Mitochondrial Oxygen Consumption by the Foreskin and its Fibroblast-rich Culture

*Fatma Al-Jasmi,¹ Thachillath Pramathan,¹ Adnan Swid,² Bahjat Sahari,² Harvey S. Penefsky,³ Abdul-Kader Souid¹

Objectives: This study investigated the feasibility of using a phosphorescence oxygen analyser to measure cellular respiration (mitochondrial O₂ consumption) in foreskin samples and their fibroblast-rich cultures.

Methods: Foreskin specimens from normal infants were collected immediately after circumcision and processed for oxygen consumption. Foreskin cultures were prepared and fibroblast respiration was measured. Oxygen consumption was determined as a function of time from the phosphorescence decay of the Pd (II) meso-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin.

Results: In sealed vials containing a foreskin specimen and glucose, O₂ concentration decreased linearly with time, confirming the zero-order kinetics of O₂ consumption by cytochrome oxidase. Cyanide inhibited O₂ consumption, confirming that the oxidation occurred mainly in the mitochondrial respiratory chain. The rate of foreskin respiration (mean ± SD) was 0.074 ± 0.02 μM O₂ min⁻¹ mg⁻¹ (n = 23). The corresponding rate for fibroblast-rich respiration (mean ± SD) was 9.84 ± 2.43 μM O₂ min⁻¹ per 10⁷ cells (n = 15). Fibroblast respiration was significantly lower in a male infant with dihydrolipoamide dehydrogenase gene mutations, but normalised with the addition of thiamine or carnitine.

Conclusion: The foreskin and its fibroblast-rich culture are suitable for assessment of cellular respiration. Further investigation is needed to determine the clinical utility of foreskin specimens to detect disorders of impaired cellular bioenergetics.

Keywords: Oxygen; Mitochondria; Foreskin; Respiration; Fibroblasts; Dihydrolipoamide dehydrogenase; Thiamine; Carnitine.

ADVANCES IN KNOWLEDGE
- This study demonstrates the feasibility of using foreskin samples to measure cellular respiration (cellular mitochondrial oxygen consumption).

APPLICATION TO PATIENT CARE
- Foreskin specimens and their fibroblast-rich cultures can be used to screen for disorders of impaired cellular bioenergetics.

- This study shows the potential use of fibroblast-rich respiration to predict responses to therapeutic interventions.

¹Department of Paediatrics, College of Medicine & Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates
²Tawam Hospital, Al Ain, United Arab Emirates
³Faculty of Medicine & Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates

*Corresponding Author e-mail: aljasmif@uaeu.ac.ae
CLINICIANS FREQUENTLY USE SKIN and muscle biopsies for investigating mitochondrial disorders. The skin is also used for generating fibroblasts, which are easily obtained and used for repetitive biochemical analyses and research purposes. Circulating lymphocytes are also available and suitable for these measurements. More novel approaches for monitoring cellular bioenergetics have been reported recently. 

Al-Jasmi et al. described the use of a phosphorescence oxygen analyser to measure lymphocyte respiration in patients. Their method was based on previously published principles. The lymphocytes from patients were shown to be suitable for the screening of certain mitochondrial disorders. The same analytical tool is applied here to measure respiration in foreskin samples and their fibroblast-rich cultures. The results demonstrate that the foreskin tissue permits accurate determination of cellular mitochondrial O₂ consumption.

The primary aim of this study was to investigate the use of the phosphorescence oxygen analyser to measure cellular respiration (mitochondrial O₂ consumption) in foreskin specimens and their fibroblast-rich cultures. The secondary aim was to utilise the foreskin to screen for metabolic disorders that impair cellular respiration (mitochondrial O₂ consumption and accompanying adenosine triphosphate [ATP] synthesis). The hypothesis was that the foreskin and its fibroblast-rich culture can be utilised for assessment of cellular metabolic fuels and their energy conversion processes.

Methods

The following reagents and solutions were used. A Pd (II) complex of meso-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin was obtained from Porphyrin Products (Logan, Utah, USA). Minimum essential medium (MEM Alpha Modification), phosphate-buffered saline (PBS), fetal bovine serum, trypsin, penicillin, streptomycin and lyophilised collagenase (cat. no. 17018-029) were obtained from Invitrogen Corporation (Carlsbad, California, USA). The thiamin HCl injection (100 mg/mL, 300 mM, m.w. 161.2) was obtained from APP Pharmaceuticals (Division of Fresenius Kabi, Schaumburg, Illinois, USA). The levocarnitine injection (1.0 g/5 mL, 1.24 M, m.w. 161.2) was obtained from Sigma-Tau Pharmaceuticals, Inc. (Gaithersburg, Maryland, USA).

Two mg of the Pd phosphor were dissolved in 1.0 mL of distilled water (dH₂O) and stored at -20°C. A glucose oxidase solution was prepared in dH₂O (10 mg/mL) and stored at -20°C. One M of sodium cyanide (NaCN) was prepared in dH₂O; the pH was adjusted to ~7.0 with 12N HCl and stored at -20°C. PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.4 mM KH₂PO₄, pH 7.4) containing 10 mM glucose was stored at 4°C.

Foreskin specimens (22–34 mg) were collected from normal infants (<6 months of age) immediately after circumcision and stored at 4°C in 50 mL MEM supplemented with penicillin and streptomycin for <24 hours until O₂ analysis or culture. For O₂ measurement, the foreskin samples were placed in 1-mL O₂ vials and processed as described below. For culture, the foreskin specimens were incubated at 37°C in a sterile vial containing PBS with 2.0 mg/mL collagenase. After tissue disintegration (typically in 2–3 hours), the samples were transferred to 25-cm² tissue culture flasks containing 10 mL MEM with fetal-bovine serum, penicillin, and streptomycin. For passage, the cells were washed with 5 mL PBS and then treated at 37°C with 2 mL of 0.25% trypsin (w/v in PBS) for 5 minutes. The flasks were inspected for cell detachment; 1.0 mL of fetal-bovine serum and 5.0 mL of MEM with fetal-bovine serum, penicillin and streptomycin were then added. The suspension was split between 2–4 flasks, depending on the cell concentration.

Fibroblast-rich cultures were harvested at confluence. Cells were washed with PBS and treated at 37°C with 0.25% trypsin for 5 minutes. The cells were then collected in MEM with fetal-bovine serum, penicillin and streptomycin and centrifuged at 1,000 g (25°C for 5 minutes). The pellet was suspended in 1.3 mL of Pd solution (PBS, 3 µM Pd phosphor, 0.5% fat-free albumin, and 5 mM glucose) and processed for O₂ measurement. Thiamin or carnitine was added to confluent fibroblast-rich cultures and the cells were harvested after 24 hours. Cell count and viability were determined by light microscopy using a haemocytometer under standard trypan blue staining condition. Only trypan blue-negative cells (>95%) were counted.
is worth emphasising that foreskin samples should be immediately placed in a large volume (e.g., 50 mL) of ice-cold medium and stored at 4°C until processing. This procedure produces better sample viability.

Sample collection from all participants was approved by the institutional review board for protection of human subjects. Informed consent was obtained for each patient. Ethical permission was granted by the Al Ain Medical District Human Research Ethics Committee (16th April 2012, Protocol No. 11/59).

The rate of cellular respiration was determined using a phosphorescence analyser to measure the concentration of dissolved oxygen as a function of time.\(^{10}\) This method is based on the principle that \(\text{O}_2\) quenches the phosphorescence of a palladium phosphor.\(^{11}\) The Pd (II) derivative of meso-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin had a maximum absorption at 625 nm and a maximum phosphorescence emission at 800 nm.

Samples were exposed to light flashes (10 per second) from a pulsed light-emitting diode array with a peak output of 625 nm. Emitted phosphorescent light was detected by a Hamamatsu photomultiplier tube (Hamamatsu, Japan) after first passing it through a wide-band interference filter centered at 800 nm. Amplified phosphorescence was digitised at 1–2 MHz using an analogue/digital converter (PCI-DAS 4020/12 I/O Board) with 1 to 20 MHz outputs. Pulses were captured at 1.0 MHz.

The phosphorescence decay rate \(1/\tau\) was characterised by a single exponential \(I = Ae^{-t/\tau}\), where \(I = \text{Pd phosphor phosphorescence intensity}\). The values of \(1/\tau\) were linear with dissolved \(\text{O}_2\): \(1/\tau = 1/\tau_0 + k_q[\text{O}_2]\), where \(1/\tau_0\) is the phosphorescence decay rate in the absence of \(\text{O}_2\) and \(k_q\) is the second-order \(\text{O}_2\) quenching rate constant in sec\(^{-1}\) \(\mu\text{M}^{-1}\).\(^{11}\)

Cellular respiration was measured at 37°C in 1-mL sealed vials containing PBS, 3 \(\mu\text{M}\) Pd phosphor,

### Table 1: Respiration of foreskin samples and their fibroblast-rich cultures from normal infants

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Rates of Respiration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foreskin*</td>
<td>((\mu\text{M O}_2) min(^{-1}) mg(^{-1}))</td>
</tr>
<tr>
<td>Mean ± SD (n)</td>
<td>0.074 ± 0.02 (23)</td>
</tr>
<tr>
<td>Median (range)</td>
<td>0.074 (0.04–0.13)</td>
</tr>
</tbody>
</table>

\* The samples were stored at 4°C in MEM for <24 hours before \(\text{O}_2\) measurement and culture.

### Table 2: Respiration of foreskin samples, fibroblasts and lymphocytes from patients

<table>
<thead>
<tr>
<th>Gender</th>
<th>Age</th>
<th>Clinical presentation</th>
<th>Rates of respiration</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>1yr</td>
<td>DLD homozygous gene mutation (c.1436A&gt;T)</td>
<td>Foreskin: Not done</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduced PDHc activity in fibroblasts</td>
<td>Fibroblast: 5.7 ± 1.4 (n = 6)(^{7})</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(P &lt;0.0001)</td>
</tr>
<tr>
<td>M</td>
<td>2m</td>
<td>Congenital lactic acidosis</td>
<td>0.076</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.6 ± 0.3 (n = 3)(^{7})</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(P = 0.002)</td>
</tr>
<tr>
<td>F</td>
<td>15m</td>
<td>Global developmental delay</td>
<td>Not done</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10.1 ± 0.4 (n = 2)(^{7})</td>
</tr>
</tbody>
</table>

\(\text{DLD} = \text{dihydrolipoamide dehydrogenase; PDHc = pyruvate dehydrogenase complex; SD = standard deviation; n = number of repeats using independence samples; P = P-value.}\)

The lymphocyte and fibroblast respiration is expressed in \(\mu\text{M O}_2\) min\(^{-1}\) per 10\(^7\) cells. The foreskin respiration is expressed in \(\mu\text{M O}_2\) min\(^{-1}\) per mg. The results in Table 2 are in comparison to Table 1.

\(^{7}\)The fibroblasts were from a skin biopsy. The \(P\) value is for fibroblast respiration in normal infants versus fibroblast respiration in the patient.

\(^{1}\)The fibroblasts were from a skin biopsy. The \(P\) value is for lymphocyte respiration in age-matched normal individuals versus lymphocyte respiration in the patient.\(^{4}\)
Mitochondrial Oxygen Consumption by the Foreskin and its Fibroblast-rich Culture

0.5% fat-free albumin, and 5 mM glucose. The respiratory substrates were endogenous metabolic fuels supplemented by glucose. O2 concentration (calculated using the equation 1/τ = 1/τº + k[O2]) decreased linearly with time, indicating the kinetics of mitochondrial O2 consumption was zero-order. The rate of respiration (k, in µM O2/min) was, thus, the negative of the slope d[O2]/dt. NaN inhibited respiration by at least 96%, confirming oxygen was mainly consumed by the mitochondrial respiratory chain. A programme was developed using Microsoft Visual Basic 6 and Access Database 2007 (Microsoft Corp., Redmond, Washington, USA), and Universal Library (Measurement Computing Corporation, Norton, Massachusetts, USA) components.12,13

Calibration with β-D-glucose plus glucose oxidase was performed as follows. Glucose oxidase catalyses the oxidation reaction of β-D-glucose + O2 to D-glucono-1,5-lactone + H2O2. The reactions contained PBS + 3 µM Pd phosphor, 0.5% fat-free albumin, 50 µg/mL glucose oxidase, and 0-500 µM β-glucose. The value of k, the negatives of the slope of 1/τ versus [β-glucose], was 101.1 sec-1 µM-1.

Data were analysed using Statistical Package for the Social Sciences (SPSS), Version 19 (IBM Corp., Chigaco, Illinois, USA). The Mann-Whitney nonparametric test (2 independent variables) was used to compare treated and untreated samples.

Results

The rates of foreskin and fibroblast respiration are shown in Table 1, and representative runs are shown in Figure 1A. A summary of all studied samples is shown in Figure 1B and Table 1. For comparison, the reference values for lymphocyte respiration are also shown in Table 1.10 The rate of respiration (k, µM O2 min-1 mg-1) in foreskin samples that was determined within one hour of circumcision was 0.076 ± 0.01 (coefficient of variation [Cv] = 14%; n = 8). For samples that were stored at 4º C in MEM for 16 to 23 hours, the respiration rate was 0.074 ± 0.025 (Cv = 33%, n = 16, P = 0.697), and for samples that were stored at 4º C in MEM for >24 hours the rate was 0.033 ± 0.023 (Cv = 70%, n = 2, P = 0.012). Thus, the samples were stable for up to 23 hours.

An infant with congenital lactic acidemia (plasma lactate 18 mM; normal range <1.5) was investigated. His plasma lactate decreased to 6–8 mM within one week of thiamin treatment (500 mg/Kg). The pyruvate dehydrogenase complex (PDHc) activity in fibroblasts was low (2.7 mU/UCS; controls = 9.7 to 36 mU/UCS; this test was done on a clinical sample analysed by Stichting Klinisch-Genetisch Centrum, Nijmegen, Netherlands. Sequencing the dihydrolipoamide dehydrogenase (DLD) gene showed homozygous c.1436A>T (p. Asp 479 Val) (Refseq accession number NM_000108). The respiration rate of the infant's lymphocytes and fibroblasts was consistently low [Table 2]. His fibroblasts were then treated with various concentrations of thiamin (cofactor of PDHc) and carnitine over 24 hours, as patients with impaired pyruvate dehydrogenase complex are typically treated with these agents. The value of k, (µM O2 min-1 mg-1) with 100 µM thiamin was 4.3, with 200 µM thiamin 7.1, and with 400 µM thiamin 8.6. The value of k, with 50 µM carnitine was 5.1, with 100 µM carnitine 8.6, and with 200 µM carnitine 8.8.

Another patient with congenital lactic acidosis, a 2-month-old infant, was also investigated. His plasma lactate was 17.9 mM and cerebrospinal fluid lactate 8.27 mM (normal = 0.86–2.19). His foreskin respiration (0.076 µM O2 min-1 per mg) and lymphocyte respiration (1.0 µM O2 min-1 per 107 cells) was normal, while his fibroblast (from a foreskin sample) respiration was low (5.6 ± 0.3 µM O2 min-1 per 107 cells, n = 3, P = 0.002).

A 15-month-old female presented with global developmental delay and failure to thrive. The urine organic acid analysis showed elevated 2-ketoglutaric acid and lactate levels. Plasma alanine was mildly elevated. The lymphocyte respiration was low (0.62 µM O2 min-1 per 107 cells) while the fibroblast respiration was normal (10.1 ± 0.4 µM O2 min-1 per 107 cells, n = 2).

Discussion

The main finding in this study is the consistency of the rate of cellular respiration in foreskin samples (Cv = 27%) and their fibroblast-rich cultures (Cv = 25%) [Table 1]. These variations are significantly lower than that of the lymphocytes (Cv = 45%) [Table 1], which are typically more fragile than the ectodermal tissue. Therefore, the foreskin appears to be a reliable source of cells for measuring cellular respiration. The other important observation is the relative stability of foreskin samples over several hours. These features make the foreskin suitable for...
metabolic disorder screening.

The term cellular bioenergetics covers all of the biochemical processes involved in the energy conversion. Cellular respiration (mitochondrial oxygen consumption), on the other hand, implies the generation of metabolic fuels, delivery of metabolites and O₂ to the mitochondria, oxidation of reduced metabolic fuels with passage of electrons to O₂, and synthesis of ATP. Thus, impaired cellular bioenergetics entails an interference with any of these critical processes. To our knowledge, this report is the first to show the feasibility of measuring cellular respiration in the foreskin. The procedure is relatively simple, and the tissue is stable at 4°C for several hours after circumcision. The clinical applications of using foreskin samples include the immediate reading of cellular respiration, as well as processing the tissue for fibroblast-rich cultures, with a success rate of >90%.

The rates of oxygen consumption by fibroblasts and lymphocytes differ markedly ($P < 0.001$); the lymphocyte rate is about 5 times lower than that of the fibroblast [Table 1]. Due to their in vitro stability, fibroblasts appear to be more reliable for measuring respiration than circulating lymphocytes.

The infant with the homozygous DLD gene mutations (c.1436A>T), and reduced PDHc activity, had low rates of fibroblast and lymphocyte respiration. These results confirm the suitability of using these types of tissues in the screening for this disorder. Thiamine and carnitine supplements are recommended for patients with impaired pyruvate dehydrogenase complexes. Therefore, these agents were tested on the PDH-deficient patient. Fibroblast respiration normalised with the addition of thiamin or carnitine (see Results section). Thus, the potential response to these therapeutic interventions could be predicted in vitro.

In one patient with congenital lactic acidosis, the foreskin and lymphocyte respiration rates were normal, while the fibroblast respiration was low. The 15-month-old female with global developmental delay of undetermined aetiology had low lymphocyte respiration, but normal fibroblast respiration [Table 2]. The source of these discrepancies is unclear, but could reflect heteroplasmy; thus, respiration should always be determined in multiple tissue sources.

In one study, 50% of patients with confirmed respiratory chain defects had abnormal measurements in muscle and lymphocyte samples, 45% in muscle samples only and 5% in lymphocyte samples only. Pearson’s syndrome, on the other hand, consistently expresses abnormalities in the

---

**Figure 1 Panels A & B:** Rates of cellular respiration of foreskins and foreskin cultures from healthy infants. **Panel A:** Representative runs of a foreskin sample (33 mg) and its fibroblast-rich culture (1.2 x 10⁷ cells) from the same infant. The O₂ measurements were performed at 37°C in 1-mL sealed vials of PBS supplemented with 5 mM glucose, 3 µM Pd phosphor and 0.5% fat-free bovine serum albumin. The rates of respiration ($k$, in µM O₂ min⁻¹) were set as the negative of the slopes of [O₂] versus time. The slopes were calculated from the best-fit curve ($R^2 > 0.920$). The values of $k_c$ in µM O₂ min⁻¹ per mg (foreskin) and µM O₂ min⁻¹ per 10⁷ cells (fibroblast-rich culture) are also shown. The additions of 5 mM NaCN and 50 µg/mL glucose oxidase are shown. Glucose oxidase (catalyses the reaction of D-glucose + O₂ to D-glucono-δ-lactone + H₂O₂) depleted the remaining O₂ in the solution. The depletion of O₂ after the addition of glucose oxidase confirmed that the halt of respiration following the cyanide injection occurred despite available O₂ in the solution. **Panel B:** The values of $k_c$ for all studied foreskins (white circles) and foreskin cultures (black circles) are shown. The short horizontal lines on the y-axis reflect the mean values.
lymphocytes. These results again highlight the need for investigating several tissues.

Cellular respiration is a reliable indicator of mitochondrial function [Figure 1]. This important biomarker is underutilised, mostly due to the traditional limitations of the polarographic method. Polarography (Clark-type \( \text{O}_2 \) electrode) and spectroscopy have been used as analytical methodologies for measuring \( \text{O}_2 \) consumption by fresh lymphocytes. Reported rates of lymphocyte respiration (all in \( \text{nmol O}_2 \text{ min}^{-1} \text{ per 10}^7 \text{ cells} \)) include 3.5 ± 0.5, 2.0 ± 0.07 (varied by the cell density), and 1.0 ± 0.2 (in equine lymphocytes). More recently, the fluorescence and phosphorescence \( \text{O}_2 \) sensors have permitted relatively simple and accurate monitoring of respiration in clinical samples of small quantities.

Measurements of mitochondrial respiration in digitonin-permeabilised fibroblasts and in isolated mitochondria from muscle specimens using phosphorescent microplates have been reported. Other analytic instruments for assessing cellular bioenergetics are also described. Of note, the phosphorescence oxygen analyser used here was calibrated using the Clark electrode, and validated for measurements of respiration in various cells and tissues.

**Conclusion**

This study demonstrates the feasibility of using the foreskin and its fibroblast-rich culture to measure cellular mitochondrial \( \text{O}_2 \) consumption. This tissue is dispensable and relatively stable for several hours; it is therefore ideal for screening. Muscle and skin biopsies, on the other hand, are relatively invasive and available in minute quantities. These more precious samples are thus more suitable for confirmatory biochemical or molecular testing. The clinical utility of foreskin samples in detecting disorders of impaired cellular bioenergetics, however, requires further investigation.

**References**