Changes in mitochondrial respiration in the human placenta over gestation

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Abstract

Introduction: Placental mitochondria are subjected to micro-environmental changes throughout gestation, in particular large variations in oxygen. How placental mitochondrial respiration adapts to changing oxygen concentrations remains unexplored. Additionally, placental tissue is often studied in culture; however, the effect of culture on placental mitochondria is unclear.

Material and methods: Placental tissue was obtained from first trimester and term (laboured and non-laboured) pregnancies, and selectively permeabilized to access mitochondria. Respirometry was used to compare respiration states and substrate use in mitochondria. Additionally, explants of placental tissue were cultured for four, 12, 24, 48, or 96 hours and respiration measured.

Results: Mitochondrial respiration decreased at 11 weeks compared to earlier gestations, and increased from 12 weeks compared to 11 weeks of gestation ($p=0.05–0.001$). In term placentae, oxidative phosphorylation (OXPHOS) through mitochondrial complex IV ($p<0.001$), the relative proportion of OXPHOS CI ($p<0.001$), and the total capacity of the respiratory system ($p=0.003$) were higher compared to first trimester. Respiration was increased ($p\leq0.006–0.001$) in laboured compared to non-laboured placenta. After four hours of culture, respiration was depressed compared to fresh tissue from the same placenta and continued to decline with time in culture. Markers of apoptosis were increased, while markers of autophagy and mitochondrial biogenesis were decreased, after four hours of culture.

Discussion: Respiration alters over gestation and with labour. Decreased respiration at 11 weeks may relate to onset of maternal blood flow, and increased respiration as a result of labour may be an adaptation to ischaemia-reperfusion. At term, mitochondria were more susceptible to changes in respiratory function relative to first trimester when cultured *in vitro*, perhaps reflecting changes in metabolic demands as gestation progresses. Metabolic plasticity of placental mitochondria has relevance to placenta-mediated diseases.

Introduction

The placenta performs many key functions throughout gestation, transporting nutrients, gases and waste between maternal and fetal systems, and modifying maternal systems to maintain the pregnancy [1]. During gestation the placenta undergoes significant growth and structural changes; initially sustaining the fetus in a low-oxygen environment before the onset of maternal blood flow, then in the third trimester delivering oxygen rich blood to support the exponential rise in energy demands of the growing fetus [2-6].

Mitochondria have an array of roles within the placenta, including the generation of ATP, synthesis of proteins, the urea cycle, lipid synthesis, steroid synthesis, and regulation of programmed cell death. Placental mitochondria must adapt to these shifting roles including changes in the energetic demands of the placenta throughout gestation. However, information about temporal changes in mitochondrial function with gestation is lacking.

The syncytiotrophoblast is formed through the fusion of underlying cytotrophoblasts. Cytotrophoblast mitochondria have a more classical morphology, whereas syncytiotrophoblast mitochondria are smaller and have an irregular shape and cristae structure potential related to their role in progesterone production [7-10]. Both the syncytiotrophoblast and cytotrophoblasts also have important roles in metabolic reactions and transport to support the fetus, and mitochondria are likely to be critical organelles in these processes [11].

While the placenta receives maternal blood through the uterine spiral arteries, until about weeks 10–12 of gestation these vessels are blocked by plugs of trophoblast that restrict the flow of maternal red blood cells, resulting in a low oxygen environment (~2.7 kPa) during fetal organogenesis [2,12]. After 10 to 12 weeks of gestation, full maternal blood flow to the placenta is

commences as the trophoblast plugs clear and oxygen tension rises three fold (~8 kPa) then falls at term (~5.4 kPa) [2,12]. Over this transition in oxygenation heat shock proteins and antioxidant enzymes appear to be required to support the placenta [12-14]. Placental oxygen consumption in the second and third trimesters is required for the high levels of protein synthesis and transport needed to support the growing fetus [5,6]. The placenta is also subjected to ischemic events during uterine contractions with labour. While it has been suggested that mitochondrial oxidative phosphorylation may vary with gestation [1], how placental mitochondrial respiration is affected by fluctuating oxygen availability is unknown.

Because mitochondria are central to many tissue functions, mitochondrial dysfunction is often implicated in disease states [15]. Obesity and pregnancy related pathologies such as preeclampsia and intrauterine growth restriction (IUGR) impact placental mitochondrial function [10,16-19]. Multiple studies of preeclampsia and IUGR have shown increases in mitochondrial content [16,19-22], indicating placental mitochondrial biogenesis is initiated in reaction to adverse conditions [23]. In addition, placental tissues are also routinely held *in vitro* to study aspects of placental biology. However, *in vitro* culture likely leads to experimental artefacts, especially over extended timeframes in oxygen conditions that differ from those *in vivo* [14,24-27]. Altered mitochondria function following *in vitro* manipulation may have implications when testing bioenergetics function of placentae.

Given the importance of placental mitochondrial respiration in both healthy and pathological pregnancies, and the use of *in vitro* models to investigate placental function, understanding how mitochondria respond is critical. This study has investigated the respiration of mitochondria in healthy tissue from first trimester and term pregnancies to gain a detailed understanding of how respiration responds to the different stages of gestation and to *in vitro* culture.

Materials and methods

Materials, tissue collection and culture

All chemicals were purchased from Sigma Aldrich, Australia unless noted. This study was approved by the Auckland Regional Ethics Committee, New Zealand and the Queensland Government Human Research Ethics Committee, Australia. All tissues were obtained following written informed consent. First trimester placental tissues were obtained from 19 pregnancies (7.2–13.0 weeks of gestation; gestation determined by ultrasound measurement of crown-rump length and cardiac activity) following elective surgical termination, and rinsed briefly in PBS within 10 mins of delivery. Tissues were immediately transferred to histidine-tryptophan-ketoglutarate transplant solution (Essential Pharmaceuticals LLC, USA) on ice and transported to the laboratory. Term placental tissues were collected from 28 normal singleton pregnancies (i.e. no known pathologies; Supplementary table 1) and processed as above within 15 min of delivery following elective caesarean section or vaginal delivery. Tissue was then washed in the laboratory in PBS and dissected into approximately 50 mg samples.

High-resolution respirometry

The plasma membrane of tissue samples were permeabilized with 50 µg/mL saponin in 1 mL BIOPS solution (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2 \cdot 6H_2O$, 20 mM taurine, 15 mM Na_2 phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol, 50 mM MES, pH 7.1) for 30 min at on ice, washed twice for 10 minutes on ice in MiR05 respiration medium (0.5 mM EGTA, 3 mM $MgCl_2 \cdot 6H2O$, 60 mM K-lactobionate, 20 mM Taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM Sucrose, 1 g/L BSA essentially fatty acid free, pH 7.1) and blot-dried before measuring wet

weight. To measure mitochondrial respiration, 10–20 mg wet weight of tissue was added to an Oxygraph-2k respirometers (Oroboros Instruments, Austria) chamber containing MiR05 at 37°C.

The activity of specific respiratory states was determined by the addition of substrates and ETS inhibitors [28,29]. Two different substrate-uncoupler-inhibitor titration (SUIT) protocols were used to assess mitochondrial function (Figure 1): For **SUIT protocol one** (Figure 1A), pyruvate (5mM), glutamate (10mM) and malate (2mM) were added to determine complex I (CI) mediated LEAK respiration, then oxidative phosphorylation (OXPHOS) through CI was stimulated by the addition of ADP (1–5mM). Cytochrome c (10µM) was then added to test the integrity of the outer mitochondrial membrane. Rotenone (1µM) was added to inhibit CI. Succinate (10mM) addition then stimulated OXPHOS through complex II (CII), and this was followed by titration of the uncoupler carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone (FCCP) or carbonyl cyanide m-chloro phenyl hydrazone (CCCP; 1mM) to investigate electron transport system (ETS) capacity with CII mediated flux alone. The addition of antimycin A inhibited complex III (CIII), and residual oxygen consumption was measured (indicating non-ETS respiration). N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride (TMPD; 0.5mM) and ascorbate (2mM) were added to determine OXPHOS through complex IV.

SUIT protocol two (Figure 1B) differed by the addition of succinate directly after cytochrome c, to elevate OXPHOS through complexes I and II (OXPHOS CI,II). Titrations of CCCP were used to investigate the ETS capacity with CI and CII (ETS CI,II). Rotenone and antimycin A were added to inhibit complexes I and III, respectively, followed by TMPD and ascorbate.

Respirational fluxes were corrected for residual oxygen consumption (ROX), where CI and CIII were fully inhibited. For SUIT protocol two, OXPHOS CII was calculated as the difference between respiratory flux in OXPHOS with CI and CII substrates (glutamate, malate, pyruvate and succinate), and respiratory flux with saturating ADP and CI supporting substrates (glutamate, malate and

pyruvate). The flux control ratio (*FCR*) for oxygen flux in different respiratory states was calculated as OXPHOS at that state as a proportion of the ETS maximum capacity. The LEAK control ratio was calculated as LEAK respiration flux with CI substrates, as a fraction of OXPHOS capacity with CI substrates and saturating ADP. The respiratory control ratio (*RCR*) was calculated as OXPHOS capacity with CI substrates and saturating ADP as a fraction of LEAK respiration with CI substrates. The reserve respiratory capacity was calculated as the difference between the ETS CI,CII and OXPHOS CI,CII, and this represents the apparent ETS excess capacity following uncoupling.

Mitochondrial content

Mitochondrial content was determined by real-time PCR normalizing two mitochondrial genes (mtF3163 GCCTTCCCCCGTAAATGATA, mtR3260 TTATGCGATTACCGGGCTCT and MTRT2_f TCCTCCTATCCCTCAACCCC, MTRT2_r CACAATCTGATGTTTTGGTTAAAC) to a nuclear gene (MTBA_f AGCGGGAAATCGTGCGTGAC, MTBA_r AGGCAGCTCGTAGCTCTTCTC). DNA was extracted via isopropanol precipitation. Briefly, approximately three mg of tissue was incubated at 55^{o}C overnight in lysis buffer with proteinase K then protein was predicted by ammonium acetate. DNA was predicated by isopropanol and washed with 70% ethanol. DNA concentration was measured on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Australia). PCR reactions were run with 10 ng of DNA and QuantiNova SYBR Green PCR Kit (Qiagen, Australia) following the manufacturer's instructions on a QuantStudio 3 Real-time PCR Thermal Cycler (Thermo-Fisher, Australia), and mitochondrial content was calculated using the $2^{-\Delta Ct}$ method.

Tissue culture

Explants of placental tissue (first trimester Supplementary table 2; term 36.4–39.6 weeks gestation) of approximately 400 mg wet weight were cultured in 12-well plates in Advanced DMEM/F12

medium (Gibco, Australia) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin streptomycin (Gibco) at 37°C with 5% CO_2 for four, 12, 24, 48, or 96 hours [28,29].

Western blot

Primary antibodies used in Western blot analyses were B-cell CLL/lymphoma 2 (BCL2; 2870; Cell Signaling), nuclear respiratory factor 1 (NRF1; 12381; Cell Signaling), microtubule associated protein 1 light chain 3 alpha (LC3; 4108; Cell Signaling) and glutathione peroxidase 1 (GPx; ab108427; Abcam). All primary antibodies were used at a concentration of 1 in 1,000, and secondary antibody at a concentration of 1 in 5,000. Proteins were extracted from whole placental tissue and Western blot analysis was conducted as previously described [28,29].

Statistical analysis

Statistical analysis was performed using Graph Pad, PRISM version 6 (GraphPad, USA). A minimum of four individual placentae were included per experimental group. Data were analysed by the D'Agostino and Pearson test to determine normality, and parametric or non-parametric tests used where appropriate. Changes in respiration and mitochondrial content across early gestation were determined by Kruskal-Wallis. Differences between first trimester and term tissue respiration were determined by Mann-Whitney test for respiratory flux and flux control factors, and t test for comparison of spare respiratory capacity in term tissue. Changes in respiration in laboured and non-laboured placentae were determined by t-test. The effects of *in vitro* culture on mitochondrial function in first trimester and term placenta were determined by ANVOA or Kruskal-Wallis test. Changes in protein expression term placentae after *in vitro* culture were determined by paired t test. For comparison of respiration in first trimester and term placentae, pooled results from first trimester (n=13) and term (n=10) were compared.

Results

Respiration across early gestation

Respiration was monitored in tissues from ~7–13 weeks of gestation. In most respiratory states there was a relative decrease at 11 weeks and an increase after 12 weeks of gestation (p=0.05–0.001; Figure 2). Normalising respiration to the maximum uncoupled respiratory capacity revealed that oxidative phosphorylation (OXPHOS) through mitochondrial complex I (CI) as a proportion of maximum respiration was greater after 12 weeks and that OXPHOS through complex II (CII) as a proportion of maximum respiration was significantly lower after 12 weeks relative to tissues collected before 11 weeks of gestation (Figure 2B). Mitochondrial content was significantly (p=0.042) increased by 2.42 fold after 12 weeks when compared to 7–10 weeks but not different at 11 weeks (Figure 2C). When OXPHOS was normalized to mitochondrial content there was a significant (OXPHOS CI p=0.013; OXPHOS CII p=0.018; OXPHOS CIV p=0.024) decrease in relative respiration after 11 weeks (Figure 2C).

Respiration in first trimester and term placenta

Respiration in non-laboured first trimester and term tissue was similar in several respiratory states, however mitochondrial respiration increased in term tissue in specific states (Figure 3). OXPHOS CI and OXPHOS CII, non-phosphorylating respiration, and the electron transport system (ETS) maximum capacity were not significantly different between first trimester and term tissue. However, OXPHOS through complex IV (CIV) was significantly (p<0.001) higher in term placentae (fold change 1.60), and residual oxygen consumption was significantly (p<0.001) lower, decreasing by 49% (Figure 3A). OXPHOS CI as a proportion of respiratory capacity, and OXPHOS CI relative to OXPHOS CII were

respectively 41% and 48% higher in term tissues ($p<0.001$; Figure 3B). Reserve respiratory capacities were 3.79 fold higher in term tissues ($p=0.003$; Figure 3B).

To further explore the involvement of the different mitochondrial complexes, term placentae were also investigated with the SUIT protocol two (Figure 1B), where ETS was determined without inhibition of CI. Reserve respiratory capacity was significantly ($p=0.001$) increased when CI and CII were assayed together, compared to CII alone (Figure 3C).

Effects of labour on respiration

Mitochondrial respiration increased by 37% and 73% ($p\leq0.006–0.001$) in all tested states in labour delivered to non-laboured placenta, whereas non-ETS respiration did not change (Figure 4A). OXPHOS CI exhibited the greatest proportional change (fold change 1.73); similarly, OXPHOS CI was also increased in proportion to the respiratory capacities of laboured placentae (fold change 1.16; $p=0.022$; Figure 4B). OXPHOS capacity through mitochondrial complex I as a fraction of non-phosphorylating LEAK respiration (*RCR*) was increased in laboured placentae (fold change 1.26; $p=0.002$; Figure 4B).

Effects of in vitro culture

Oxygen flux was similar to fresh tissue after four hours in vitro culture. By 12 hours of culture (first trimester; term 24 hours) a decrease was apparent in most respiratory states ($p<0.05–0.001$; Figure 5A and B). However, CIV mediated flux did not decrease significantly in term tissues (fold change 0.62–0.94), but did in the first trimester tissues (fold change 0.42–0.54; Figure 5A and B). In term tissues, CI flux as a proportion of OXPHOS respiratory capacity decreased with culture duration (Figure 5D and E). Complex-specific respiration was also normalised to respiration through CI and CII

(SUIT protocol two). In fresh tissues, the fractional contribution of CI and CII to the ETS was greater (complex I: 0.37±0.09; complex II: 0.41±0.11), but in cultured tissues CII became increasingly dominant ($p<0.05$–0.0001; Figure 5E). An increase in the cytochrome c flux control factor, indicative of mitochondrial damage, was observed in term tissue after 24 hours, but not until 96 hours in first trimester tissue (Figure 5F). To further investigate potential consequences of mitochondrial damage following *in vitro* culture, markers of cellular processes linked to mitochondrial function were investigate in term tissue after four hours in culture. BCl2 (anti-apoptosis marker; $p=0.001$), NRF1 (regulator of mitochondrial biogenesis; $p<0.001$), and in the ratio of LC3 I/II (autophagy marker; $p=0.030$) were significantly decreased. No change was seen in the antioxidant GPx-1 (Figure 6).

Discussion

Here we present data illustrating dynamic changes in human placental mitochondrial respiration throughout gestation, and in mitochondrial respiration in tissues derived from laboured birth and Caesarean section. We show that respiration changes over gestation, as on comparison the mitochondrial complex utilisation differs between first trimester and term tissues. Mitochondria in skeletal muscles have been shown to adapt their respiration in response to training [30,31] and mitochondria in the fetal brain also respond to hypoxia [32]. Placental mitochondria are reported to be altered in disease states [10,16-18,20-22], many of which are thought to include hypoxia/reperfusion insults [10]. As the oxygen pressure increases three fold between 10 and 12 weeks of gestation when maternal blood flow to the placenta is established [12], placental mitochondria will likely experience profound changes in functional requirements [33].

When provided with excess oxygen *in vitro*, most respiratory states of first trimester and term tissues were similar (e.g. OXPHOS CI and CII, and LEAK), suggesting minimal differences in mitochondria respiratory behaviour in the placenta over gestation. However, term placenta had 60%

greater mitochondrial respiration through CIV, and a 0.4 fold higher proportional flux through OXPHOS CI, as well as nearly seven fold increased reserve capacity with CI+CII than with CII alone. Respiratory flux through CI accepts electrons from NADH + H^+, while CII accepts electrons from succinate to form $FADH_2$ with ~300 mV less reducing power than NADH. Hence, CI supported respiratory chains transfer a predicted 10 protons (NADH: CI 4 H^+, CIII 4 H^+, CIV 2 H^+), relative to the six protons from CII driven chains ($FADH_2$: CII 0 H^+, CIII 4 H^+, CIV 2 H^+). This effectively represents a greater efficiency of ATP production though CI, and decreased CI flux represents a substantial loss of OXPHOS capacity. The lower proportionate O_2 flux that is supported by CI in the first trimester perhaps indicates a greater reliance on succinate, which can be derived from γ-amino butyric acid and glutamate metabolism [34].

Placental mitochondria are in the immediate zone of gas transfer and theoretically supply the bulk ATP to placental tissues that mediate fetal growth, nutrient transport, and ion regulation [7,33]. Bustamante et al. [35] found a low respiratory control ratio (*RCR*; 1.1±0.4) in syncytiotrophoblast mitochondria using substrates for CI and suggest that these mitochondria have impaired respiratory efficiency. Conversely, using succinate as a substrate (OXPHOS CII), De Los Rios Castillo et al. [7] found a *RCR* of ~7.1 in syncytiotrophoblast, suggesting strong coupling of mitochondrial respiration and ATP synthesis. In the current study, the *RCR* in the relevant tissue (laboured term) was 4.2±0.7 at OXPHOS CI. In both previous reports mitochondria were isolated from tissue and the observed differences may be due to assaying isolated mitochondria, as opposed to mitochondria retained within tissue. Isolated mitochondria can lose cristae structure which may impact OXPHOS, although activity of mitochondrial complexes is maintained [7].

During early human pregnancy, placental oxygen pressures are low as the maternal supplied circulation is incomplete. Following unplugging of the uterine spiral arteries at 10–12 weeks of gestation, oxygen pressures rise steeply from ~20 mm Hg (2.5%) to ~60 mm Hg (8.5%). Over this

time there are changes in placental antioxidant function, amniotic pH, and PCO_2 in the exocoelomic cavity [3,12-14,36]. In the current study, mitochondrial respiration and content also followed a distinct pattern that is potentially related to the onset of maternal blood flow. At ~11 weeks of gestation mitochondrial respiration decreased, and then rose sharply from 12–13 weeks of gestation. The syncytiotrophoblast has low levels of antioxidants, and mitochondria in this tissue are particularly sensitive to oxygen-mediated damage [14]. The morphology of syncytiotrophoblast mitochondria changes between 6–14 weeks of gestation, with cristae structure becoming less ordered and their numbers diminished at 9–10 weeks the recovering after 12 weeks [12]. These changes potentially reflect the changed mitochondrial respiration we report here, which may be related to the onset of maternal circulation. Increased mitochondrial biogenesis might also be initiated at this time with enhanced antioxidant defences [23]. Indeed, oxidative stress and endogenous antioxidant function are positively correlated with gestational age [12]. We found that mitochondrial content did not change between ~11 weeks and earlier gestations but was increased after 12 weeks, indicating that mitochondrial biogenesis is initiated after maternal blood flow has been established. Therefore, the decrease in respiration may be indicative of oxidative damage which leads to tissue adaptation to the increased oxygen supply via increased mitochondrial content and respiration, as is seen in other tissues [31,32].

Mitochondrial content was lower in early gestations (below ~11 weeks) compared to later in the first trimester. Glycolytic or polyol-based metabolic pathways are utilised by the placenta in early gestation when oxygen is limited, and placental mitochondria may therefore be less important in the production of ATP during this stage [1,36,37]. Alternatively, as OXPHOS is 15 fold more efficient at synthesising ATP than substrate level phosphorylation, a greater load may be placed on fewer mitochondria in early gestation.

Culture conditions may be damaging, as *in vitro* culture of term tissue at standard O_2 pressures showed markers of increased autophagy and apoptosis after 24 hours, and a marker of decreased mitochondrial biogenesis after only four hours. Conversely, no evidence of mitochondrial damage was observed in first trimester tissue until 96 hours. Term tissue mitochondrial respiration also showed greater depression *in vitro* than first trimester, suggesting that first trimester tissues are more tolerant to oxidative stress. These results contrast with previous findings that early gestational tissues may be more prone to oxidative damage *in vitro* [14]; however, mitochondrial activity in the Watson et al. [14] study was investigated over 7–15 weeks of gestation, and did not extend to term tissue. Placental tissue can maintain metabolically active functions after extended time *in vitro* [14,38], and it is possible that this is achieved through a switch to anaerobic metabolism.

In this present study all mitochondrial respiratory states were increased in placentae subject to vaginal deliveries and the *RCR* increased in laboured placenta, suggesting improved coupling to ATP production and/or decreased oxidative damage, as a decrease in the *RCR* often coincides with elevated oxidative stress. However, previous workers have reported decreased placental ATP [39] and increased markers of placental oxidative stress [40-43] with labour. Uterine contractions will constrict blood flow, and lead to placental ischaemia-reperfusion [2]. Therefore increased mitochondrial respiration resulting from labour may represent an acute response that induces mitochondrial adaptations and antioxidant defences [23].

Conclusion

Placental mitochondria are responsive to different *in vivo* and *in vitro* environments. Components of the ETS respond variably throughout gestation and the overall respiratory output of the mitochondria changes in response to gestational age and with labour. Of note, OXPHOS CI had the greatest relative increase over early gestation and with labour, and was also increased

proportionally in term compared to first trimester placenta. Oxygen levels also fluctuate over early gestation [12] and during labour [2], as maternal blood flow to the placenta is established or interrupted. Variations in OXPHOS CI flux may relate to changes in oxidative stress loads, as CI appears to be more sensitive to damage than other ETS components [44], and CI appears to be most labile to damage *in vitro* placental culture.

Oxygen tensions and demands of fetal growth increase between the first and third trimesters with shifts from the utero-placental development to fetal-placental growth [6,45], and these changing placental roles may lead to distinct mitochondrial requirements. The required metabolic plasticity of placental mitochondria in healthy tissue may provide insights to how mitochondria respond in pathological settings.

Acknowledgments

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Supplementary table 1. Patient characteristics for term placental collection.

Maternal age (years)	30.44±5.27
Maternal BMI	27.71±5.35
Gestation (weeks)	39.01±1.12
Baby weight (g)	3857.56±355.88
Placental weight (g)	657.49±99.06

Mean±standard deviation; BMI=body mass index

Supplementary table 2. Gestational ages of first trimester placentae used in tissue culture experiments.

Gestation (weeks)	Culture time (hours)
7.2	0
8.3	0
8.5	0
8.5	0
10.2	0
10.3	0
11.0	0
11.5	0
12.0	0
12.1	0
13.0	0
7.2	4
8.3	4
8.5	4
8.5	4
10.2	4
10.3	4
11.0	4
11.5	4
12.0	4
12.1	4
13.0	4
8.0	12
10.3	12
11.0	12
11.5	12
12.0	12
13.0	12
9.1	14
10.3	14
10.6	14
11.0	14
11.5	14
12.0	14
13.0	14
9.1	24
9.2	24
9.5	24
11.3	24
9.2	48
11.3	48
9.1	48
9.5	48
10.6	48
8.5	96
8.6	96
10.2	96
12.1	96

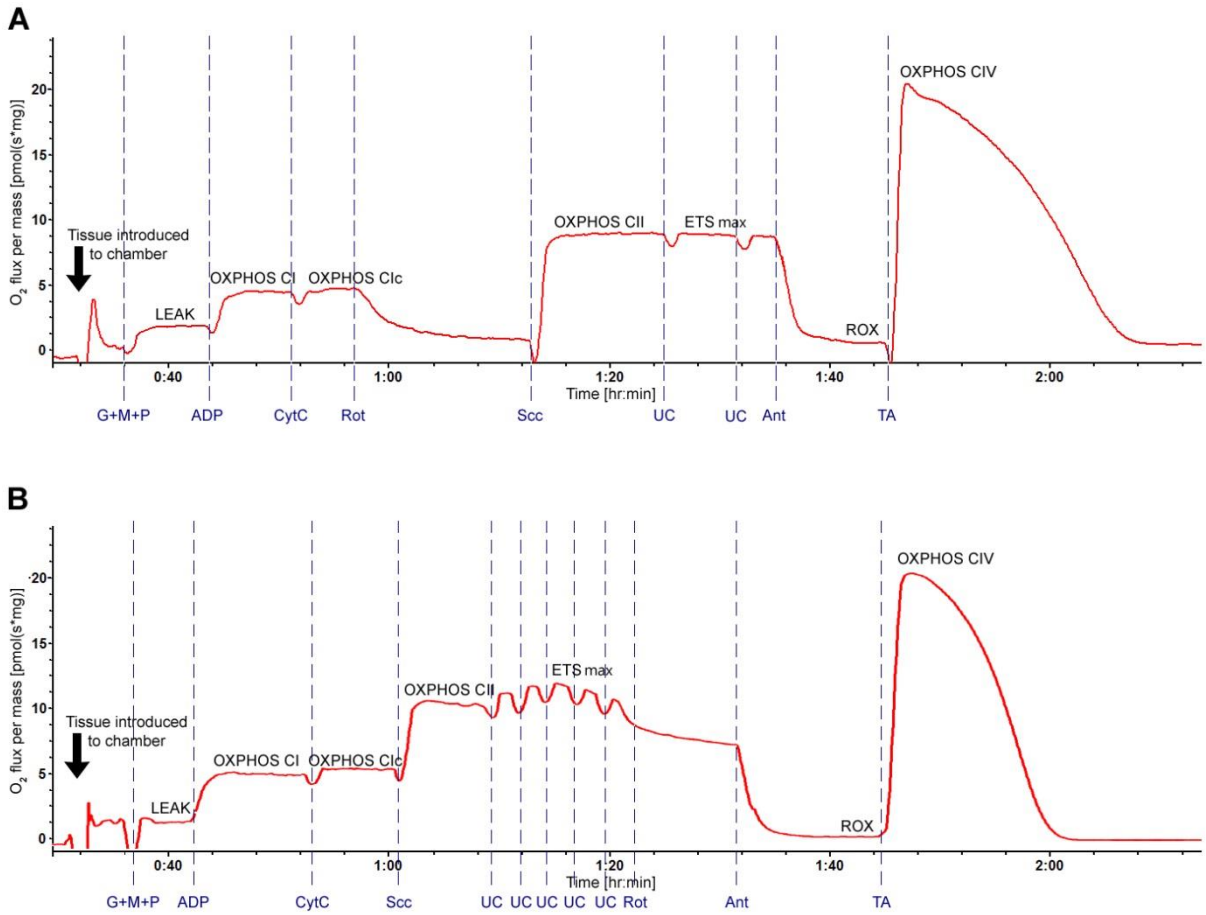
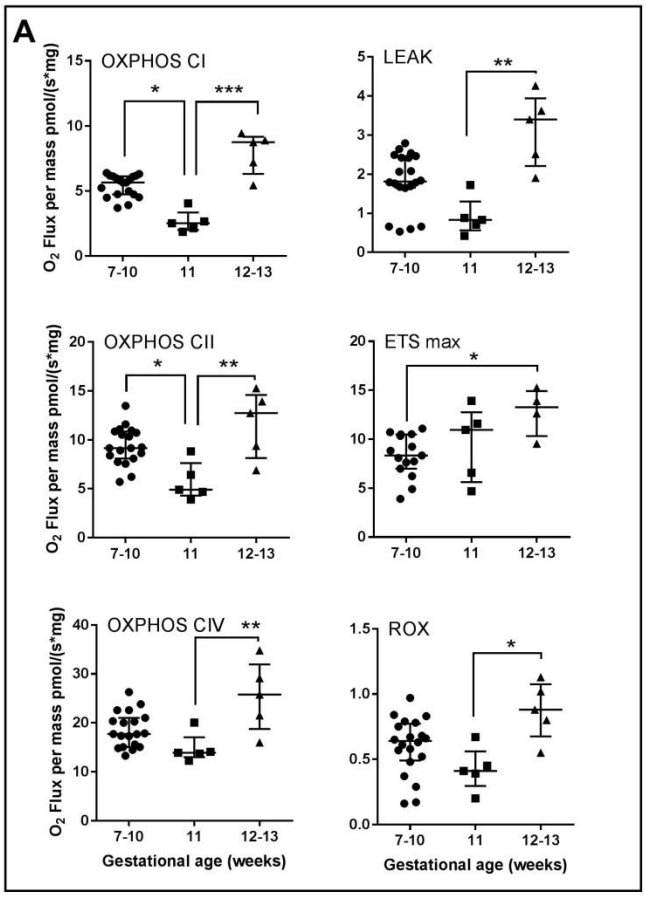
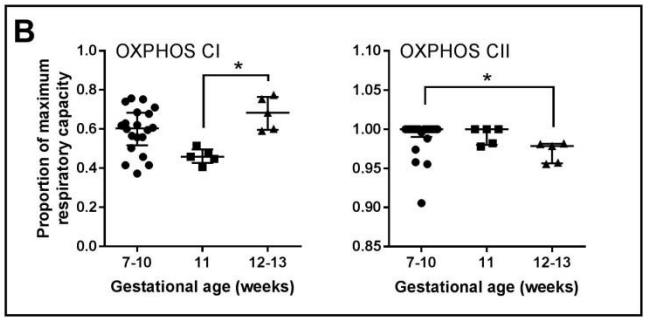


Figure 1. Substrate-uncoupler-inhibitor titration (SUIT) protocols. Representative oxygraph traces of oxygen flux relative to tissue mass, vertical hashed lines show the introduction of substrates or inhibitors. (A) SUIT protocol one, (B) SUIT protocol two. In each plot: LEAK=non-phosphorylating respiration; OXPHOS CI, CII and CIV=oxidative phosphorylation through mitochondrial complex I, II and IV, respectively; ETS max=electron transfer system maximum capacity; ROX=non-ETS respiration; G+M+P=glutamate, malate and pyruvate; CytC=cytochrome c; Rot=rotenone; Scc=succinate; UC=uncoupler, carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone or carbonyl cyanide m-chloro phenyl hydrazone; Ant=antimycin A; TA= N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride and ascorbate.


A
OXPHOS CI
LEAK
OXPHOS CII
ETS max
OXPHOS CIV
ROX
O2 Flux per mass pmol/(s*mg)
7-10
11
12-13
Gestational age (weeks)


B
OXPHOS CI
OXPHOS CII
Proportion of maximum respiratory capacity
7-10
11
12-13
Gestational age (weeks)

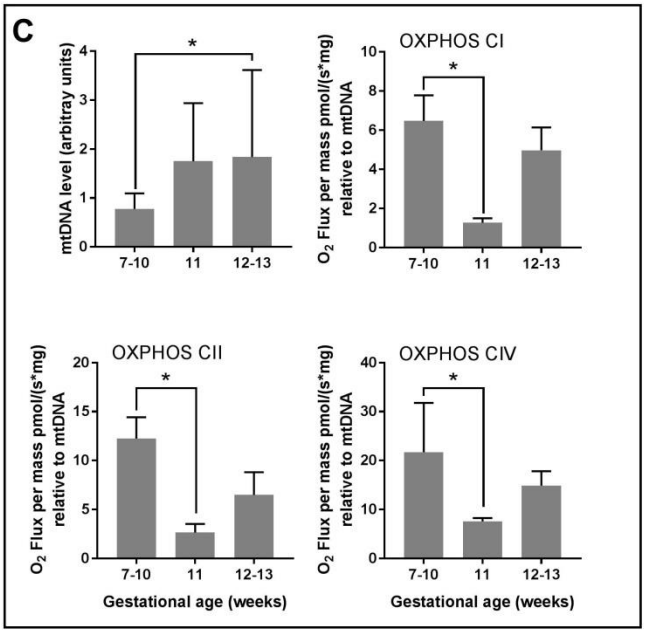

C
mtDNA level (arbitray units)
OXPHOS CI
OXPHOS CII
OXPHOS CIV
O2 Flux per mass pmol/(s*mg) relative to mtDNA
7-10
11
12-13
Gestational age (weeks)

Figure 2. Respiration varies across early gestation. (A) Mitochondrial and non-electron transfer system respiration, (B) flux control factors, (C) mitochondrial content and OXPHOS CI relative to mitochondrial content in first trimester tissues ordered by gestation. OXPHOS=Oxidative phosphorylation; LEAK=non-phosphorylating respiration; ETS max=electron transfer system maximum respiration; ROX= non-electron transfer system respiration. *≤0.05; **≤0.01; ***≤0.001. Significance determined by Kruskal-Wallis test. Data are presented as median ± interquartile ranges.

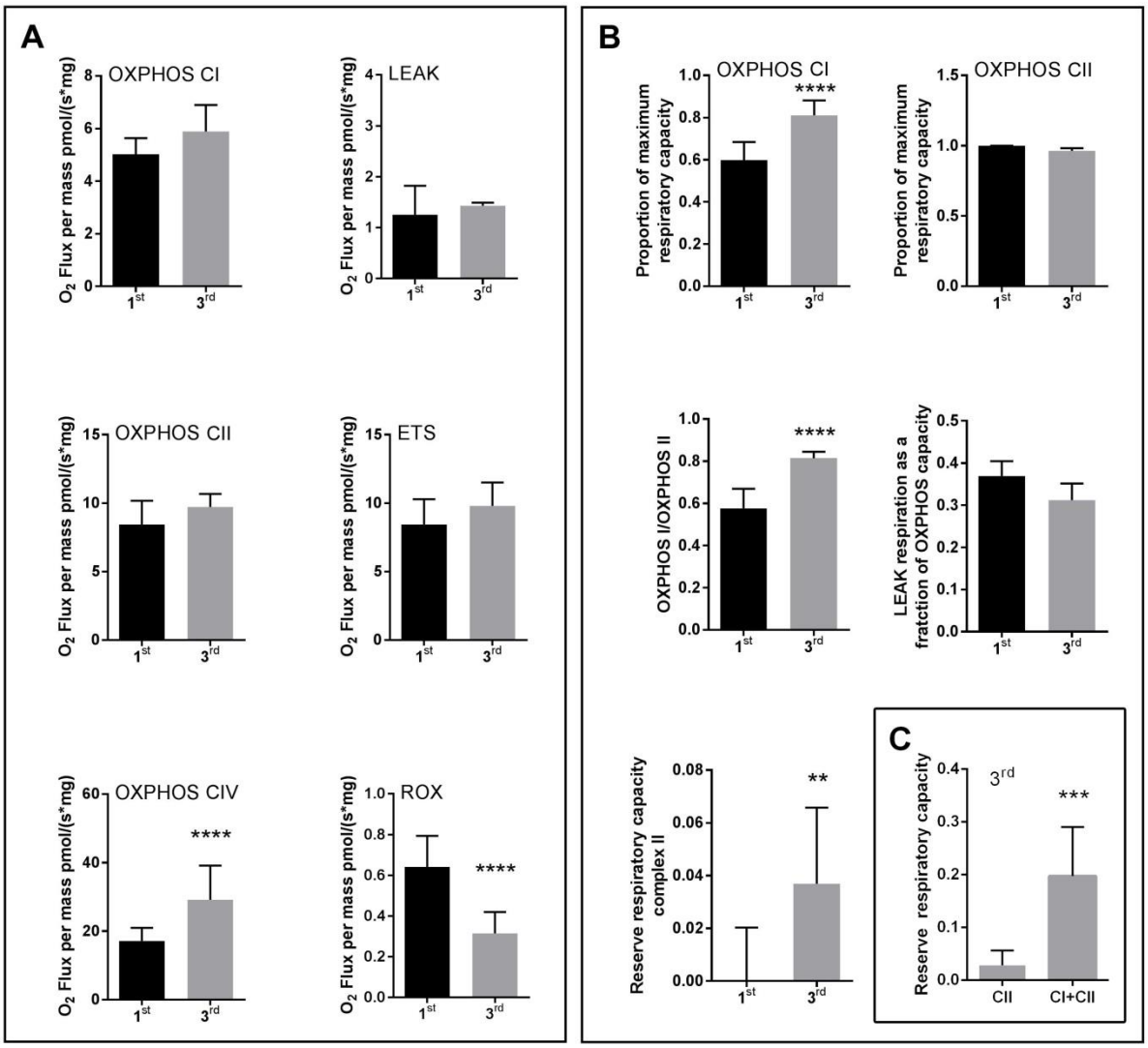


Figure 3. Respiration differs between first trimester and term tissue. (A) Mitochondrial and non-electron transfer system respiration; Oxidative phosphorylation with substrates for mitochondrial complexes I (OXPHOS CI) and CII, non-phosphorylating respiration (LEAK) and electron transfer system maximum respiration (ETS max) were not significantly different between first trimester and term tissue. OXPHOS CIV was significantly increased, and non-electron transfer system respiration (ROX) was significantly decreased, in term compared to first trimester tissue. (B) Flux control factors; OXPHOS CI as a proportion of maximal respiratory capacity, OXPHOS CI as a proportion of OXPHOS CII, and spare respiratory capacity were significantly increased in term compared to first trimester tissue. OXPHOS CII as a proportion of maximal respiratory capacity and LEAK were not significantly different between first trimester and term tissue. (C) In term tissue, reserve respiratory capacity at

mitochondrial complex I+II (CI+II; uncoupled respiration with glutamate, malate, pyruvate, succinate, and FCCP or CCCP) was significantly increased compared to spare respiratory capacity at complex II (CII; uncoupled respiration with glutamate, malate, pyruvate, succinate, FCCP or CCCP, and rotenone). *≤0.05; **≤0.01; ***≤0.001; ****≤0.0001. Significance determined by Mann-Whitney test for respiratory flux and flux control factors, and t test for comparison of spare respiratory capacity in term tissue; n=30 first trimester, n=13 tem; 1^{st}=first trimester; 3^{rd}=term. Data are presented as median ± interquartile ranges for differences between first trimester and term tissue, and mean ±s.d. for comparison of spare respiratory capacity in term tissue.

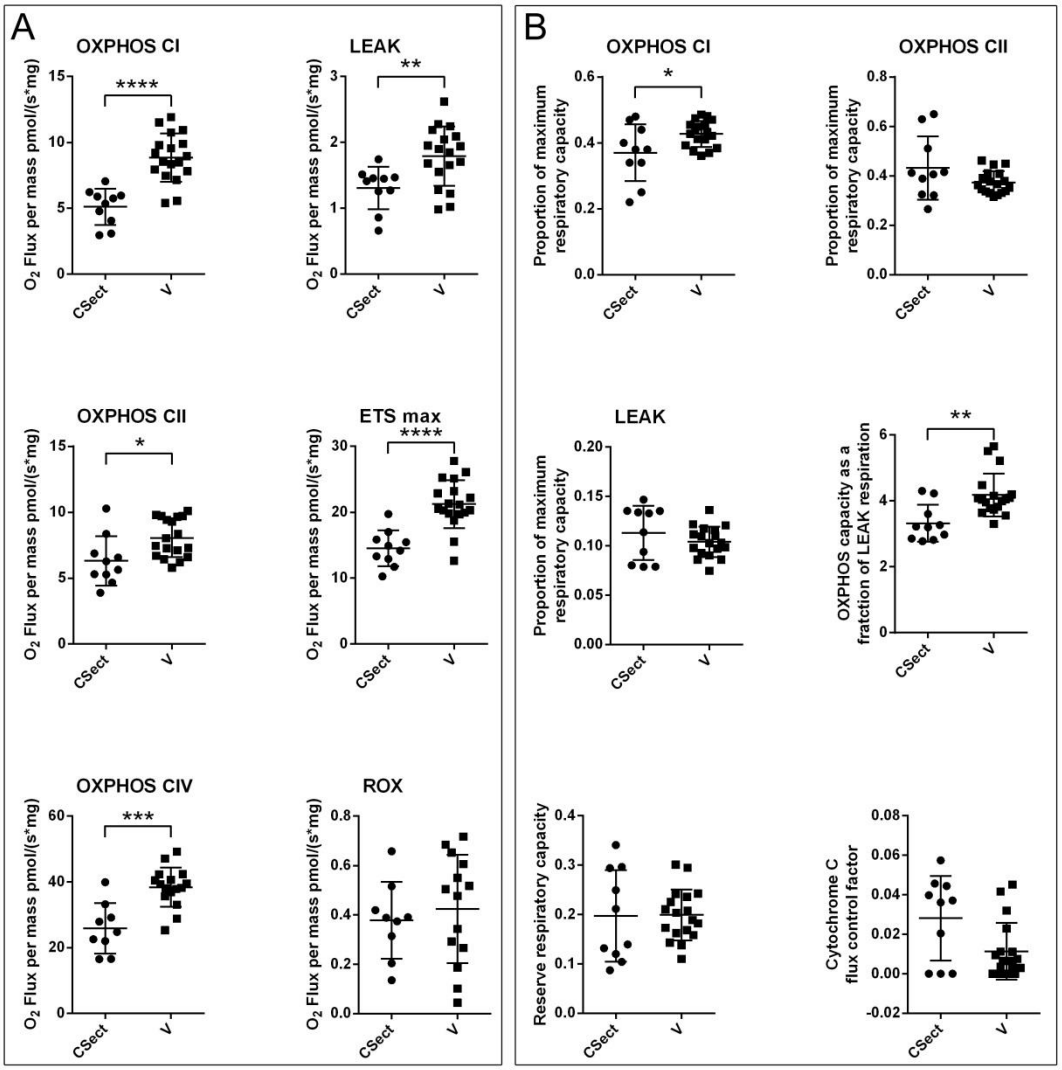
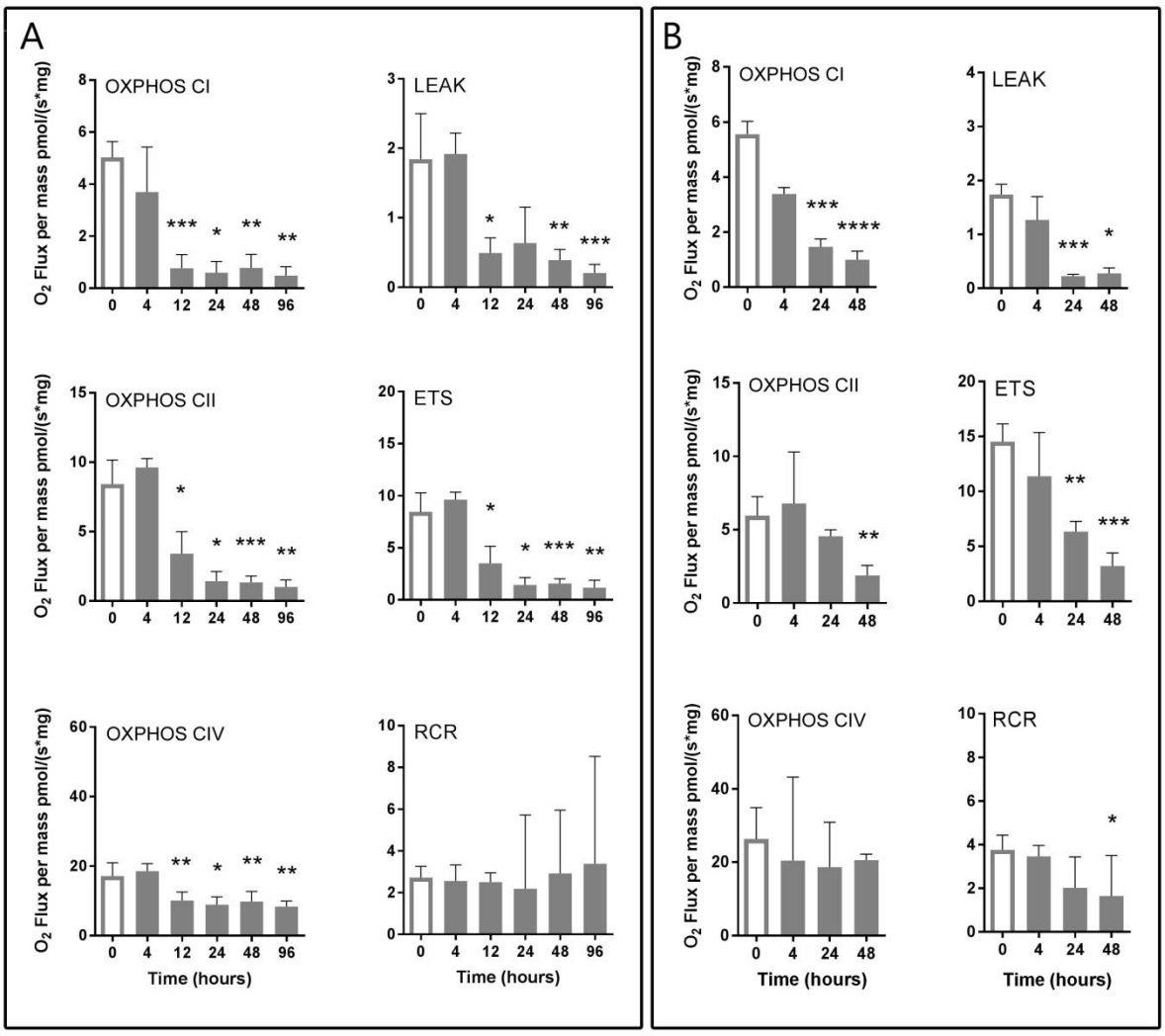
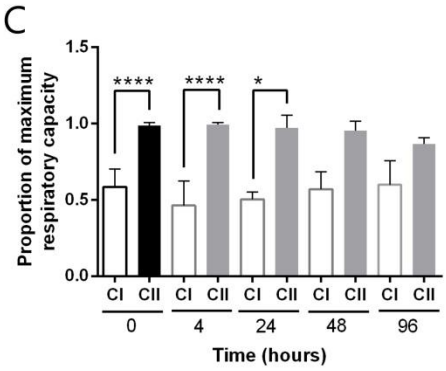
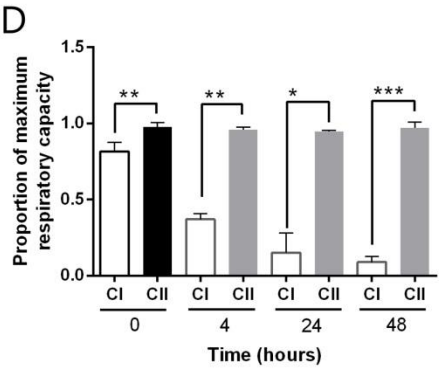
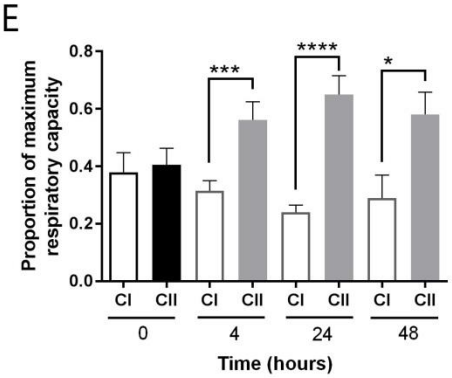


Figure 4. Respiration is increased by labour. (A) Oxidative phosphorylation (OXPHOS) through all mitochondrial complexes, LEAK respiration, and ETS max respiration were significantly increased in vaginal birth (v) compared to Caesarean section (CSect). Non-electron transfer system respiration (ROX) did not change. (B) OXPHOS CI as a proportion of maximal respiratory capacity, and OXPHOS as a proportion LEAK respiration were significantly increased in vaginal birth compared to Caesarean section. Other flux control factors did not change. OXPHOS=Oxidative phosphorylation; LEAK=non-phosphorylating respiration; ETS max=electron transfer system maximum respiration; ROX= non-electron transfer system respiration. *≤0.05; **≤0.01; ***≤0.001; ****≤0.0001. Significance determined by t-test. Data are presented as mean ± standard deviation.


A
OXPHOS CI
LEAK
OXPHOS CII
ETS
OXPHOS CIV
RCR
O2 Flux per mass pmol/(s*mg)
Time (hours)
B
OXPHOS CI
LEAK
OXPHOS CII
ETS
OXPHOS CIV
RCR
O2 Flux per mass pmol/(s*mg)
Time (hours)


C
Proportion of maximum respiratory capacity
CI CII
0 4 24 48 96
Time (hours)


D
Proportion of maximum respiratory capacity
CI CII
0 4 24 48
Time (hours)


E
Proportion of maximum respiratory capacity
CI CII
0 4 24 48
Time (hours)

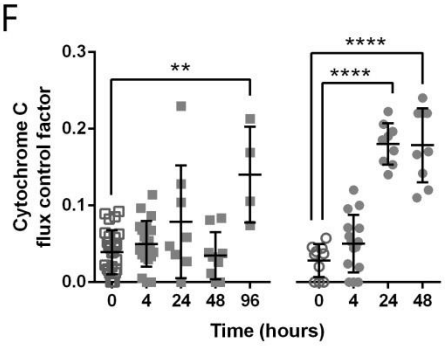

F
Cytochrome C flux control factor
0 4 24 48 96 0 4 24 48
Time (hours)

Figure 5. *In vitro* culture decreases mitochondrial function to different degrees in first trimester and term placenta. (A) By 12 hours, first trimester placenta respiration rates were depressed regardless of substrate or respiration state. (B) In term placenta, oxidative phosphorylation through complex I (OXPHOS CI) decreased with time in culture, OXPHOS CII remained intact until 24 hours and OXPHOS CIV remained intact for 48hrs. (C) In first trimester placenta, OXPHOS CI decreased in a similar proportion relative to complex II over time. (D) When compared within the same substrate-uncoupler-inhibitor titration protocol (SUIT protocol one), term tissue OXPHOS CI was significantly higher than OXPHOS CII at all time points. OXPHOS CI decreased after 24 and 48 hours relative to fresh placenta. (E) A second SUIT protocol tested maximal respiratory capacities for combined OXPHOS CI and OXPHOS CII flux in term placenta, and while OXPHOS CI and OXPHOS CII contributions were similar in fresh term placenta, after 4 hours OXPHOS CI flux was depressed relative to OXPHOS CII flux. (F) The cytochrome c flux control factor increased after 24 and 48 hours culture, relative in term placenta (circles), and after 96 hours of culture in first trimester tissues (squares). 1^{st}=first trimester; 3^{rd}=term; OXPHOS=oxidative phosphorylation; LEAK=non-phosphorating respiration with complex I substrates; ETS= electron transfer system maximum respiration; RCR=respiratory acceptor control ratio; CI=oxidative phosphorylation through mitochondrial CI as a proportion of maximal respiratory capacity; CII=oxidative phosphorylation through mitochondrial complex II as a proportion of maximal respiratory capacity. *≤0.05; **≤0.01; ***≤0.001; ****≤0.0001. Significance determined by Kruskal-Wallis (A–E) or ANVOA (F). Data are presented as median ± interquartile ranges for A–E, and mean ± standard deviation for F.

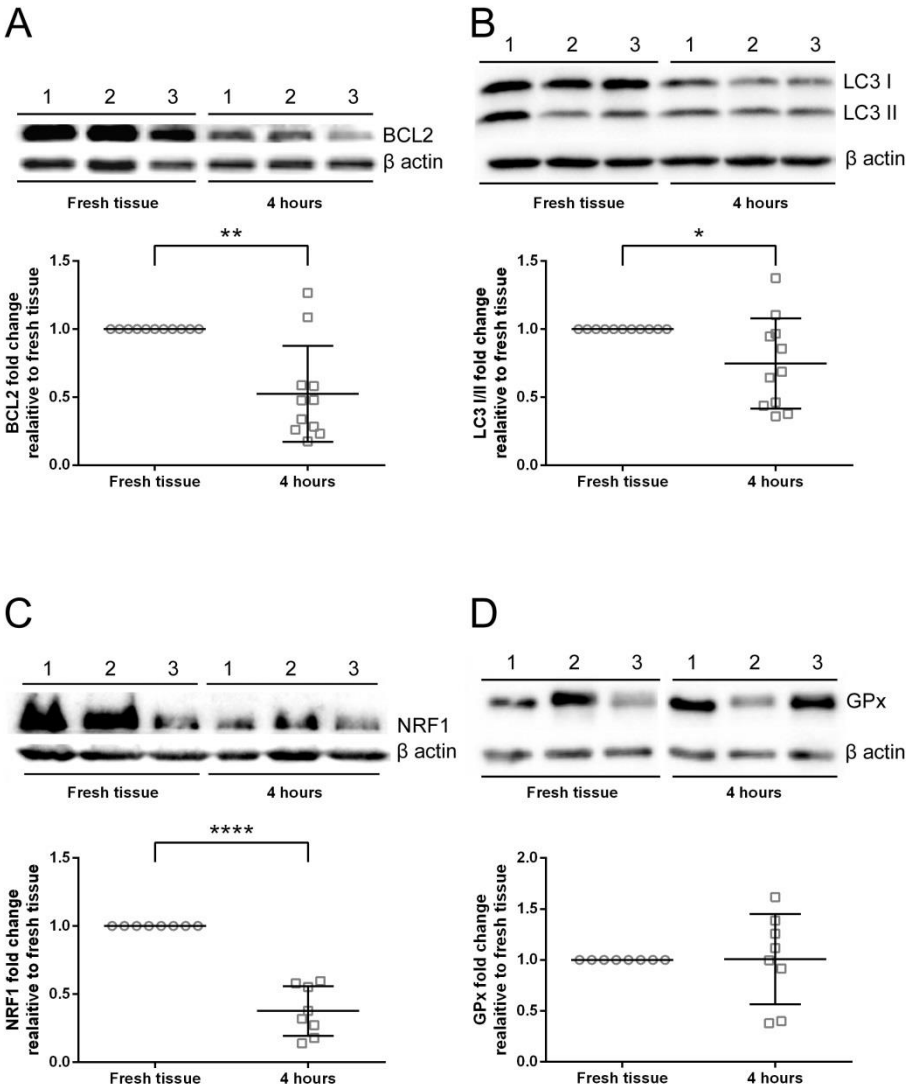


Figure 6. Protein expression changes in term placentae after four hours *in vitro* culture. Western blot analysis of protein levels of anti-apoptotic (A; BCL2), autophagy (B; LC3 I to II ratio), mitochondrial biogenesis (C; NRF1), and endogenous antioxidant (D; GPx) markers in whole term tissue. Plots show full densitometry data, above plots are representative images of three samples. Compared to fresh placenta, after four hours *in vitro* culture protein levels of (A) BCL2, (B) LC3 I/II and (C) NRF1 decreased significantly; (D) GPx did not change significantly. BCL2=B-cell CLL/lymphoma 2; LC3=microtubule associated protein 1 light chain 3 alpha; NRF1=nuclear respiratory factor 1; GPx=glutathione peroxidase 1. *≤0.05; **≤0.01; ****≤0.0001. Significance determined by paired t test. Data are presented as mean ± standard deviation.

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