Video Article

Evaluating the Role of Mitochondrial Function in Cancer-related Fatigue

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Abstract

Fatigue is a common and debilitating condition that affects most cancer patients. To date, fatigue remains poorly characterized with no diagnostic test to objectively measure the severity of this condition. Here we describe an optimized method for assessing mitochondrial function of PBMCs collected from fatigued cancer patients. Using a compact extracellular flux system and sequential injection of respiratory inhibitors, we examined PBMC mitochondrial functional status by measuring basal mitochondrial respiration, spare respiratory capacity, and energy phenotype, which describes the preferred energy pathway to respond to stress. Fresh PBMCs are readily available in the clinical setting using standard phlebotomy. The entire assay described in this protocol can be completed in less than 4 hours without the involvement of complex biochemical techniques. Additionally, we describe a normalization method that is necessary for obtaining reproducible data. The simple procedure and normalization methods presented allow for repeated sample collection from the same patient and generation of reproducible data that can be compared between time points to evaluate potential treatment effects.

Video Link

The video component of this article can be found at https://www.jove.com/video/57736/

Introduction

Fatigue is a prevalent and distressing condition that has a negative impact on the quality of life of cancer patients[1]. To this date, cancer fatigue remains poorly defined and relies only on subjective reporting by patients[2]. Therefore, there is an urgent need to identify an easily adaptable diagnostic laboratory test to objectively characterize fatigue in the clinical setting[3,4].

Multiple underlying mechanisms, including mitochondria dysfunction, have been proposed to cause fatigue[5]. Mitochondria are the powerhouse organelles, providing 95% of cellular energy needs via oxidative phosphorylation, and play an important role in calcium signaling, apoptosis, immune signaling, and regulation of other intracellular signaling events[6]. Accordingly, impaired mitochondrial bioenergetics and defects in energy production may contribute to fatigue. Supporting this hypothesis, previous studies have observed mutations in mitochondrial DNA in patients with chronic fatigue syndrome[7]. While it remains unclear whether the pathophysiological origin of fatigue lies within the central nervous system or peripheral tissues, such as skeletal muscles[8,9], there is currently no direct method to accurately assess mitochondrial dysfunction related to fatigue in live, respiring cells.

Using peripheral blood mononuclear cells (PBMCs) to study mitochondrial function offers several advantages. First, PBMCs are readily available in the clinical setting using standard phlebotomy and can be isolated quickly using basic laboratory techniques. Second, blood collection is less invasive than collecting other tissues such as a muscle biopsy. Thus, blood samples can be collected from the same patient repeatedly over time, which facilitates longitudinal assessment of treatment effects. Interestingly, mitochondrial function in PBMCs appeared to be well correlated with kidney mitochondrial status in an animal model[10]. Furthermore, immune cell mitochondria have been used as a proxy for detecting systemic changes under different disease conditions[11,12]. Mitochondria in circulating immune cells are particularly sensitive to changes in immune functions and immune signaling molecules such as cytokines[13,14,15]. For example, it has been observed that PBMCs from patients with acute rheumatic inflammatory diseases exhibit high baseline oxygen consumption[14]. In contrast, oxygen consumption was reduced in PBMCs isolated from patients with systemic inflammatory conditions including sepsis[16]. Under inflammatory conditions, free radicals produced by dysfunctional mitochondria may further contribute to elevated oxidative stress and prolonged inflammation[17]. The central role of mitochondria in energy production as well as in oxidative stress suggests the potential utility of using mitochondrial function as a proxy for studying fatigue in cancer patients [13].

Previous studies examining mitochondrial function utilized biochemical techniques, mitochondrial membrane potential measurement, or isolation of specific cell populations that may not be readily adaptable in the clinical setting[5,14,18]. In recent years, the development of extracellular flux assays has allowed researchers to easily and accurately examine changes in oxygen consumption rate (OCR) in response to automated injections of respiratory inhibitors[19,20,21,22]. However, most of these studies are designed for specific cell types and the large high-throughput

format may not be applicable in a clinical setting. In this manuscript, we describe an optimized protocol for examining mitochondrial function for clinical use.

Protocol

The current study (NCT00852111) was approved by the Institutional Review Board (IRB) of the National Institutes of Health (NIH), Bethesda, Maryland. Participants enrolled in this study were euthymic men, 18 years of age or older, who were diagnosed with non-metastatic prostate cancer with or without prior prostatectomy and were scheduled to receive external beam radiation therapy (EBRT). Potential participants were excluded if they had a progressive disease that could cause significant fatigue, had psychiatric disease within the past five years, had uncorrected hypothyroidism or anemia, or had a second malignancy. Individuals who used sedatives, steroids, or non-steroidal anti-inflammatory agents were also excluded. Healthy control blood samples were obtained at the NIH Department of Transfusion medicine from healthy donors under an IRB-approved protocol (NCT00001846). All participants are recruited at the Magnuson Clinical Research Center at the NIH. Signed written informed consents were obtained prior to study participation.

1. Mitochondrial Function Measurement Preparation (Day 1 of the Experiment)

1. Hydrate Sensor Cartridge (see **Table of Materials**; approximate duration: 5 min).
    1. Remove Sensor Cartridge from package. Add 200 µL Calibrant solution into each well of the utility plate, then fill each moat with 400 µL Calibrant solution.
    2. Return Sensor plate to the utility plate, which now has Calibrant solution in it. Hydrate the Cartridge in a non-CO_2 37 °C incubator overnight.
    NOTE: Sensor Cartridge needs to be hydrated for a minimum of 4 h and a maximum of 72 h.

2. Clinical Sample Preparation (Day 2 of the Experiment)

1. Measure fatigue using the 13-item Functional Assessment of Chronic Illness Therapy - Fatigue (FACIT-F)[2,23,24] at baseline (prior to EBRT initiation), midpoint and completion of EBRT, and 1-year post-EBRT.
    1. Use a 0 - 4 scale for each item response, where a 0 represents "not at all" and a 4 indicates that the respondent relates to the corresponding statement "very much." Total scores should range from 16 - 53, with lower scores reflecting high fatigue intensity.
    2. Define fatigue as a FACIT-F score lower than 43, with a FACIT-F score of ≥43 indicating absence of or not clinically-meaningful fatigue[2].
    NOTE: A FACIT-F score of 43 best divides fatigue scores of cancer patients and the general population[2].
    3. Include the following 13 items in the FACIT Fatigue Scale: 1) I feel fatigued; 2) I feel weak all over; 3) I feel listless ("washed out"); 4) I feel tired; 5) I have trouble starting things because I'm tired; 6) I have trouble finishing things because I am tired; 7) I have energy; 8) I am able to do my usual activities; 9) I need to sleep during the day; 10) I am too tired to eat; 11) I need help doing my usual activities; 12) I am frustrated by being too tired to do the things I want to do; and 13) I have to limit my social activity because I am tired.

2. Isolate PBMC from freshly collected blood samples (approximate duration: 1 h).
    1. Collect 8 mL of blood into a Mononuclear Cells Preparation Tube (see **Table of Materials**).
    NOTE: Blood samples should be processed within 2 h of collection. The quality of PBMCs may be compromised if blood samples are processed more than 2 h after sample collection.
    2. Centrifuge at 1,750 x g for 30 min at room temperature (18 - 25 °C). Transfer the cloudy layer to a 15 mL conical tube. Add up to 15 mL of PBS and invert 5 times.
    3. Centrifuge at 300 x g for 15 minutes at 4 °C. Carefully remove and discard the supernatant without disturbing pellet. Re-suspend the pellet by adding up to 10 mL PBS, and invert 5 times.
    4. Centrifuge at 300 x g for 10 min at 4 °C. Discard liquid supernatant and re-suspend cells in 1 mL PBS. Transfer the PBMCs to a 1.5 mL microfuge tube.
    5. Centrifuge at 610 x g for 10 minutes. Carefully remove supernatant and resuspend pellet in complete RPMI-1640 (RPMI-1640 supplemented with 10% FBS, 10 mM Penicillin/Streptomycin).

3. Coat cell plates for non-adherent cells (approximate duration: 30 min).
    1. Prepare cell and tissue adhesive solution (see **Table of Materials**) by diluting the stock solution in 0.1 M sodium bicarbonate pH 8.0. The working solution should be at 3.5 µg/cm^2 of surface area.
    NOTE: This solution contains polyphenolic proteins extracted from the marine mussel, *Mytilus edulis*. These proteins are key components of the glue secreted by the mussel to anchor itself to surfaces. We find that Cell-Tak works the best with PBMCs.
    2. Add 100 µL of the diluted adhesive solution to each well and incubate for at least 20 min at room temperature. Wash three times with DI water and air dry.

3. Mitochondrial Function Measurement

1. Preparation of Assay Media (approximate duration: 10 min).
NOTE: Assay media must be freshly prepared on the day of the experiment.
    1. Add L-glutamine, pyruvate, and glucose to base media (the same constituents as Dulbecco's Modified Eagle's Medium (DMEM), but without any sodium bicarbonate, glucose, glutamine, or sodium pyruvate) to make assay media. Warm media up to 37 °C, then adjust pH to 7.4.

NOTE: Concentrations of L-glutamine, pyruvate, and glucose are usually the same as concentrations in normal growth media, but can be adjusted based on the assay.

2. Prepare PBMCs for the Mito Stress test (approximate duration: 2 h).
    1. Plate enough PBMCs from Step 2 into each well to reach 80 - 90% confluency. In our experience, 1.5 x 10^5 cells/well yielded the most consistent results.
    NOTE: Wells A and H are background wells and should only contain assay media with no cells. In wells B to G, we recommend plating at least 3 - 6 wells per patient sample in order to account for outliers.
    2. Spin plates down at 200 x g for 2 min to allow cells to adhere to the bottom of the wells. Wash the cells once with Assay media. This step removes serum and sodium bicarbonate from the growth media.
    NOTE: In our experience, the washing step typically removes 20 - 30% of cells.
    3. Add 180 µL Assay media into each well, including background wells A and H. Incubate in a non-CO_2 37 °C incubator for 45 - 60 min.

3. Running the Mito Stress Test
    1. Reconstitute drugs in the Mito Stress Kit with assay media as follows:
        1. Oligomycin: Add 252 µL assay media into the vial to generate a stock solution at 50 µM.
        2. FCCP: Add 288 µL of assay media into the vial to generate a stock solution at 50 µM.
        3. Antimycin A/Rotenone: Add 216 µL of assay media into the vial to generate a stock solution at 25 µM.

    2. Vortex reconstituted drugs for approximately 1 minute. Dilute into working solutions.
    NOTE: Concentrations of the working solutions should be titrated and pre-determined prior to the experiment depending on the cell type. For PBMCs, we find that 1 µM of Oligomycin, 1 µM FCCP, and 0.5 µM antimycin A/Rotenone work the best.
    3. Pipette the drugs into each port in the sensor cartridge sequentially:
        1. Port A: Pipette 20 µL of 10 µM Oligomycin, for a final concentration in each well of 1 µM.
        2. Port B: Pipette 22 µL of 10 µM FCCP, for a final concentration in each well of 1 µM.
        3. Port C: Pipette 25 µL of 5 µM antimycin A/rotenone for a final concentration in each well of 0.5 µM.

4. Select "Mito Stress Test" on an extracellular flux instrument. Follow instrument prompt and insert the sensor cartridge. The instrument will automatically perform sensor calibration. Insert cell plate after sensor calibration, and the instrument will finish the rest of the assay. Oxygen dynamics is measured at 530 nm (excitation)/650nm (emission), and proton concentration is measured at 470 nm (excitation)/ 530 nm (emission).

4. Normalization of Mitochondrial Function Data

1. Prepare Cell Proliferation Assay solution (approximate duration: 5 min).
    1. Add 48 µL nucleic acid stain (500x) and 240 µL background suppressor to 11.7 mL PBS (2x). The nucleic acid stain is a cell-permeant DNA-binding dye, and the background suppressor is a masking dye which blocks dead cells or cells with compromised cell membrane integrity from being stained. The combination of the DNA-binding dye and the background suppressor ensures that only live cells are stained.
    2. Add equal volume of 2x working solution (180 µL) directly into the medium in each well after the Mito Stress Test has completed.

2. Incubate the cells in the presence of cell proliferation assay solution in a 37 °C incubator for 45 - 60 min. Quantify the number of live cells (fluorescent) on a plate reader at 508 nm (excitation)/527 nm (emission) and normalize OCR data (approximate duration: 10 min).
NOTE: in addition to the number live cells, users may also choose to normalize their data with the total number of cells, amount of nucleic acid, protein concentrations, *etc*.

Representative Results

The Mito Stress Test relies on measuring oxygen consumption rate (OCR) after sequential injection of various respiratory inhibitors to map a complete mitochondrial profile. OCR measurements after each drug injection can be used to calculate the following parameters related to mitochondrial health: **Basal OCR** is first measured before any drug injection to assess oxygen consumption needed to meet resting level ATP demand. Basal respiration is calculated by subtracting non-mitochondrial respiration rate from baseline OCR prior to oligomycin injection. Next, oligomycin is injected to inhibit the proton channel of the ATP synthase (complex V). The subsequent drop in OCR after oligomycin injection represents **ATP production-related oxygen consumption**. FCCP (Carbonyl cyanide 4-[trifluoromethoxy] phenylhydrazone), an ionophore used to disrupt the proton gradient and mitochondrial membrane potential, is then used to elicit maximal oxygen consumption. **Maximal respiration** is calculated by subtracting non-mitochondrial respiration from the maximum OCR after FCCP injection. **Spare respiratory capacity** is the difference between maximal and basal respiration. Lastly, antimycin A (complex III inhibitor) and rotenone (complex I inhibitor) are injected at the same time to shut off the electron transport chain. By definition, **non-mitochondrial respiration** is independent from the electron transport chain (*e.g.,* non-mitochondrial NADPH oxidase in macrophages, *etc*.) and is represented as OCR values after antimycin A/rotenone injection. **Coupling efficiency** describes the fraction of basal mitochondrial oxygen consumption used for ATP synthesis, and is calculated as 100% x (ATP production-related OCR)/(basal respiration rate).

Cell density for each culturing condition should be optimized before performing a mito stress test. In our experience, 80 - 90% confluency is achieved by plating 1.5 x 10^5 cells/well of freshly isolated PBMCs from human blood (**Figure 1A**). This plating density accounts for the washing step described in step 3.2.3. In addition to determining the optimal plating density, FCCP titration must be performed to determine the concentration needed to generate maximal OCR. At the FCCP concentrations tested - 0.125 µM, 0.25 µM, 0.5 µM, 1 µM, and 2 µM - OCR increased with FCCP concentration reaching a plateau at 1 µM, but decreased at 2 µM (**Figure 1B**). This indicates that 1 µM is the optimal FCCP concentration that should be used for examining PBMC mitochondrial function.

We tested mitochondrial function of the same batch of cells from the same blood sample collected from a healthy donor at various time points after PBMC isolation. Basal oxygen consumption as well as maximal OCR decreased as early as 3 h and continued to decrease at 5 and 8 h after PBMC isolation (**Figure 2A**). After 3 h, maximal respiration elicited by FCCP injection (time points 7 - 9) did not exceed basal OCR, suggesting that even in live cells, mitochondria spare respiratory capacity decreases rapidly over time. Extracellular acidification rate (ECAR) was simultaneously measured on an extracellular flux instrument. Since oligomycin inhibits mitochondrial ATP synthase, glycolysis is recruited within minutes to meet the energy demand and to compensate for the lack of ATP production via oxidative phosphorylation, as demonstrated by the rapid increase in ECAR after oligomycin injection. As early as 3 h after PBMC isolation, there was a decrease in ECAR as well as OCR (**Figure 2B**). Although we did not observe any notable change in cell viability at 1, 3, 5, and 8 h after PBMC isolation, mitochondrial function decreased rapidly, suggesting that timing is key to capture the mitochondrial profile of live, respiring PBMCs.

Patient sample collections tend to occur at various times on separate days; thus, it is crucial to normalize the data to compare different samples and time points. While other normalization methods such as protein assay and nucleic acid quantification may be used, we find that staining live cells with a DNA-binding dye and quantification of green fluorescent cells in each well is the most efficient and reliable normalization method. Representative data of PBMCs collected from two different healthy donors on two separate days are shown in **Figure 3**. Inset is a representative image of a cell plate well showing total cells (phase contrast) and live cells stained with the DNA-binding dye (green fluorescent) after mito stress test. Basal oxygen consumption rate, as well as oxygen consumption after each drug injection normalized to live cells were similar in two different healthy non-fatigued donors (**Figure 3**).

Representative mitochondrial function data of a fatigued subject (FACIT-F score <43) with prostate cancer (red) and an age/gender/race-matched non-fatigued healthy donor (blue) are shown in **Figure 4**. Although we did not observe any difference in the basal OCR (**Figure 4B**), the fatigued subject exhibited decreased maximal oxygen consumption as well as spare respiratory capacity compared to the control (**Figure 4C,D**). Fatigue appeared to be related to the reduction in spare respiratory capacity, because there were no differences found in non-mitochondrial oxygen consumption (**Figure 4E**), ATP-related oxygen consumption (**Figure 4F**), or coupling efficiency (**Figure 4G**). Baseline data (prior to any drug injection) and maximal respiration after FCCP injection can be visualized using an energy phenotype plot, which reveals the preferred pathway (OCR oxidative phosphorylation versus ECAR glycolysis) in the presence of increased energy demand (**Figure 4H**). Empty squares indicate basal energy phenotype and solid squares indicate the energy phenotype in response to maximal ATP demand. The distance between basal (empty square) and maximal (solid square) indicates spare capacity, whereas the slope indicates cellular preference towards oxidative phosphorylation versus glycolysis. As shown in **Figure 4H**, spare capacity in the fatigue subject decreased compared to the non-fatigued healthy control. In addition, the energy phenotype in response to increased ATP demand skewed towards glycolysis in the fatigued subject compared to the healthy control (**Figure 4H**).

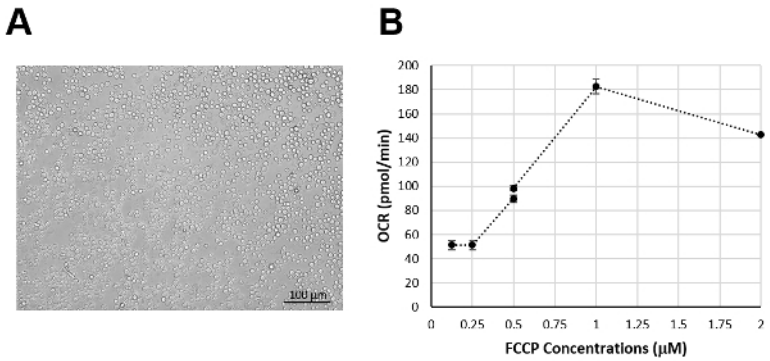

Figure 1: PBMC plating density and FCCP dose-response curve. **(A)** Freshly isolated PBMCs were plated at 1.5 x 10^5 cells/well in CellTak-coated cell culture miniplates. After washing and media change, cells were evenly distributed at 80 - 90% confluency. Scale bar = 100 µm.**(B)** OCR increased with increasing concentrations of FCCP at 0.125 µM, 0.25 µM, 0.5 µM, reaching peak OCR at 1 µM. OCR dropped at 2 µM, indicating that 1 µM is the optimal dose to elicit maximal respiration in PBMCs.Error bars indicate the standard deviation of 3 sequential measurements after the initial drug injection. Please click here to view a larger version of this figure.

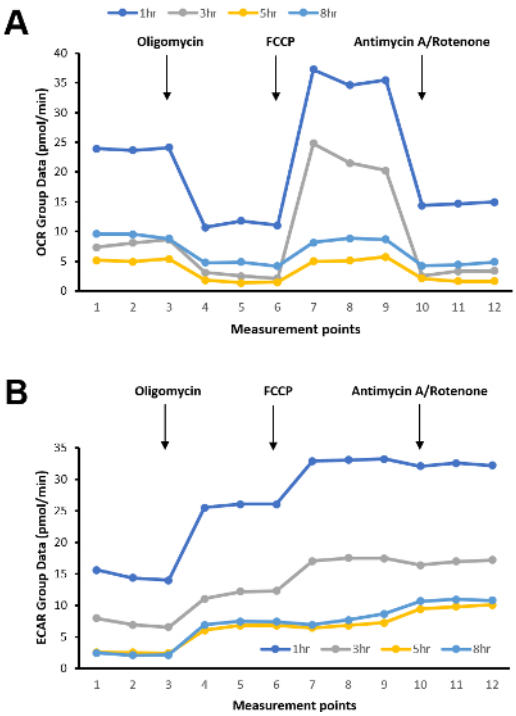

Figure 2: Mitochondrial respiration decreases over time in freshly isolated PBMCs. OCR **(A)** as well as ECAR **(B)** decreased rapidly over time after PBMC isolation. Please click here to view a larger version of this figure.

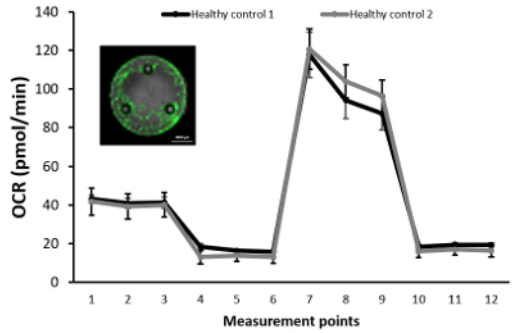

Figure 3: Representative mitochondrial respiration data with normalization. Mito stress test was performed using fresh PBMCs isolated from two different healthy volunteers on different days and normalized using a fluorescent nucleic acid stain quantified on a fluorescent plate reader. Inset: a representative image of live cells (green) and total cells (phase contrast) in a well. Scale bar = 1,000 µm Please click here to view a larger version of this figure.

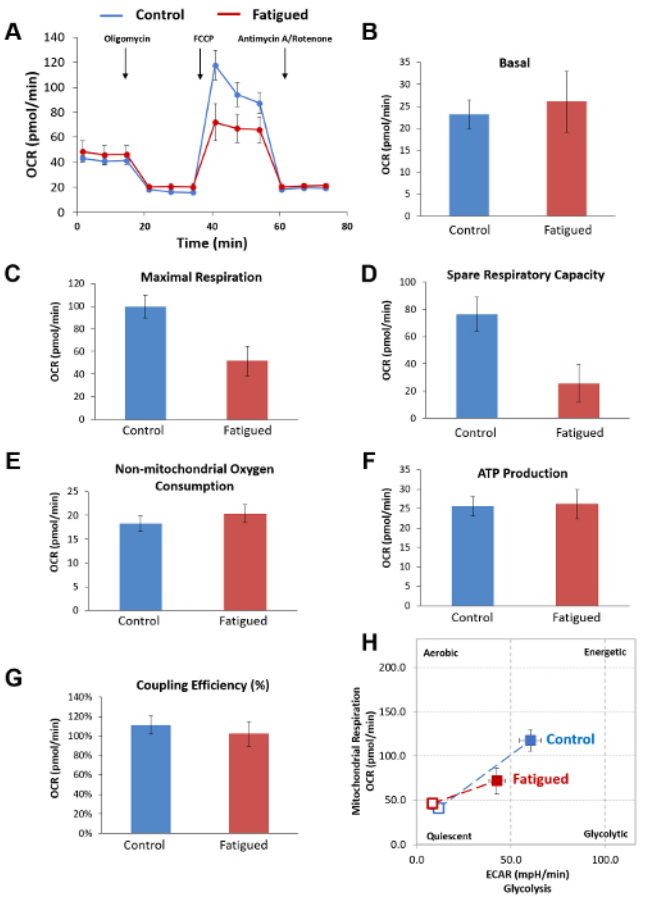

Figure 4: Representative analysis of a fatigued subject compared to a healthy control. **(A)** Representative Mito Stress test OCR graph of a fatigued subject compared to an age/gender/race-matched non-fatigued healthy control. Bar graphs of basal OCR **(B)**, maximal respiration **(C)**, spare respiratory capacity **(D)**, non-mitochondrial oxygen consumption **(E)**, ATP production **(F)**, and coupling efficiency **(G)** are shown. The energy phenotype plot of the fatigued subject and healthy control is shown **(H)**. Empty squares indicate baseline energy phenotype, solid squares represent stressed energy phenotype measured after FCCP injection. Error bars indicate standard deviation of 3 different wells for each study participant. Please click here to view a larger version of this figure.

Discussion

Fatigue in cancer patients is a debilitating condition that is not well defined or characterized[1]. Diagnosis of fatigue entirely relies on subjective reporting and there is no current diagnostic standard or treatment for this condition, largely due to a lack of understanding in its pathobiology[2]. Of the proposed mechanisms underlying fatigue in cancer patients, impairment in mitochondrial function is one of the most therapeutically targetable pathways. Therefore, we developed a quick and practical method for measuring mitochondrial function in clinical samples that can be used to proactively identify patients at-risk for developing cancer treatment-related toxicities including fatigue, so that early management can be instituted.

The extracellular flux technology in combination with sequential injection of respiratory inhibitors allow for assessment of mitochondrial functional status and has been used for both *in vitro* and *in vivo* models[19,20,21]. We expanded the existing body of work and developed a method optimized for examination of mitochondrial dysfunction in clinical samples. An advantage of the study is the utilization of the compact extracellular flux analyzer. As we have demonstrated, timing of the experiment is crucial after isolation of fresh PBMCs from human blood. While the larger format (96-well or 24-well format) is the ideal choice for a high throughput experiment, the compact extracellular flux system, which contains 8 wells, allows for individual assessment of each patient sample[19]. This method is more practical and cost-effective (both in regard to the instrument itself and to the reagents used to perform the assay), because sample collection from different patients tend to occur at various times on different days.

The utilization of PBMCs to assess mitochondrial function in fatigued cancer patients has several advantages: 1) biopsy of tissues such as skeletal muscles may be impractical at times; 2) PBMCs are readily available and can easily be isolated using cell preparation tubes, which offers a practical advantage in a clinical setting; and 3) we have demonstrated that mitochondrial function decreases after freshly isolated cells have been in culture for more than 3 hours and isolating specific cell types typically takes several hours (more than 3 hours). PBMCs can be prepared within an hour, thus ensuring the accuracy of measuring real-time mitochondrial function. While we recommend using PBMCs for the initial clinical investigation in previously uncharacterized patient populations, other cell types such as T lymphocytes, platelets, neutrophils, and monocytes can also be used with the current protocol after optimization of plating density and respiratory inhibitor concentrations[15,25,26].

Prior to testing mitochondrial function in a new system, it is important to first determine the optimal cell density and FCCP concentrations. In our experience, plating 1.5 x 10^5 cells per well ensures that the cell density after plating and the washing step remains 80 - 90% confluent. In addition, it is crucial to determine the optimal FCCP concentrations for every cell type. A range of FCCP concentrations should be tested and the FCCP concentration that produces maximal OCR without compromising the health of a cell should be used. At the concentrations tested, 1 µM of FCCP resulted in maximal respiration in PBMCs. Another critical step is the timing of the experiment, as mitochondrial respiration drops precipitously 3 hours after PBMC isolation. This means that the mito stress test should be performed within 3 hours after sample collection to accurately capture mitochondrial function in a clinical sample. It is worth pointing out that many researchers only have access to frozen samples. We have previously tested PBMCs after freezing and thawing, and the mitochondrial functions of these cells are greatly compromised. Therefore, we recommend using freshly isolated PBMCs in clinical studies, when it is feasible.

Another important aspect of generating reproducible data is the method of data normalization. While some laboratories have had success using BCA protein quantification to normalize mitochondrial respiration data, we find that it is not always feasible especially at a low cell density. The normalization method described in this protocol relies on the linear correlation between cell numbers and the fluorescence emission of the dye-nucleic acid complexes, and can accurately quantify 10 to 50,000 cells[27]. In addition, normalization using a combination of fluorescent nucleic acid stain and a fluorescent plate reader allows for rapid quantification of live cells after completion of the experiment. In addition, this method does not require trypsinization of adherent cells or using a cell scraper.

A caveat of the protocol is that we do not separate PBMCs into specific cell types before performing mitochondrial functional analysis. As we have demonstrated, mitochondrial respiration decreases rapidly over time after PBMC isolation. This suggests that differences between patients may be inaccurate if the experiment was performed after isolating different cell populations, which usually takes a few hours. Even though studying mixed populations such as PBMCs does not reveal important cell type-specific information, information obtained from experiments using PBMCs can help guide future mechanistic investigations focused on specific cell types. PBMCs serve as a proxy for systemic mitochondrial dysfunction, which is relevant to systemic disorders such as cancer fatigue[10,28]. The protocol described in the current manuscript only provides a crude measurement of mitochondrial dysfunction without pinpointing the cause of the observation. Therefore, findings using this technology and procedure should be interpreted with caution and must consider the multifactorial nature of fatigue, such as its co-occurrence with other behaviors (*e.g.,* depression, sleep, cognitive impairment) and conditions (*e.g.,* cardiopulmonary status, polypharmacy). Future studies should examine alterations in mitochondrial functions in specific cell types (*e.g.,* natural killer cells, T lymphocytes, and monocytes) and in various clinical populations (*e.g.,* fatigued/non-fatigued cancer patients and healthy controls). While it is beyond the scope of the current manuscript, we will determine the associations between cancer fatigue severity and mitochondrial dysfunction, as well as the correlation between mitochondrial function measurements and physical tests of fatigue such as a 6-minute walk test, in future investigations. Furthermore, future studies will also examine mitochondrial dysfunction in other cancer types in order to determine whether fatigue across various cancer types is associated with mitochondrial dysfunction. In conclusion, we have developed a protocol optimized for a quick assessment of general mitochondrial dysfunctions using clinical samples. Further mechanistic investigations can be performed to pinpoint the involvement of specific pathways in this debilitating condition.

Disclosures

The authors have nothing to disclose.

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References

1. Berger, A. M. *et al.* Cancer-Related Fatigue, Version 2.2015. *Journal of the National Comprehensive Cancer Network.* **13** (8), 1012-1039, (2015).
2. Feng, L. R., Dickinson, K., Kline, N., & Saligan, L. N. Different phenotyping approaches lead to dissimilar biologic profiles in men with chronic fatigue following radiation therapy. *Journal of Pain and Symptom Management.* **52** (6), 832-840, (2016).
3. Feng, L. R. *et al.* Clinical Predictors of Fatigue in Men With Non-Metastatic Prostate Cancer Receiving External Beam Radiation Therapy. *Clinical Journal of Oncology Nursing.* **19** (6), 744-750, (2015).
4. Feng, L. R., Wolff, B. S., Lukkahatai, N., Espina, A., & Saligan, L. N. Exploratory Investigation of Early Biomarkers for Chronic Fatigue in Prostate Cancer Patients Following Radiation Therapy. *Cancer Nursing.* **40** (3), 184-193, (2017).
5. Myhill, S., Booth, N. E., & McLaren-Howard, J. Chronic fatigue syndrome and mitochondrial dysfunction. *International Journal of Clinical and Experimental Medicine.* **2** (1), 1-16, (2009).
6. Pernas, L., & Scorrano, L. Mito-Morphosis: Mitochondrial Fusion, Fission, and Cristae Remodeling as Key Mediators of Cellular Function. *Annual Review of Physiology.* **78** (1), 505-531, (2016).
7. Vecchiet, L. *et al.* Sensory characterization of somatic parietal tissues in humans with chronic fatigue syndrome. *Neuroscience Letters.* **208** (2), 117-120, (1996).
8. Jones, D. E. J., Hollingsworth, K. G., Taylor, R., Blamire, A. M., & Newton, J. L. Abnormalities in pH handling by peripheral muscle and potential regulation by the autonomic nervous system in chronic fatigue syndrome. *Journal of Internal Medicine.* **267** (4), 394-401, (2010).
9. Feng, L. R., Suy, S., Collins, S. P., & Saligan, L. N. The role of TRAIL in fatigue induced by repeated stress from radiotherapy. *Journal of Psychiatric Research.* **91** 130-138, (2017).
10. Karamercan, M. A. *et al.* Can peripheral blood mononuclear cells be used as a proxy for mitochondrial dysfunction in vital organs during hemorrhagic shock and resuscitation? *Shock.* **40** (6), (2013).
11. Ijsselmuiden, A. J. J. *et al.* Circulating white blood cells and platelets amplify oxidative stress in heart failure. *Nature Clinical Practice Cardiovascular Medicine.* **5** 811, (2008).

12. Kong, C.-W. *et al.* Leukocyte mitochondrial alterations after cardiac surgery involving cardiopulmonary bypass: Clinical correlations. *Shock.* **21** (4), 315-319, (2004).
13. Straub, R. H. The brain and immune system prompt energy shortage in chronic inflammation and ageing. *Nature Reviews Rheumatology.* **13** 743, (2017).
14. Kuhnke, A., Burmester, G., Krauss, S., & Buttgereit, F. Bioenergetics of immune cells to assess rheumatic disease activity and efficacy of glucocorticoid treatment. *Annals of the Rheumatic Diseases.* **62** (2), 133-139, (2003).
15. Kramer, P. A., Ravi, S., Chacko, B., Johnson, M. S., & Darley-Usmar, V. M. A review of the mitochondrial and glycolytic metabolism in human platelets and leukocytes: Implications for their use as bioenergetic biomarkers. *Redox Biology.* **2** (Supplement C), 206-210, (2014).
16. Garrabou, G. *et al.* The Effects of Sepsis on Mitochondria. *The Journal of Infectious Diseases.* **205** (3), 392-400, (2012).
17. Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., & Malik, A. B. Reactive oxygen species in inflammation and tissue injury. *Antioxidants & Redox Signaling.* **20** (7), 1126-1167, (2014).
18. Adrie, C. *et al.* Mitochondrial Membrane Potential and Apoptosis Peripheral Blood Monocytes in Severe Human Sepsis. *American Journal of Respiratory and Critical Care Medicine.* **164** (3), 389-395, (2001).
19. Traba, J., Miozzo, P., Akkaya, B., Pierce, S. K., & Akkaya, M. An optimized protocol to analyze glycolysis and mitochondrial respiration in lymphocytes. *Journal of Visualized Experiments.* (117), e54918, (2016).
20. Nicholls, D. G. *et al.* Bioenergetic profile experiment using C2C12 myoblast cells. *Journal of Visualized Experiments.* (46), e2511, (2010).
21. Van den Bossche, J., Baardman, J., & de Winther, M. P. J. Metabolic characterization of polarized M1 and M2 bone marrow-derived macrophages using real-time extracellular flux analysis. *Journal of Visualized Experiments.* (105), e53424, (2015).
22. Boutagy, N. E. *et al.* Using isolated mitochondria from minimal quantities of mouse skeletal muscle for high throughput microplate respiratory measurements. *Journal of Visualized Experiments.* (104), e53216, (2015).
23. Yellen, S. B., Cella, D. F., Webster, K., Blendowski, C., & Kaplan, E. Measuring fatigue and other anemia-related symptoms with the Functional Assessment of Cancer Therapy (FACT) measurement system. *Journal of Pain and Symptom Management.* **13** (2), 63-74, (1997).
24. Yost, K. J., Eton, D. T., Garcia, S. F., & Cella, D. Minimally important differences were estimated for six Patient-Reported Outcomes Measurement Information System-Cancer scales in advanced-stage cancer patients. *Journal of Clinical Epidemiology.* **64** (5), 507-516, (2011).
25. Kang, J.-G., Wang, P.-y., & Hwang, P. M. in *Methods in Enzymology.* Vol. 542 eds Lorenzo Galluzzi & Guido Kroemer 209-221 Academic Press, (2014).
26. Chacko, B. K. *et al.* Methods for defining distinct bioenergetic profiles in platelets, lymphocytes, monocytes, and neutrophils, and the oxidative burst from human blood. *Laboratory Investigation.* **93** (6), 690-700, (2013).
27. Jones, L. J., Gray, M., Yue, S. T., Haugland, R. P., & Singer, V. L. Sensitive determination of cell number using the CyQUANT® cell proliferation assay. *Journal of Immunological Methods.* **254** (1), 85-98, (2001).
28. Hartman, M.-L. *et al.* Relation of mitochondrial oxygen consumption in peripheral blood mononuclear cells to vascular function in type 2 diabetes mellitus. *Vascular Medicine (London, England).* **19** (1), 67-74, (2014).

