

Evaluation of ATP production in frozen brain samples

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Introduction. Respirometry-based evaluation of mitochondrial function in the clinical settings is challenging, often requiring long workdays and high flexibility from the operator, ultimately limiting the throughput. In 2020, Acin-Perez et al introduced respirometry in frozen samples (RIFS)(1), showing that the electron transport capacity (ETS) remains largely preserved after a period of deep freezing, retaining up to 95% of that in fresh tissue. In contrast, ATP production (OXPHOS) was lost as freeze-thawing disrupts mitochondrial membrane integrity and thereby dissipates the proton electrochemical gradient required for ATP synthesis. Similarly, frozen samples do not respond to TCA intermediates but rather need direct stimulation with NADH for complex I-driven electron transport (1). Hence, the use of frozen biobank samples can provide valuable diagnostic insights, while overcoming the challenges with the flexibility of operator working with fresh clinical samples. However, when starting work on frozen biobank samples from healthy brains regions, we observed retained responses to TCA substrates and an ability produce ATP in homogenates. Here, we briefly discuss our findings and potential pitfalls regarding incorporating RIFS protocols in routine diagnostical procedures.

Methods. Brain samples were collected from patients undergoing surgical resection of glioblastomas at Aarhus University Hospital, Skejby. Adjacent tumour-free brain tissue was removed as part of the surgical approach, and this tissue was snap-frozen and then kept on -70 °C until they were used for respirometry. Fresh brain samples were obtained from C57BL6/J mice immediately after euthanasia and processed in parallel with snap-frozen brains.

On the day of the experiments, frozen samples were submerged in preservation medium (250 mM sucrose, 10 mM Trizma base, 3 mM EGTA) and allowed to thaw at room temperature for 15-30 minutes. Further sample preparation was done as previously described (2). Briefly, the tissue was homogenized and centrifuged in a second preservation medium (250 mM sucrose, 5 mM Trizma base), after which the supernatant was collected for respirometry.

High-resolution respirometry was measured with an oxygraphy (O2K High-Resolution respirometry, Oroboros Instruments, Austria) using a combination of pyruvate, malate, and glutamate to stimulate complex I (N-pathway), and succinate for complex II (S pathway). Complex I was inhibited with rotenone, and oligomycin was used to evaluate LEAK in the S-pathway. Membrane integrity was tested with cytochrome C. The protocol was as follows:

Sample – pyruvate (5mM), malate (10mM), glutamate (2mM) – ADP (1.25mM or until saturated) - cytochrome C (10 μ M) – succinate (10mM) – rotenone (0.5 μ M) – oligomycin (5nM) – antimycin A (2.5 μ M)

A small number of samples were also run with NADH and FCCP to test respiratory responsiveness and indirectly evaluate membrane integrity. Oxygen flux was normalized to protein concentration and corrected for residual oxygen consumption (ROX).

Results and discussion. Frozen brain samples responded to the TCA-dependent substrates and showed an increased oxygen consumption after titration

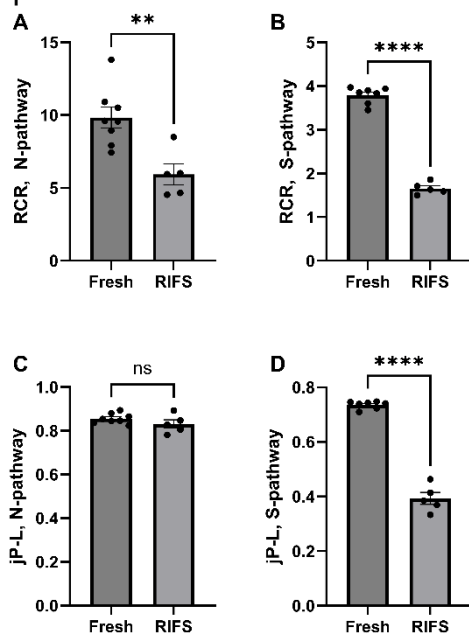


Fig 1. Respiratory control ratios (RCRs) and P-L control efficiencies (jP-L) in the N (A, C) and S-

of ADP (5.9-fold increase). There was minimal increase in OXPHOS upon titration of cytochrome c (5.7%, data not shown). Similarly, uncoupling with FCCP, increased oxygen flux 1.9-fold in frozen samples, showing preserved respiratory reserve capacity. Together, these findings suggest that mitochondrial function and membrane integrity were retained following the freeze-thaw procedures. Importantly, frozen brains did respond to NADH, but to a much lesser extent than RIFS protocols in other tissues, such as heart and liver homogenates (data not shown). NADH does not cross the inner mitochondrial membrane but interacts directly with complex I when the membrane integrity is compromised. These results suggest that the freeze-thawing procedure increased inner membrane permeability in

only a fraction of the mitochondria.

Respiratory control ratios (RCR) were significantly lower in RIFS samples than in fresh tissue (Fig 1 A, B). However, the P-L control efficiency (jP-L), which expresses the OXPHOS capacity relative to LEAK respiration, did not differ in the N-pathway between fresh and RIFS samples (Fig 1C). On the other hand, the jP-L for the S-pathway was significantly decreased (Fig 1D). We attribute this to the fact that

succinate is readily available for both mitochondria with intact membranes and those with disturbed integrity, which then leads to a sharp increase in ETS when complex II is stimulated. Hence, flux measured after stimulating complex II should not be interpreted as a true OXPHOS, but represents both OXPHOS and maximum ETS. Accordingly, the measured LEAK with oligomycin cannot be considered a true LEAK, either. In other words, complex II-driven OXPHOS cannot be measured in these samples. Importantly, though, in the brain, the complex I-activity has been shown to be more dominant the NS-pathway than complex II (3), so isolated changes in complex I could still be highly relevant for diagnostic purposes.

The brain is membrane-rich and may thus be protected against freezing/thawing-associated membrane damage, possibly due to formation and preservation of synaptosomes (4,5). We also speculate whether the brain, being rich in fat, might somehow be insulated from damage. The consistency and degree of integrity loss should be studied further, for example through specific isolation of mitochondrial subpopulations (4,5).

Because frozen brain tissue appears to contain a mixed population of mitochondria with different degrees of membrane integrity, using protein concentrations to normalize fluxes, might lead to an underestimation of actual activity in the intact mitochondria. Normalization of actual ATP-production would thus require a measurement of the percentage of intact mitochondria. A requirement could be that membrane potentials are measured in parallel with oxygen consumption, using stepwise permeabilization in order to correct for any differences in abilities to maintain the electrochemical gradient. We aim to study this approach further.

Conclusion. Here, we show that mitochondrial membrane integrity and ATP production are partly preserved in frozen brain homogenates. To our knowledge, this is the first report of an approach that enables estimation of complex I-driven OXPHOS in frozen brain samples, without specific preparation of synaptosomes. This concept can improve throughput and feasibility of performing routine diagnostic screenings of mitochondrial disorders affecting the brain.

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