cristae morphology of human syncytiotrophoblast mitochondria: role for complex V, *The Journal of biological chemistry* 286(27) (2011) 23911-23919.
- [28] N. Klusch, B.J. Murphy, D.J. Mills, Ö. Yildiz, W. Kühlbrandt, Structural basis of proton translocation and force generation in mitochondrial ATP synthase, *Elife* 6 (2017) e33274.
- [29] L.D. Zorova, V.A. Popkov, E.Y. Plotnikov, D.N. Silachev, I.B. Pevzner, S.S. Jankauskas, V.A. Babenko, S.D. Zorov, A.V. Balakireva, M. Juhaszova, S.J. Sollott, D.B. Zorov, Mitochondrial membrane potential, *Analytical biochemistry* 552 (2018) 50-59.
- [30] P. Dimroth, G. Kaim, U. Matthey, Crucial role of the membrane potential for ATP synthesis by F(1)F(o) ATP synthases, *The Journal of experimental biology* 203(Pt 1) (2000) 51-9.
- [31] O. Flores-Herrera, S. Olvera-Sanchez, M. Esparza-Perusquia, J.P. Pardo, J.L. Rendon, G. Mendoza-Hernandez, F. Martinez, Membrane potential regulates mitochondrial ATP-diphosphohydrolase activity but is not involved in progesterone biosynthesis in human syncytiotrophoblast cells, *Biochimica et biophysica acta* 1847(2) (2015) 143-152.
- [32] F. Martinez, S. Olvera-Sanchez, M. Esparza-Perusquia, E. Gomez-Chang, O. Flores-Herrera, Multiple functions of syncytiotrophoblast mitochondria, *Steroids* 103 (2015) 11-22.
- [33] L. Tilokani, S. Nagashima, V. Paupe, J. Prudent, Mitochondrial dynamics: overview of molecular mechanisms, *Essays in biochemistry* 62(3) (2018) 341-360.
- [34] W.-X. Ding, X.-M. Yin, Mitophagy: mechanisms, pathophysiological roles, and analysis, *Biological chemistry* 393(7) (2012) 547-564.
- [35] S.L. Archer, Mitochondrial Dynamics — Mitochondrial Fission and Fusion in Human Diseases, *New England Journal of Medicine* 369(23) (2013) 2236-2251.
- [36] V. Eisner, M. Picard, G. Hajnóczky, Mitochondrial dynamics in adaptive and maladaptive cellular stress responses, *Nature Cell Biology* 20(7) (2018) 755-765.
- [37] J. Nedergaard, B. Cannon, [1] Overview—Preparation and properties of mitochondria from different sources, *Methods in Enzymology*, Academic Press 1979, pp. 3-28.
- [38] J.M. Skehel, Preparation of extracts from animal tissues, *Protein Purification Protocols*, Springer 2004, pp. 15-20.

[39] J.L. Frendo, P. Therond, J. Guibourdenche, J.M. Bidart, M. Vidaud, D. Evain-Brion, Modulation of copper/zinc superoxide dismutase expression and activity with in vitro differentiation of human villous cytotrophoblasts, *Placenta* 21(8) (2000) 773-81.