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Mitochondrial Function in Previously Frozen Specimens: The New Respirometry

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Abstract

Mitochondrial function is essential to meet energy demand and to coordinate cellular function. Mitochondria are often referred as the powerhouse of the cell, but besides converting nutrients that we eat into energy that can be used by the cell, mitochondria also play an important role in intercellular signaling.

Keywords

Mitochondria • Specimens • Electron transport chain

Description

Nutrients that we eat break down into small molecules that can be used either to enter catabolic or anabolic processes. In the catabolic processes, nutrients break down into small molecules that are used to fuel the mitochondrial Electron Transport Chain (ETC) to either produce ATP or generate heat. On other hand, the anabolic processes involve building up molecules than can be used by the cells as storage or for structural purposes. The balance between catabolism and anabolism define the metabolic signature in every tissue [1-4]. There are highly energy demanding, oxidative tissues, such as heart and brown adipose tissue where mitochondria are very abundant and active; whereas in other tissues, such as white adipose tissue, mitochondria are less active in energy production. In every tissue, independently of their energy demand, the ETC is also required for all other aspects of mitochondrial-dependent cell metabolism. For this reason, measuring oxygen consumption, namely respirometry, is the gold standard measurement of mitochondrial function.

When measuring respiration using the traditionally established protocols there is always the limitation that fresh tissue (collected the day of the assay) or living cells are required. The reason for that is that freeze-thawing samples leads to membrane damage resulting in cytochrome c release from the intermembrane space, which makes electron transport chain inefficient as cytochrome c is an essential electron, carry. In addition to that, by damaging the mitochondrial membranes, respiration uncouples from proton pumping and energy production. For these reasons, respirometry in previously frozen samples stored in biobanks, which are essential for translational research, become largely unfeasible. Several attempts have been made to cryopreserved samples. In these approaches, samples were collected and frozen in different solutions trying to preserve the structure and integrity of the mitochondrial membrane. However, the outcome of these methodologies is very variable and not standardized [5-9].

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Despite the fact that electron transport chain and proton pumping processes uncouple when the samples are frozen, the mitochondrial electron transport complexes remain intact and functional [10, 11]. For that reason, the alternative methods used to assess mitochondrial function in previously frozen samples have been to measure spectrophotometric enzymatic assays that provide information of the activity of each individual ETC complexes, or the combination of CI+III or CII+III. To measure enzymatic activities, protocols often use supraphysiological concentration of some reagents and non-physiological electron donors and acceptors. Although these measurements were successfully used in a relatively high-throughput manner to diagnose primary mitochondrial diseases, namely diseases caused by a primary defect in electron transport chain function [12-14], they cannot provide a single measurement of the coordinated function of the electron transport chain function working at more physiological rates. Taken together, these observations suggest that spectrophotometric assays might be less sensitive to detect defects in mitochondrial architecture and complexes ultrastructure, such as the ones associated with age-related cardiovascular metabolic diseases.

Mitochondrial complexes and supercomplexes can be isolated from frozen samples and separated in native gels. These complexes and supercomplexes preserve their enzymatic activity and more interestingly, mitochondrial super complexes respire. This works proves that ETC is not destroyed by freeze-thawing and that previously frozen sample respirometry is feasible. The challenge is then to convert the assay performed in isolated mitochondrial supercomplexes to previously frozen samples, without the need to isolate the ETC components.

We have recently published a new approach named Respirometry In Frozen Samples (RIFS) that reconstitutes maximal mitochondrial respiration in previously frozen samples [15, 16]. Using RIFS, we measure maximal oxygen consumption of the ETC by providing physiological electron donors and acceptors. It is important to consider that in previously frozen samples the natural provider of electron equivalents, the tricarboxylic acid (TCA) cycle, is not directly feeding the ETC. For that reason, we need to provide electron donors directly to the ETC to bypass the components that are lost during the freeze-thaw and correct for variable permeabilization of mitochondrial membranes, when necessary. The protocol is straightforward in almost all cases with the exception of tissues that contain high proportion of fiber and collagen such as skeletal muscle or tissues that are rich in membranes such as brain. In these tissues, an optimization step was required that included either enzymatic digestion (skeletal muscle) or additional membrane permeabilization (brain) for the reagents and electron donors, particularly NADH, to be accessible to mitochondria.

RIFS preserves 90-95% of the maximal respiratory capacity in frozen samples and can be applied to isolated mitochondria, cells, and tissue homogenates with high sensitivity. RIFS formulation allows for simultaneous

measurements of respiration driven by electron entry through Complex I and II, while determining Complex IV individual activity. The simplified sample preparation, particularly with homogenates, and the 96 well format of the Seahorse XF96 Analyzer allows using significantly less biological material. RIFS does not require preparation of mitochondria or the use of detergents or plasma membrane permeabilizers, which simplifies the methodology and minimizes changes induced by partial or over permeabilization due to the use of detergents. Acin-Perez et.al provides validation in mouse, zebra fish and human samples. Another advantage of using total tissue lysates is that it accounts for tissue specific mitochondrial function, where, for example, soleus had significantly higher respiration than quadriceps as previously described [17]. In homogenates and cell lysates, respirometry can also be normalized to mitochondrial content to provide a measure of respiration per functional unit. In additional to traditional methods to measure mitochondrial mass, we have recently described a high throughput MitoTracker Deep Red (MTDR) protocol that requires minimal biological sample that can be run in parallel to RIFS and used to normalize respiration.

Furthermore, we demonstrated that highly oxidative tissues such as heart, BAT and brain showed higher mitochondrial respiration rates per milligram of tissue whereas WAT, known for its low mitochondrial content, showed the lowest rates. These results allow to conclude that RIFS points to physiological differences depending on the energy and metabolic demand of the tissue. Since RIFS can be used in cells in culture and those isolated from human blood, this methodology opens the door to clinical based research in bioenergetic health monitoring in large populations.

When comparing RIFS to traditional methods used in frozen samples such as spectrophotometrical enzymatic assays, the latter provides information about the maximal activity of the individual complexes or some of them combined, whereas RIFS allows for an integrative measure of the ETC from Complex I or II to the natural electron acceptor that is oxygen. Another advantage of RIFS is the amount of sample needed to perform the assay (10 times less) and the sensitivity to specific inhibition of the ETC complexes. In addition, the simplicity of the assay where the number of reagents needed is minimal in comparison to spectrophotometrical assays.

One of the initial concerns when freezing samples was how immediate the freezing process has to be, since dealing with clinically relevant samples might involve a delay from collecting the samples from the patient to the freezing and storage. For that reason, different freezing procedures were tested for their capacity to preserve maximal respiratory rate when samples are not immediately flash frozen in liquid nitrogen. Samples placed on ice for 0.5-3 hours or placed at -20 rights after collection preserved the integrity and function of the mitochondrial complexes.

There are numerous applications where RIFS can be used considering all the samples that have been stored for a long time in a freezer. It is well known that mitochondria have been proposed as a key target of environmental toxins. For that reason, all applications targeted to direct screening of compounds leading to mitochondrial toxicity can benefit from RIFS and can have a functional impact of environmental exposures within a large population [18,19].

RIFS has the limitation that it measures maximal respiration but it does not allow to measure coupled respiration or ATP synthesis. However, it has been demonstrated that disease related phenotype correlated with decrease in maximal respiration [20-23] and that defects in coupled respiration are mostly related to some mechanism of uncommon drug toxicity [24].

Conclusion

In summary, impaired mitochondrial function has been shown to contribute to development and progression of various diseases. There was an unmet need to clinically monitor mitochondrial function; however, until now this was near impossible due to the requirement of immediate processing of living tissue samples. With the development of RIFS to measure mitochondrial function in previously frozen samples, we have opened up the possibility of clinical monitoring of mitochondrial function in large population, of samples collected at remote sites or retrospectively in samples residing in tissue biobanks.

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