THE EFFECT OF CRYOPRESERVATION ON MITOCHONDRIAL FUNCTION IN HUMAN SKELETAL MUSCLE

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ABSTRACT

The Effect of Cryopreservation on Mitochondrial Function in Human Skeletal Muscle Tissue

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The analysis of mitochondrial function is fundamental in the diagnosis of muscle pathologies. Long term preservation of human tissues for functional analysis is a frontier challenge in diagnostics but has potential benefits for inter-laboratory and interdisciplinary analyses. High resolution respirometry of permeabilized muscle fibers allows for quantification of mitochondrial function ex-vivo from small biopsy samples. Scientific studies on respirometry of cryopreserved muscle tissues have seldom been performed. In this study we investigated mitochondrial respiration of DMSO cryopreserved muscle tissue and compared oxygen flux rates to freshly harvested muscle from the same biopsy sample. The purpose of this study is to test the functional capacity of mitochondria in cryopreserved muscle tissue and to compare respirometry to fresh muscle mitochondrial tissue. Cryopreserved muscle showed robust coupled state 3 mitochondrial respiration comparable to fresh muscle. However, the magnitudes of state 3 (ADP-driven) respiration rates for complex I substrates with malate and glutamate and complex I and II with addition of succinate were lower than that of fresh muscle tissue. The significant increase in state 3 respiration upon addition of cytochrome c indicates that cryopreservation causes results in cytochrome c loss and potential disruption of the inner mitochondrial membrane. Optimization of the cryopreservation method will permit long-term storage of samples, allow mitochondrial function and energy metabolism to be analyzed and provide the potential for exchanging samples between research centers.

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CHAPTER 1: INTRODUCTION

Investigation of mitochondrial function under physiological and pathological conditions has been an objective in the field of mitochondrial physiology and bioenergetics. Direct assessment of mitochondrial coupled respiration and ATP synthesis rates as well as uncoupled electron transport capacity provides critical information on mitochondrial structure and integrity and remains an important screening process for the presence of mitochondrial abnormality and alterations due to environmental factors such as diet, exercise training and environmental toxins, as well as intrinsic changes associated with aging. High resolution respirometry of permeabilized muscle fibers allows for quantification of mitochondrial function ex vivo from small biopsy samples. However, a major limitation in the study of mitochondrial function is that it requires analyses to be performed soon after biopsy to guarantee stable mitochondria. Furthermore, thorough investigation of multiple steps in electron transport, substrate supply and the functionality of individual respiratory chain complexes I-V often require intricate and time consuming experiments that may lead to deterioration of the mitochondria. Time limitations impede the performance of large scale muscle bioenergetics and studies distinctive of mitochondrial defects in various diseases. Consequently, the current methods may slow or limit rapid creation of a database with various samples with well defined pathologies that may be translatable in mitochondrial research. Comparison of cryopreservation techniques have yet to be studied however, most studies employ similar freezing and thawing rates of cryopreservation of cells and tissues (Nukala et al, 2006; Kuznetsov et al, 2003). Comparison of cryopreserving agents and concentrations has been conducted in human cells. Various natural and synthetic compounds are currently being used as cryoprotective additives such as alcohols, amino acids, yeast extract and skim milk. Of the lot, glycerol and DMSO are

the most widely used cryoprotective agents (Nukala et al, 2006; Kuznetsov et al, 2003; Fuller et al 1989). Previous studies have compared both cryoprotective agents and results show that DMSO is clearly more effective at protecting human cells and tissues than glycerol (Kuznetsov et al 2003). DMSO is a more penetrating agent causing greater dehydration thereby minimizing freezing injury. Other studies have applied cryopreservation in human reproductive medicine, aquaculture and in the conservation of endangered species. However, few studies have investigated the effects of cryopreservation on mitochondrial function in human muscle tissue. Preserving and stabilizing fully intact and functional muscle mitochondria after the freeze-thaw process is a challenge met by scientists in mitochondrial physiology and bioenergetics. Therefore, long term preservation of human tissue for functional analysis is a frontier challenge in diagnostics and has the potential if successful for inter-laboratory and interdisciplinary analysis.

OBJECTIVES

The purpose of this study was 1) To evaluate the efficacy of a method of cryopreservation of muscle tissue on freshly isolated rat muscle tissue and 2) To evaluate the effect of cryopreservation on mitochondrial respiration in cryopreserved-thawed muscle samples obtained from Greenland Expedition in 2004 led by Dr. Jorn Helge and Dr. Bengt Saltin from the Copenhagen Muscle Research Centre and compare to matching freshly harvested muscle tissue previously analyzed from the same study in 2004 and 3) To determine the effect of the cryoprotectant agent DMSO on mitochondrial respiration on freshly isolated rat muscle tissue.

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HYPOTHESES

It was hypothesized that mitochondrial respiratory oxygen flux rates are comparable in magnitude between cryopreserved and freshly isolated human skeletal muscle samples obtained from the Greenland Expedition in 2004. Control experiments using rat muscle tissue show that cryoprotective agent, DMSO has no deleterious effect on mitochondrial function independent of the cryopreservation procedure.

CHAPTER 2: REVIEW OF LITERATURE

Mitochondria are ubiquitous in eukaryotic cells and one of their more important functions is to produce ATP via oxidative phosphorylation to provide energy for cellular homeostasis (Watts and Kline 2003). Mitochondria also play a significant role in cell apoptosis in senescence and neurodegenerative diseases (Lemasters et al 1999; Kunz et al, 1999; Nasseh et al, 2006; Nicholls et al 2000).The mitochondria are host to many biochemical pathways, including β-oxidation, Krebs' citric acid cycle and parts of the urea cycle, and thus, play a pivotal role in cellular biochemistry. The relationship between mitochondria and human disease has been identified only recently, but has now become one of the most rapidly expanding areas of human pathology. Mitochondrial disorders may be a consequence of inherited defects of either the nuclear or mitochondrial genomes or, alternatively, may be due to endogenous or exogenous environmental toxins. Long term preservation of tissue and cell suspensions for functional analysis is a challenge often met by scientists and researchers in the clinical study of metabolic diseases and in the basic research of mitochondrial physiology. Therefore the analysis of mitochondrial oxidative phosphorylation is fundamental in the diagnosis and screening procedure of mitochondrial pathologies and in the basic research in mitochondrial physiology.

Mitochondria function is traditionally analyzed after isolating mitochondria from tissues and cells (Lee et al 1995). However, this process requires relatively large amounts of tissue and is consequently not suitable for clinical testing of small biopsies. A second method involves chemically penetrating the fibers and cells that sequentially enables the investigation of mitochondria directly in the biopsy sample without isolating the organelles themselves (Kuznetsov et al 2003). Nevertheless the major limitation in

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the study of mitochondrial function is that it must be performed immediately after obtaining a biopsy sample to ensure mitochondrial intactness. Freezing is widely used for banking and presentation of cells, tissues, organs and embryos. But freezing is also extremely useful for rapidly stopping or slowing metabolic and chemical reactions in cells. Rapid freezing of isolated cells is easily achieved by lowering the freezing point of intracellular water by the addition of cryoprotectant or anti-freezes. However, the approach is not easily feasible with relatively thick samples of tissues particularly muscle tissues. High freezing ratios are difficult to achieve because freezing time increases with the thickness of the tissue. One way to ensure proper freezing of tissues is to permeabilize fiber bundles in a sample of tissue. This technique has been successful in various mitochondrial studies and in only one report in skeletal muscle of the rat (Kuznetsov et al 2003); and can be applied to detect oxygen consumption of muscle tissues. Otherwise, experimental preparations of isolated mitochondria from frozen tissues or cell suspensions often consist of broken mitochondria and fragmented membranes (Lee et al 1995). This precludes diagnostic accuracy of functional analyses following long term storage of isolated mitochondria. To overcome such limitations, research has been undertaken to optimize methods to preserve, specifically to cryopreserve mitochondria without affecting the structural or functional aspects of isolated mitochondria maintaining viability of tissues after freezing and thawing (Nukala et al 2006; Kuznetsov et al 2003). Recently, several cryopreservation protocols have been used successfully for long term preservation of blood cells, heart and liver tissues in clinical settings (Kuznetsov et all 2003). However, no successful study on cryopreservation of muscle tissue in human skeletal muscle has ever been reported in the literature. The following will focus on the principles of the cryopreservation method, the effects of cryopreservation on mitochondrial function and compare experimental

results involving mitochondrial function using freshly isolated mitochondria and cryopreserved mitochondria.

Mitochondria-The Universal Energy 'Currency' of Cells

Discovered more than 100 years ago, mitochondria are now acknowledged as the site of respiration-dependent ATP synthesis, the universal energy 'currency' of the cell. Each mitochondrion is made up of an outer membrane, which is permeable, and an inner membrane that is less permeable and projects inward into the mitochondrial matrix, forming folds known as cristae. The cristae house proteins that form the respiratory complexes, referred to as the electron transport chain (ETC), and are responsible for the synthesis of adenosine triphosphate (ATP). The other regions of the mitochondria are the intermembrane space that contains enzymes involved in nucleoside synthesis, and the gel-like matrix, that contains a high concentration of enzymes, ions, organic molecules as well as the DNA of the mitochondrial genome and the components necessary for synthesizing its own proteins.

Oxidative Phosphorylation

Mitochondria, the cellular energy organelle, generate ATP through the oxidative phosphorylation system (OXPHOS). OXPHOS is defined as the oxidation of fuel molecules by oxygen as a final electron acceptor and the concomitant transduction of this energy into ATP is the final process of the complicated biochemical network involved in cellular energy production. The OXPHOS molecular system, which is embedded in the lipid bilayer of the mitochondria's inner membrane consist of electron acceptors, CoEnzyme Q and cytochrome c and five multi-subunit protein complexes (I-V). This sophisticated system comprises about 70 nuclear genes products and 13 mitochondrial

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gene products. Briefly, carbon sources enter mitochondria as pyruvate (from glucose), amino acids, or fatty acids. In the mitochondrial matrix, pyruvate is converted into acetyl-CoA by pyruvate dehydrogenase to form one molecule of NADH, an electron carrier (*figure 2*). Fatty acids are first converted into a fatty acyl-CoA and then via the β-oxidation cycle to acetyl-CoA producing one $FADH_2$, one NADH and one acetyl per cycle. The citric acid cycle (Krebs cycle) is the terminal pathway for all mitochondrial oxidations and the major producer of NADH. Succinate is converted to fumarate producing $FADH_2$ by succinate dehydrogenase, which is part of complex II in the electron transport chain. Fumarase converts fumarate to malate and malate dehydrogenase regenerates oxaloacetate to form a third NADH in the process. The oxidations are coupled to phosphorylation by respiration, a function of the electron transport chain.
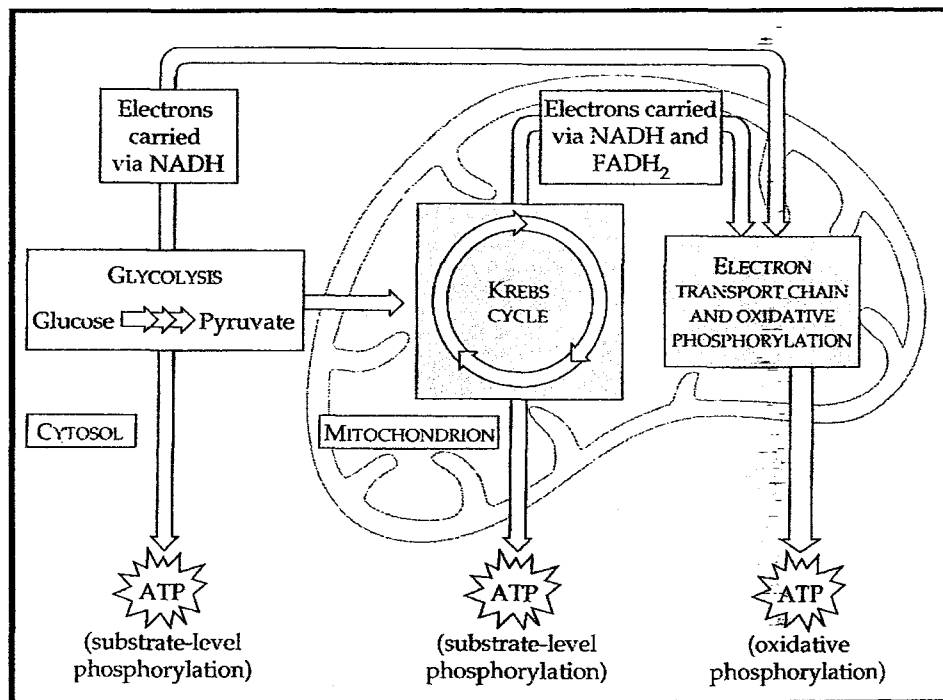
Illustration 1: Overview of cellular respiration. Gycolysis occurring outside the mitochondria whereas the Krebs cycle and electron transport chain (ETC) are located inside the mitochondria. NADH and $FADH_2$ transfer electrons from glycolysis and the Krebs cycle to the ETC. The ETC in turn converts the chemical energy to drive the oxidative phosphorylation (Campbell 1999).

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Electron Transport Chain

The mitochondria are able to generate most of the cell's ATP via the OXPHOS system, however, any impairment of the organelle's ability to produce ATP can have disastrous consequences, not only due to the loss of ATP, but also due to indirect impairment of downstream functions, such as maintenance of cellular calcium homeostasis, membrane integrity, etc. Electrons are systematically passed through the ETC (*figure 2*), beginning when electrons derived during glycolysis, transported in the form of NADH and $FADH_2$ and transferred to complex I (NADH dehydrogenase) and complex II (succinate dehydrogenase) respectively, in the inner membrane. These complexes are responsible for catalyzing the transfer of electrons to coenzyme Q (ubiquinone) that carries the electrons to complex III (ubiquinone-cytochrome c reductase), which subsequently shuttles them to cytochrome c, a peripheral protein bound to the outer face of the inner membrane. The electrons are received at complex IV (cytochrome c oxidase), where 4 electrons are transferred to O_2, forming H_2O in the process. Complex II (succinate dehydrogenase) mediates the transfer of electrons from succinate to coenzyme Q and utilizes $FADH_2$.
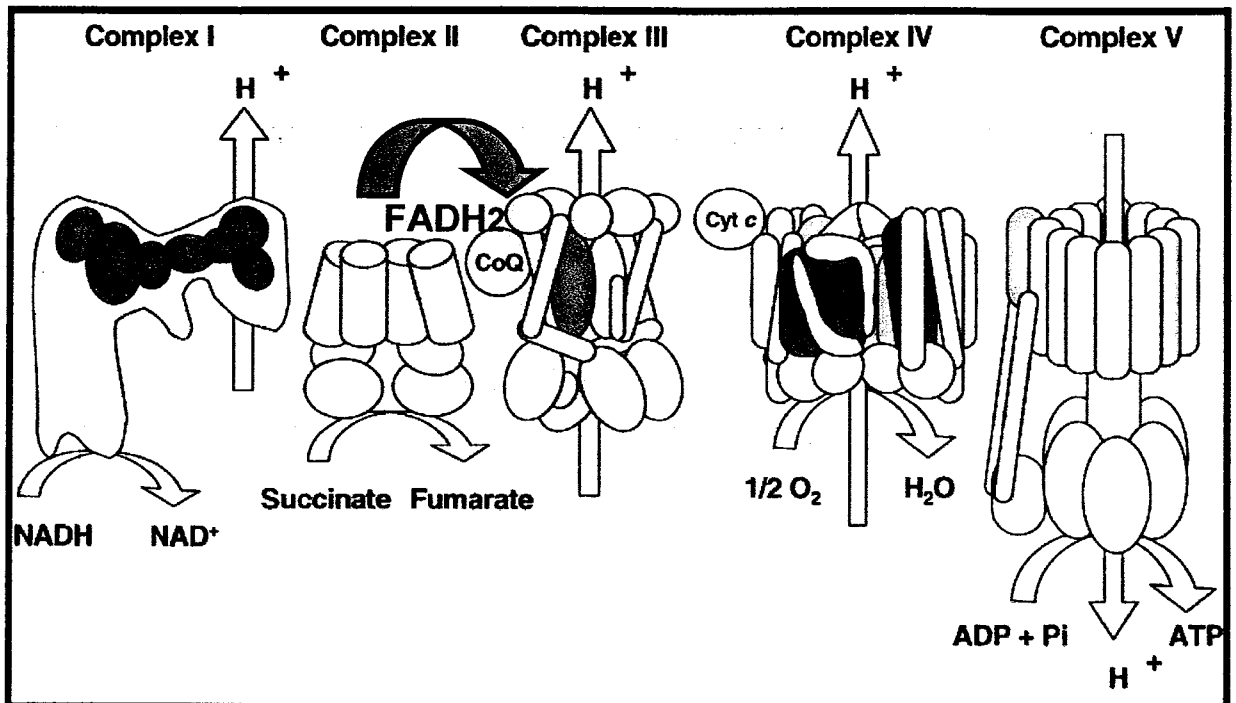
Illustration 2: Respiratory chain complexes. In each nuclear subunit the colors denote: blue = complex I; green = complex II; red = complex IV; yellow = complex V; P_i = inorganic phosphate; cyt c = cytochrome c; C_oQ = coenzyme Q; $FADH_2$ and NADH shuttle their cargo of high energy electrons to their respective complexes (NADH to complex I; $FADH_2$ to CoQ) (Zeviani et al 2004)

As electrons are passed through each complex, protons are transferred from the matrix

to the intermembrane space, generating an electrical potential and a proton gradient

across the inner membrane. Excess protons in the intermembrane space are

transported back across the matrix down their concentration gradient through complex V

(ATP synthase). The stored energy from the proton gradient used to drive the synthesis

of ATP molecules. Once ATP is formed, it is then transported out of the matrix and into

the cytosol in exchange for a molecule of ADP entering into the mitochondrion via the

adenine nucleotide translocase system (ANT). Therefore, from the supramolecular

structure of the mitochondria, it is known that the energy production lies in the electron

transport chain and subsequent oxidative phosphoylation. Notably, energy production in

mitochondria requires not only a full assembly of functional proteins at the level of the

inner mitochondria membrane, but also a bidirectional flow of information and

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coordinated assembly process between the nuclear and mitochondrial genomes to adjust energy production in tissues to different energetic demands. Consequently, different mutations in the mitochondrial DNA, nuclear DNA-encoding subunits, components or regulators of the ETC can produce a plethora of OXPHOS diseases (DiMauro and Schon, 2003)

ATP Synthase

Understanding the stoichiometric properties of oxidative phosphorylation depends upon a thorough understanding of the molecular machine, ATP synthase, the enzyme that actually makes ATP. It works like an ion pump running in reverse; it uses the energy from the proton gradient to power the ATP synthesis. Embedded within the inner mitochondrial membrane, electron microscopic studies have revealed that ATP synthase consists of 2 domains: and intramembranous F_0 domain, and a catalytic F_1 domain which consists of five different subunits. Negative staining techniques have revealed that the two sections are joined to each other by means of a stalk-like structure (Tyler, D, 1992), in other words, a cylinder within the inner mitochondrial membrane, a knob protruding into the mitochondrial matrix and an internal rod connecting the two. When hydrogen ions flow through the cylinder, they cause the cylinder and the rod to rotate, bringing about a conformational change in the knob, activating catalytic sites where ADP and inorganic phosphate combine to make ATP.

H⁻ H⁻ H⁺ H⁺
H⁺ H⁺ H⁺

INNER
MITOCHONDRIAL
MEMBRANE

Cylindrical rotor
Rod

H⁺ ← Knob containing
catalytic sites

ADP
C_i

ATP

Rotation of γ relative to α & β

MITOCHONDRIAL MATRIX
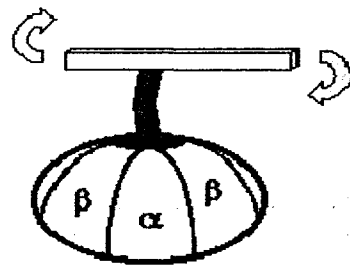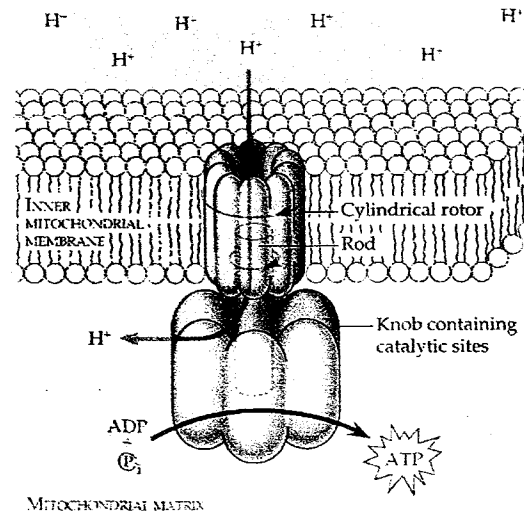
Illustration 3: (left): Rotation of the γ shaft relative to the ring of α and β subunits
http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb1/part2/f1fo.htm
(right) ATP synthase molecular machine uses energy from H⁺ gradient to drive ATP synthesis (Campbell 1999)

Mitochondria-Metabolic Pathology of the Oxidative Phosphorylation System

Aerobic production of ATP involves an intricate biochemical network of cellular

uptake, transport of substrates, their metabolic conversions and myriad accessory

systems which act in a cell-, tissue-, or system dependent manner. It is known that the

synthesis of ATP, oxidative phosphorylation is driven by the energy released during the

oxidation of electron carriers reduced by the breakdown of carbohydrates, fats and

amino acids. Mitochondrial defects are now recognized as important causes of disease.

Faulty oxidative phosphorylation may be due to the overall dysfunction of the

respiratory chain, or can be associated with single or multiple defects involving one or

more of the five respiratory chain complexes. Respiratory chain is a unique structure of

the inner membrane formed by the complementation of two separate genetic systems:

the nuclear genome and the mitochondrial genome. The nuclear genome encodes the

large majority of the protein subunits of the respiratory complexes; the mitochondrial

DNA (mtDNA) encodes the replication and expression systems, whereas the mitochondrial genome encodes the 13 respiratory complex subunits and RNA components of the mitochondrial translational apparatus. OXPHOS disorders can be classified genetically according to whether the primary defect is in the nuclear or mitochondrial genome as well as by the pathways affected. There is a considerable lack of understanding of the pathogenic mechanisms involved in the development of clinical symptoms and deterioration seen in many patients.

Some key features of OXPHOS genetics suggest that the mtDNA is found in high copy number; mutations can affect all mtDNA copies in an individual or only some copies. In the case of heteroplasmy, a threshold mutation level is required for the expression of a biochemical defect in individual cells, meaning that within a tissue, there may be mixture of respiratory competent and deficient cells (Smeitink et al, 2006). In addition, OXPHOS defects can be tissue specific, due to the variable metabolic threshold for the different OXPHOS enzymes in each tissue. An inability of mitochondria to supply sufficient ATP to meet cellular needs is often assumed to be the primary effect of mitochondrial disease mutations.

OXPHOS disease is by its nature extremely heterogenous, as well as pleiotropic in its effects. Understanding its metabolic consequences thus requires a multidisciplinary approach combining in vitro assays with in vivo studies on patients and animal models. Modern cryopreservation techniques could maintain such tissues (healthy and diseased) at maximum viability of different tissues after freezing and thawing. Long term preservation would enable transport and storage of tissue for later use or analysis and provide the potential for exchanging samples between research centers. Moreover, cryopreservation of tissues would provide the opportunity to create a database consisting of various samples with well defined pathologies and integrate various specialized analyses such as DNA analysis, proteonomics, metabolomics and

comparative studies. Moreover, cryopreservation of mitochondria will ensure long term stability but not correct any mitochondrial defects already present in tissues allowing better diagnosis and broader screening of mitochondrial abnormalities in various diseases.

High Resolution Respirometry:

A method to detect oxygen consumption in muscle biopsies

In recent years, a new collection of powerful molecular biology techniques have been applied to the analysis of the function of the mitochondrial respiratory chain and oxidative phosphorylation such as electron microscopy, immunoassays, bioluminescence and respirometry. Assessing mitochondrial function is an important area of investigation in the clinical and applied science fields. Various genetic and acquired mitochondrial disorders can alter the stoichiometric ratios of mitochondrial enzymes, rendering them impaired or non-functional. Evaluation of the electron transport chain and respiratory states can be accomplished using high resolution respirometry with optimized titration and inhibitor protocols. This method allows the researcher to isolate specific enzymes in the respiratory chain and assess their contribution to total oxygen consumption, respiratory control, and numerous other parameters.

High resolution respirometry can be used to understand and investigate pathological effects resulting in reduced respiration such as apoptosis/necrosis, kinetics of aging, mitochondrial and metabolic diseases, ischemia-reperfusion injury and oxidative stress. The major advantage to using this method is that it requires only a small amount of biopsy, which is particularly important in the diagnosis of genetic and acquired mitochondrial defects in clinical settings. Moreover, the technique allows the

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investigation of oxygen at relatively low or high metabolic levels. The major

disadvantage lies in the preparatory stages of the mitochondria, which is often laborious

and detailed, with the ultimate fate of potential damage to the mitochondria, particularly

in the case of defective or diseased mitochondria.

High resolution respirometry is an optimized application of polarographic oxygen

sensors and instrumental design of the Oroboros Oxygraph (Oroboros Instruments,

Innsbruck; and Paar, Graz, Austria) in combination with digital data acquisition and

analysis (DatLab software). The Oxygraph is a two-chamber titration-injection

respirometer with a limit of oxygen detection of up to 0.5 pmol sec-1 mL-1. In addition to

this low limit of detection, high resolution respirometry allows for measurement with only

0.04 mg mitochondrial protein, 106 endothelial cells, or 2 mg of muscle biopsy (wet

weight) in the 2 mL chamber; fundamental in the diagnosis of mitochondrial function in

health and disease (Gnaiger, E. 2001).

Accurate measurement of mitochondrial oxygen consumption over temperatures

ranging from 3° C to 37° C depend on several related features of the system, such as (1)

instrumental design allowing for minimum oxygen diffusion into a homogeneously stirred

closed chamber, (2) polarographic oxygen sensors (POS) and electronic components

which provide sufficient stability and resolution over a range of oxygen concentrations

(from 0.02 to 200 μM O2) (3) appropriate software providing mathematical signal

corrections based on standardized instrumental and chemical calibrations and (4)

resolution of nonlinear changes in the time derivative of the oxygen concentration signal

(Gnaiger, 2001). Moreover, the Oroboros Oxygraph consists of gas impermeable

materials such as chambers that are fabricated with materials possessing low oxygen

buffering capacities [polyetheretherketone (PEEK) stirrer bars versus conventional

Teflon stirrers; glass chambers instead of Perspex; Viton w-rings and titanium cannula

and stopper, all of which have minute oxygen buffering capabilities, minimizing back-

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diffusion of oxygen into the chamber under microxic conditions, and thus experimental artifact] (Gnaiger, 2001). The chambers are equipped with 2-mm cathode POS sensors, increasing sensitivity and signal-to-noise ratio, and decreasing signal drift when compared to smaller cathode diameter sensors (Gnaiger, 2001). Angular insertion of the sensors prevents the sensor from being an integral part of the chamber wall. In order to detect the oxygen concentration, the sensor must consume an amount proportional to the O2 concentration in the chamber, which explains the background oxygen flux observed in a water filled 2 mL chamber at air saturation of about $2 - 3$ pmol O_2 sec-1 mL-1 at $30 - 37°$ C (Gnaiger E, 2001 and Haller et al, 1994). Therefore, during the instrumental background tests, the DatLab software can accurately calculate the appropriate linear equations used to correct for back-diffusion of oxygen into the chamber from the various components, leak from the exterior, and sensor O_2 consumption. The DatLab calculates the time derivative of the digital O_2 concentration signal at appropriate time intervals (usually in the millisecond range) and plots it in real time, rendering oxygen 'flux' curve (Gnaiger, 2001). This flux curve can be reconverted to a digital signal, and pasted into an excel file for further analysis. For that reason, high-resolution analysis of oxygen concentration and flux remains the fundamental basis in bioenergetics of mitochondrial and cellular physiology research and offers a precise diagnostic test of mitochondrial dysfunction in small biopsy samples from human muscle; ultimately improving our fundamental understanding of mitochondrial related diseases.

Principle of Cryopreservation

To ensure reproducible results and continuity on research and biomedical processes, today's scientists are faced with the challenge of stabilizing tissues and cells for future analysis. It is a well known fact that freezing could be an ideal method for long-term preservation of living cells and tissues. Addition of proper cryoprotectant agents and a modern cryopreservation technique could maintain maximum viability of different tissues after freezing and thawing. Therefore, whole tissues or a population of cells can be stabilized by subjecting them to cryogenic temperatures, which for all practical purposes, stops time. Therefore, cryopreservation is a process in which cells or whole tissues are preserved by cooling to low sub-zero temperatures, typically the boiling point of liquid nitrogen (77 K or -196°C). At these low temperatures biological activity and biochemical processes or reactions are temporarily slowed. Advances in cryopreservation technology have led to methods that allow low temperature maintenance of a variety of cells or tissues. The technique can be applied for the preservation of microorganisms, isolated tissue cells, small multicellularorganisms, and more complex organisms such as embryos (Nalge Nunc Cryopreservation manual).

Briefly, cryopreservation methods comprises the following: 1) collection of tissues; 2) suspension of tissue in an extender solution containing cryoprotectant; 3) cooling tissue toward a low temperature; 4) storage of tissue in liquid nitrogen (-196°C) to assure long term stability; 5) warming of samples to physiological temperatures and 6) removal of the cryoprotectant agent and assays of functional to determine survival and tissue integrity. It has been shown that injury to cryopreserved tissues is caused by most of the steps numerated above, particularly during the addition of a cryoprotectant and during the freeze-thawing process (Leung, 1991).

Cryoprotective Agents (CPA)

A cryoprotectant is a chemical reagent added to the tissue solution in an attempt to prevent or minimize cryoinjuries by suppressing or reducing ice formation. Cryoprotectants are classified as permeating or non-permeating. Permeating cryoprotectants are used to reduce the salt concentration; minimize the change in cell volume; decrease the rate of ice formation; decrease the rate of water diffusion from cell to ice crystals; and increase the vitrification temperature (Leung 1991). Permeating cryoprotectants include glycerol, methanol, and dimethyl sulfoxide (DMSO). Non-permeating cryoprotectants, such as sugars (glucose, sucrose), amino acids (egg yolk, milk) are usually used in conjunction with permeating cryoprotectants and act to reduce the freezing point and increase the vitrification temperature of the extracellular medium (Leung 1991). Many compounds have been used as cryoprotective agents, either alone or in combination such as sugars, serum and solvents. There is currently no "gold standard" cryo-agent for cryopreservation, however, dimethyl sulfoxide (DMSO) and glycerol have been widely used and seem to be most effective (Kuznetsov et al, 2003; Nukala et al, 2006; He et al, 2004). Cyoprotective agents have several functions during the freezing process: encourage greater dehydration of the cells and tissue prior to intracellular freezing and penetrate the cell and delay intracellular freezing and minimize solution effects. DMSO, the most widely used cyroprotective agent, is an oxidized thioester with a chemical formula of $(CH_3)_2SO$, hence making it water soluble whereas its further oxidized form called dimethyl sulfone does not exhibit any cryoprotective properties. Permeable CPAs, like DMSO, are highly hydrophilic and act by binding to intracellular water preventing excess dehydration, reducing salt toxicity and thereby preventing formation of ice crystals inside the cell or isolated mitochondria. (Farrant, 1980). The mechanism by which DMSO interacts with the plasma membrane was

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studied by Anchordoguy et al (1991) on liposomes. Their results suggest an electrostatic

interaction between the polar sulfoxide moiety of DMSO and phospholipids bilayer.

Sucrose and other nonpermeating agents prevent membrane destabilization by direct

interactions with membrane phospholipids with the same mechanism as gylecrol,

suggesting a possible cause for the noneffectiveness of glycerol in protecting the plasma

membrane in trout spermatozoa (Ogier de Baulny et al 1997). The choice of which

cryoprotectant to use may depend upon the type of cell or tissue to be preserved.

However, DMSO is able to penetrate more readily and is usually the choice when larger

cells or tissues are to be preserved. An important aspect when using cryoprotective

agents involves the notion that it must be diluted to the desired concentration prior to its

application to cells and/or tissues. This minimizes the deleterious effects of chemical

reactions such as generation of heat, and intracellular ice formation, all of which can

have serious detrimental effects in the biological system.

Cryo-Injury by freezing and thawing

The freezing process involves a series of complex events that even today's top

scientists are unable to fully understand the underlying mechanisms. Cryobiology

studies have led to speculation on what occurs during freezing of living tissue or cells

(Fuller et al, 1989; Maas et al, 2000; Sutton et al 1991). Damage to cells or tissues

during cryopreservation are often due to solution effects, extracellular ice formation,

dehydration and intracellular ice formation. It is suggested that most of the damage

occurs during an intermediate zone of temperature, between -15°C and -60°C, that cells

must traverse twice: once during cooling and once during warming. Since water is the

fundamental component of all living matter, it must be available for the chemical

processes of life to occur, consequently cellular metabolism stops when all the water in

the system is converted to ice, that is during the freezing process. During slow cooling,

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between -5 and -10°C, ice forms in the external medium but the cells' content remains unfrozen and supercooled; presumably because the plasma membrane blocks the growth of ice crystals in the cytoplasm. The supercooled water in the cells has a higher chemical potential than that of water in the partially frozen extracellular solution, therefore water flows out of the cells and freezes osmotically. (Farrant J.,1980). In other words, as ice forms, water is removed from the extracellular environment and an osmotic imbalance occurs across the cell membrane leading to water migration out of the cell. The increase in solute concentration outside the cell, and intracellularly can be detrimental to the cell (Farrant, 1980). If the freezing rate is too slow, the water in the extracellular solution freezes and the remaining solution becomes more concentrated; intracellular water then diffuses out, which results in severe dehydration. On the other hand, if the freezing rate is too rapid, water is not lost fast enough to maintain equilibrium; too much water remains inside the cell, damage due to ice crystal formation and recrystallization during warming can occur (Farrant, 1980).

The rate of cooling has a dramatic effect on the mechanics of freezing; rapid cooling minimizes the solute concentration effect as the ice forms, but it undoubtly leads to more intracellular ice. Inversely, slow cooling results in greater loss of water from the cell and less internal ice, but increases the solution effects. Cells permeability affects the rate of water loss; therefore, the more permeable cells are able to tolerate rapid cooling better than less permeable cells (Mazur et al 1972). The notion that ice crystal formation and solution effects play a role in the cell inactivation has directed research to optimize the cooling rates that would minimize the effect of each. In normal practice a cooling rate of 1°C per minute is preferred. The use of cryoprotective additives can also minimize the detrimental effects of increased solute concentration and ice crystal formation. Additionally, maintaining frozen tissue or cells at the appropriate storage temperature and using an appropriate warming rate may minimize damage.

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Mitochondrial Respiration:

Differences in Cryoprotective Agents on Muscle Tissue

Previous studies have proposed glycerol and DMSO as most effective for cryopreservation of mitochondria (Fuller et al, 1989; De Loecker et al 1991) However, the study by Kuznetsov et al (2003) tested respiratory capacity of mitochondria in permeabilized animal muscle fibers after freezing in glycerol or DMSO based media. Results suggest that when freezing in glycerol medium the best preservation was at 20% glycerol, which gave the muscle fibers similar control rates of respiration when using succinate as a substrate for complex II electron supply in the presence of ADP and cytochrome c. Similar results were also observed when using skinned fibers in mechanical studies of muscle tension (Larsson et al 1993). However, DMSO was deemed more effective than glycerol with better preservation at concentrations between 20% and 50% with an optimal around 30%. In the literature, DMSO is often reported to have a higher cryoprotection potential (Kuznetsov et all 2003; Panoff et al, 2000) than glycerol even though some studies reveal that glycerol can be used for preservation of tissues with the added benefit of being less toxic than DMSO (Tselutin et al, 1999). Whereas other studies whose aim was to compare the cryoprotective properties of glycerol and DMSO confirm that at 10% DMSO appears to be a better cryoprotective agent than glycerol by way of diminishing intracellular ice crystallization and cellular dehydration (Lehr, H. 1971; Graham, W.)

During ADP-stimulated respiration, glycerol preserved fibers showed less than 50% respiratory activity compared to unfrozen permeabilized muscle fibers (Kuznetsov et al, 2003). However, respiratory capacities with succinate and cytochrome c after freezing at optimal glycerol or DMSO concentrations were not significantly different from control samples. Moreover, respiration of glycerol freeze thawed fibers with succinate

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as a substrate was significantly stimulated by cytochrome c, indicating a decrease in endogenous cytochrome c and a possible rupture of the outer mitochondrial membrane during freeze-thawing with glycerol (Kuznetsov et al 2003) suggesting that DMSO was the better cryoprotectant at the optimal concentration of 30% DMSO + 10mg-mL BSA. Since DMSO may have a toxic side effect on mitochondria, rendering a change in substrate linked oxygen uptake that may be due to alteration of the permeability characteristics of the inner membrane in the presence of DMSO (Fuller et al, 1989), a standard equilibrium period of 5 seconds (time between mixing fibers with cryopreservation medium and freezing) was suggested to minimize exposure to DMSO (Kuznetsov et al 2003). In mitochondrial studies it is not known whether the mitochondria is first damaged by the freeze-thawing, which leads to a drop in ATP content, ultimately causing a malfunction in the ionic pumps and a destabilization of the plasma membrane or if the plasma membrane and mitochondrial membranes are mechanically altered. Further studies are essential to determine the best method to protect the mitochondria, and evaluate the mechanisms of interaction between cryoprotectant and membranes.

Effect of Cryopreservation on Mitochondrial Function

The cryopreservation method has been extensively used to study the cellular structure of spermatozoa in reproductive studies and organ preservation for transplantation. However, cryopreservation has been applied to preserve cardiac and liver tissue and most recently delicate brain and muscle fibers for batch storage and shipment during clinical studies of metabolic diseases. Oxidative phosphorylation assays are fundamental in the study and diagnosis of mitochondrial pathologies, and is usually preformed with fresh muscle mitochondria. However, new techniques have enabled

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scientists to stabilize and fix mitochondria in permeabilized fibers by applying specific

preserving solutions and methods; nevertheless such preservation is still limited to

several hours of cold storage. Conversely, simple freezing of muscle tissues result in

significant damage to mitochondrial functional integrity by means not yet understood.

However, application of cryoprotective permeating agents or non-permeating or a

combination of both can minimize mechanical damage to the mitochondria and render

the mitochondria intact for analysis.

A research team from Austria has recently tested several procedures for

cryopreservation of permeabilized fibers isolated from different muscle types. Various

concentration of the cryoprotectant agents, glycerol and DMSO, were applied to find the

optimal one, and the freezing procedure was optimized to find the best preservation of

mitochondrial function. Moreover, results from oxidative phosphorylation assays from

cryopreserved mitochondrial preparations were comparable to that of freshly isolated,

unfrozen muscle tissue. More importantly, other mitochondrial respiratory parameters

such as, respiratory control ratios with O_2 flux rates with various substrates, were not

significantly altered during the cryopreservation stages. Results suggest that the

respiratory capacity of mitochondria from rat heart, rat soleus, were similar to that of

corresponding control values (Kuznetsov et al 2003). Moreover, rates of resting state

respiration (before the addition of ADP) and respiratory control rates (active respiration

after addition of ADP and resting state respiration) after freeze thawing were not

significantly different from the control samples (Kuznetsov et al 2003). Respiration rates

of permeabilized fresh fibers with pyruvate and malate after the addition of succinate

were similar to the rates from DMSO freeze-thawed muscle fibers, but most important,

the rates did not increase when cytochrome c was added to the protocol (Kuznetsov et

al 2003). Strengthening the notion that an optimal DMSO concentration and

cryopreservation protocol completely protects the integrity of the outer mitochondrial

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membrane and ensures complete cytochrome c conservation compared to samples cryopreserved with glycerol.

Additional analysis with creatine allows for the investigation of functional coupling between mitochondrial creatine kinase and oxidative phosphorylation during intracellular energy transfer. Freeze thawed rat heart fibers showed identical stimulatory effects as the control muscle fibers during activation of respiration, after the addition of creatine in the presence of ADP (Kuznetsov et al 2003) suggesting that the mitochondrial outer membrane structure remained intact during the cyropreservation process in freeze thawed muscle fibers.

Effect of Cryopreservation on Mitochondrial Structure and Integrity

Recently, various imaging techniques have allowed researchers the opportunity to visually study various physical features of the mitochondria such as: intactness of mitochondrial outer membrane, intracellular arrangement, and metabolic responses. Fluorescent confocal microscopy studies reveal organized and functional mitochondria in cryopreserved myocardial fibers compared to the controls (Kuznetsov et al 2003). Further imaging techniques, such as photon excitation, reveal normal metabolic responses of mitochondria in freeze-thawed fibers (various substrates are added to stimulate respiration) compared to fresh unfrozen rat heart fibers or mice skeletal muscle fibers (Kuznetsov et al 2003; 1998 respectively). Autofluorescence of mitochondrial flavoproteins indicate normal metabolic responses in freeze thawed fibers, similar to that of fresh rat myocardial muscle fibers (Kuznetsov et al, 2003) and rat skeletal muscle fibers (Kuznetsov et al, 1998). Therefore, cryopreservation does not alter the spatial organization or the functional metabolic response of the mitochondria in myocardial muscle fibers.

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The rate of ATP synthesis provides information on the proficiency of the OXPHOS system and allows the ATP/O ratio to be calculated as an important mitochondrial index (Kuznetsov et al, 2003) that may indicate a possible pathology (Luft et al 1962; Kuznetsov et al 2003). ATP production ratios from the cryopreserved samples did not differ from the control values even in the presence of ATP inhibitors (Kuznetsov et al 2003). No uncoupling of muscle mitochondria was found in the study, however, previous studies have reported DMSO as having an uncoupling effect on liver mitochondria, ultimately affecting mitochondrial function (Fuller et al 1989). It may be possible that the permeabilization fiber technique used in Kuznetsov's study (2003) allowed a faster sample transfer from the cryopreserved medium to medium without the DMSO. In this case, minimizing the amount of mitochondrial exposure to the cryoprotective agent may have resulted in less damage to the mitochondria. Therefore, freeze-thaw samples from various muscles demonstrate good preservation of mitochondrial functional integrity and a normal OXPHOS system. Cryopreservation may be employed as an alternative technique to the investigation of freshly isolated muscle preparations and will be considered important when "sharing" biopsies between various scientific research centers. Moreover, cryopreservation of functional mitochondria will ensure long term stability without affecting any mitochondrial defect that may be already present in the sample, an otherwise useful tool that may be employed in the clinical study of mitochondrial pathologies.

Cryopreservation in Brain Tissue

Previous studies have investigated the effects of cryopreservation in liver and muscle mitochondria in rabbits (Araki et al 1977), mice (Fishbein and Griffin, 1976), and rats (Tsevtkov et al 1985; De Loecker et al 1991; Kuznetsov et al 2003) but few studies have directly addressed the effects of cryopreservation on mitochondrial brain tissue. The study by Nuksala et al (2006), demonstrate that DMSO can be successfully used to cryoprotect rat cortical mitochondria which can be a possible strategy to employ for preservation of human brain mitochondria. Electron microscopy results demonstrate that fresh mitochondria and cryopreserved mitochondria have intact inner and outer mitochondria membranes (Nuksala et al 2006). Similar to Kuznetsov's study, Nukala's oxygen consumption measurements reveal no significant differences in the respiratory control ratio (RCR) between the freshly isolated mitochondria and the cryoprotected mitochondria; indicating that the electron transport system is well coupled to the oxidative phosphorylation even after freeze-thaw stress during the preservation process (Nuksala et al 2006). Other studies have reported a decrease in respiratory control ratios from isolated liver mitochondria that had been frozen for months (Fleisher 1979; Fuller et al 1989; De Loecker 1991). The reduction possibly indicates a DMSO effect on mitochondrial bioenergetics (Nukala et al, 2006). Western blotting analysis revealed no significant differences in immunoreactivity of mitochondrial membrane maker proteins (COXIV or VDAC) supporting their electron microscopy findings that the mitochondrial membrane remained intact following cryopreservation. Clayton et al (2005) have shown that the release of cytochrome c is mediated, in part at least, by mitochondrial membrane proteins Bak and VDAC forming pores rather than by non specific rupture. These results support the data by Nukala et al that cytochrome c release is due to an increased leakiness of the mitochondrial outer membrane and not due to its rupture.

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Furthermore, that cytochrome c is a low molecular weight protein (12kDa) present in the intermembrane space, loosely bound to the inner mitochondrial membrane, confirming that the leakiness is partly responsible for the high cytochrome c values during respirometric measurements (Nukala et al, 2006). In this study, 10% DMSO caused a reduction of mitochondrial bioenergetics (partly due to the loss of cytochrome c) which is in contrast to Kuznetsov and Graham 's study in which they observe cryopreserved mitochondria demonstrating functional integrity, normal ATP production and energy transfer as well as structural intactness of the outer mitochondrial membrane.

Cryopreservation in Reproductive Medicine

Cryopreservation methods are extensively used in other areas of medical research such as organ and tissue transplantation and reproductive medicine. Sperm cryopreservation is routinely performed in reproductive centers and andrology laboratories around the world. During the cryopreservation process, osmotic effects of freezing and thawing lowers the fertility capacity of the spermatozoa by damaging cell membranes, impairing sperm motility and mitochondrial processes and inducing morphological changes such as coiled tails (Nallella et al, 2004).

The freeze–thawing process and the preparation of cryopreservation media has been refined over the years, however no standard method has yet been established. The optimal rate of temperature drop during freezing and the type of cryoprotectant remains controversial. In previous studies involving conserved sperm from fish (bass), DMSO was found to be the better cryoprotectant compared to glycerol, propylene glycol, methanol, and dimethylacetamide (Jenkins et al, 2002). However, glycine is used as a non-permeating agent to cryopreserve spermatozoa, and improve post thaw motility in a variety of species, including goat (Kundu et al 2001) and fish (He et al, 2003). The

mechanism by which glycine increases post thaw motility is still unclear. The morphology of mitochondria has been reported to be affected during the freezing and thawing phases (Lahnsteiner et al 1996; Gwo et al 1992; Oiger et al, 1999; O'Connell et al 2002) of cryopreservation. It is thought that damage to the mitochondria may be partly responsible for the decrease in the percent of motile sperm as well as decreased duration of motility in post thaw sperm. The study by He and Woods (2004) report damage to the plasma membrane during the freezing and thawing stages of cryopreservation, and that the degree was concentration dependent. They report that 2.5 % DMSO concentration at a 10 minute equilibrium time may have been too low to protect the sperm from damage associated ice crystal formation during the freeze-thaw stages of cryopreservation. Therefore, optimal concentrations of CPA are required to stop biological deterioration processes and create an unlimited shelf life of tissues and cells.

Energy is essential for sperm motility and fertilization and is supplied in the form of ATP synthesized by either glycolysis in the cytoplasm or through oxidative phosphorylation (OXPHOS system) in the mitochondria, thus facilitating efficient propulsion for the sperm to reach the oocyte and for fertilization. It is thought that the ATP generated by OXPHOS in the inner mitochondrial membrane is transferred to the microtubules to drive motility (Zamboni et al, 1982; O'Connell et al 2002). Therefore, reduced motility may be attributed to damaged mitochondria. He and Woods (2004) report a decrease in ATP production with increasing DMSO concentration at both the pre-freezing and post-thaw stages; however, 5 % DMSO had a higher percentage of post thaw spermatozoa with functional mitochondria than 2.5 % DMSO. When considering post thaw plasma membrane integrity and mitochondrial function, functional mitochondria became the limiting factor for 7.5 % and 10 % DMSO; plasma membrane integrity at 2.5 % and 5 % DMSO, suggesting that concentration of DMSO must be

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identified in order to obtain a higher percentage of sperm with both intact plasma membranes and functional mitochondria (He and Woods, 2004). Further results suggested that glycine, although not providing additional protection to the plasma membranes, did significantly improve mitochondrial function and ATP content of fish sperm (striped bass). For this reason glycine increased the percentage of sperm with both intact plasma membranes and functional mitochondria at 7.5 % and 10 % DMSO but not at 5 % (He and Woods, 2004). A possible mechanism by which glycine serves to protect the plasma membranes may be attributed to the glycine receptors on the plasma membrane and that the binding of glycine molecules to the receptors triggers and signal transduction pathway which ultimately protects mitochondrial function and preserves ATP content (He and Wood, 2004). Further studies would have to monitor pathways involving glycine and their ultimate impact on mitochondrial enzymes.

In summary, cryopreservation can be used to preserved muscle tissue for long term storage without affecting mitochondrial structure and functional integrity. However, more elaborate studies are needed to quantitate the specific cryoprotective virtues of several different cryoprotective agents, specify the underlying mechanisms of the freeze-thaw phases, determine tissue specificity, and develop a "gold" standard by which the method can be used and optimized for various tissues. Nevertheless, the method allows the opportunity to create a database of various samples with well-defined pathologies accessible for comparative studies or studies to be performed in various geographical locations. Furthermore, the technique will be fundamental in the diagnosis and screening of mitochondrial injuries and pathologies in future clinical studies.

CHAPTER 3: METHODOLOGY

HUMAN BIOPSIES

Human biopsies (M. Vastus Lateralis; Deltoids; n= 35) were taken from The Greenland
Expedition study in 2004 led by Dr. Jorn Helge and Dr. Bengt Saltin from the
Copenhagen Muscle Research Centre. Freshly harvested samples were analyzed on
site and matching samples from the same muscle biopsy were dipped in DMSO
containing solution and frozen in liquid nitrogen at uniform freezing rates (1 °C/min) and
stored at -80°C until 2007.

CRYOPRESERVATION OF MUSCLE TISSUE

For cryopreservation of human muscle biopsies, tissue samples from the Greenland
Expedition were immersed in 100μL of BIOPS relaxing solution. The muscle relaxing
and preservation solution BIOPS contains 10 mM Ca-EGTA buffer, 0.1 μM free calcium,
20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl2, 5.77
mM ATP, 15 mM phosphocreatine, pH 7.1, with the addition of dimethyl sulfoxide
(10mg/mL) and fatty acid free BSA. The tissue was bathed and equilibrated with
cryopreservation solution for 5 seconds. Samples were then frozen in liquid nitrogen at
uniform freezing rates and immediately stored at -80°C.

PREPARATION OF CRYOPRESERVED PERMEABILZED MUSCLE TISSUE

Tissue samples from the Greenland Expedition were taken out of their -80°C storage in
the winter of 2007 and thawed in a water bath at 37° C, blotted with gauze to remove
any residual DMSO on the tissue and immediately transferred to a petri dish containing 1
ml BIOPS solution (10 mM Ca-EGTA buffer, 0.1 μM free calcium, 20 mM imidazole, 20

mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl2, 5.77 mM ATP, 15 mM

phosphocreatine, pH 7.1). Muscle fibers were carefully dissected using forceps until the

tissue obtained a pale pink hue, indicative of optimized fiber dissection and maximal

surface area exposure. The fibers were then agitated and permeabilized for 30 minutes

in a petri containing 2ml BIOPS and 20uL of saponin stock solution (5 mg/ml) final

concentration of 50uL/ml. Saponin, a glycoside of plant origin, is used as a detergent for

permeabilization of muscle fibers. Its hydrophobic steroid core enables it to preferentially

bind and remove cholesterol to selectively partially dissolve the sarcolemma to allow for

permeability of substrate titration and supply to the mitochondrial electron transport

system during respirometric measures. The fiber bundles were transferred to 2ml ice

cold mitochondrial respiration medium (Mir05 containing 1g/l BSA, see below) and

agitated for 10 minutes before proceeding to high resolution respirometry.

ANIMAL BIOPSIES

Adult Wister rats (n=6; male, 250-300g) were used for all control experiments.The soleus

muscle was harvested and placed in a solution of BIOPS for subsequent preparation

and analysis.

SURGICAL PROCEDURES

Male Wister rats were anaesthetized with ketamine (100 mg kg−1 intramuscularly) and

kept warm using a lamp and heating pad. Clippers were used to shave off animal fur

from the ankle region to the upper thigh region. Briefly, the soleus muscle was extracted

by cutting and pulling back the skin around the ankle. The fascia around the hamstring

was removed and an opening between the gastronemius muscle and the hamstring

muscle was created using blunt scissors. The Achilles tendon was cut and the entire

gastronemius muscle was lifted from the bone. The soleus muscle, located on the lateral

side of the gastronemius muscle, was cut, pulled away from the gastronemius muscle, and cut again at the distal end. The soleus muscle was placed in a solution of BIOPS for respirometric analyses.

PREPARATION OF CONTROL AND DMSO MUSCLE TISSUE

Rat soleus was harvested and cut into 2 pieces; one piece was designated as the control sample and the other piece as the DMSO sample. After permeabilization, the control sample was weighed and placed in one of the chambers of the oxygraph; the other sample was quickly dipped into a solution containing DMSO (excess DMSO was blotted off) and placed in the second chamber.
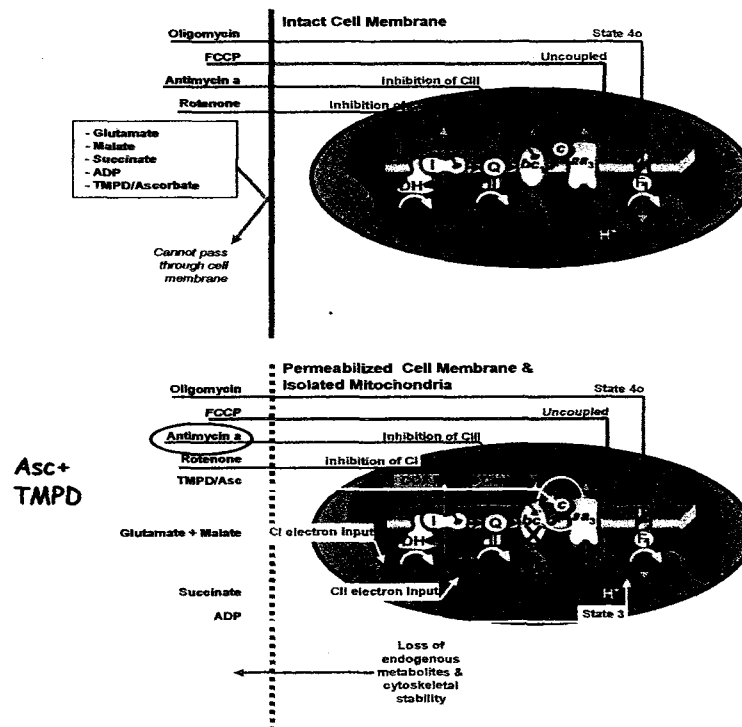

Illustration 4 : Intact Cell Membrane versus Permeabilized Cell Membrane

HIGH RESOLUTION RESPIROMETRY

Respirometric measurements were performed at 37°C using a high resolution Oroboros oxygraph as shown in figure below (Oroboros Instruments, Innsbruck; and Paar, Graz, Austria) in Mir05 (Oroboros, Austria) medium containing 0.5mM EGTA, 3mM $MgCl_2$ $6H_2O$, 60mM K-lactobionate, 20mM taurine, 10mM KH_2PO_4, 20mM HEPES, 110mM sucrose, 1g/ml BSA. Before measurements in the oxygraph, the wet weight of the tissue sample was measured and recorded. The total tissue sample was then separated into two relatively equal pieces and placed in their respective chambers of the oxygraph. The tissue samples were in the range of 2-6 mg. The respiration protocol used for the cryopreserved samples was in the order of malate, ADP, glutamate, succinate, cytochrome c. Cytochrome c test was used to evaluate the integrity of the outer mitochondrial membrane.

RESPIROMETRIC ANALYSIS

Cellular oxygen consumption is measured by high resolution respirometry with the Oroboros Oxygraph-2k in a standard configuration, with 2mL volume of the two chambers, at 37°, and 750rpm stirrer speed. The software DatLab 4 (Oroboros Instruments, Innsbruck, Austria) is used for real-time data acquisition and analysis, including calibration of the OROBO-POS polarographic oxygen sensors and on-line calculation of the time derivative of oxygen concentration. Respiration is automatically corrected for possible contribution of the polarographic oxygen sensors, back diffusion of the total apparent respiration, and chemical oxygen consumption.
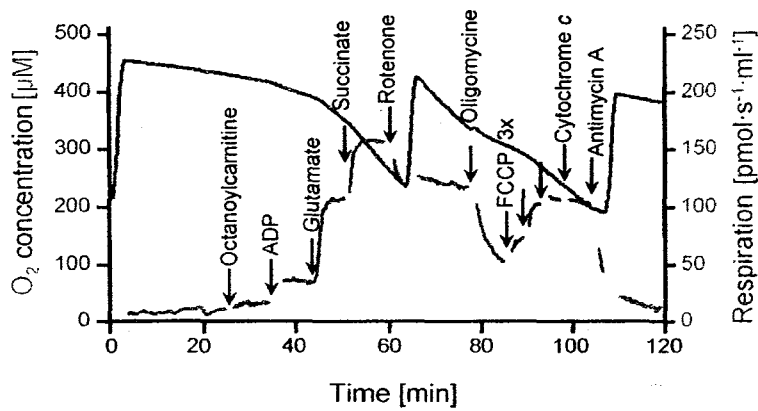
Illustration 5: Example of Oroboros Respirometric Tracing
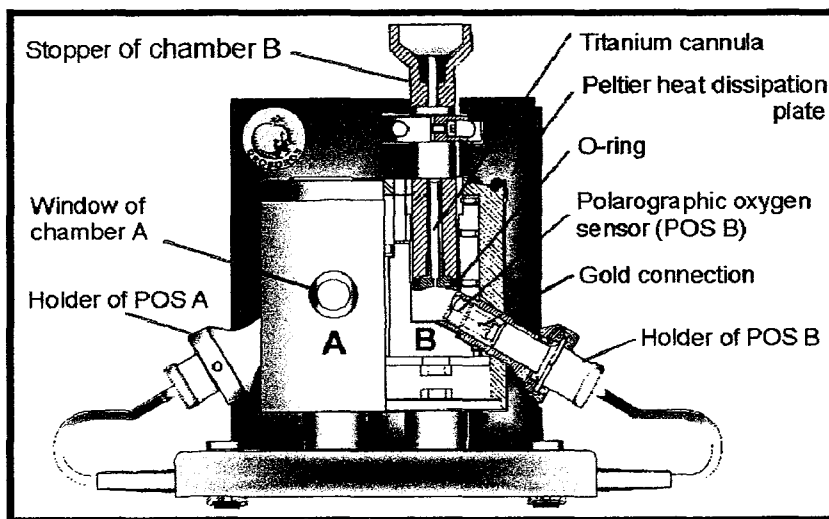
Illustration 6: The OROBOROS® Oxygraph. **A**, window into chamber A; **B**, glass chamber B; chambers are housed in a copper block that is temperature-regulated and insulated.
Polarographic oxygen sensor (POS) with butyl India rubber sleeve for sealing the POS against the glass chamber. Magnets generate a rotating electromagnetic field for driving the PEEK stirrer bars (not shown). Stopper (not shown for chamber A) with titanium cannula and conical titanium plate at the bottom, and adjustable sleeve for setting the chamber volume (Gnaiger, E. 2004)

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INSTRUMENTAL BACKGROUND TEST

Instrumental background tests are routinely carried out for sensor and chamber performance. It is performed using mitochondrial medium (MiR05) without any biological sample and the standard protocol for calibration of the oxygen sensor, yielding a calibration of the O2k chamber performance. Oxygen consumption of the sensor at air saturation is obtained after the chamber has been closed and stabilized for 10 minutes (J1 Mark). The chamber is partially opened by carefully lifting the stopper about 1cm to obtain a gas phase above the medium to which nitrogen gas is injected. Once the oxygen concentration reduced to 45%, the stopper is then tighten and closed in such a manner so as to avoid any bubbles trapped in the chamber. The flux will temporarily undershoot and thereafter stabilize to which the J2 mark is labeled on the section of stable flux (figure below). Two or three more reduced oxygen levels are performed before the linear regression can be calculated and displayed in the DatLab excel file. This linear regression plots the background oxygen flux as a function of oxygen concentration with intercept a and slope b. Instrumental background checks confirm the proper functioning of the respirometer and monitors instrumental characteristics, such as leaks due to defective sensor membrane or the O-ring on the stoppers.
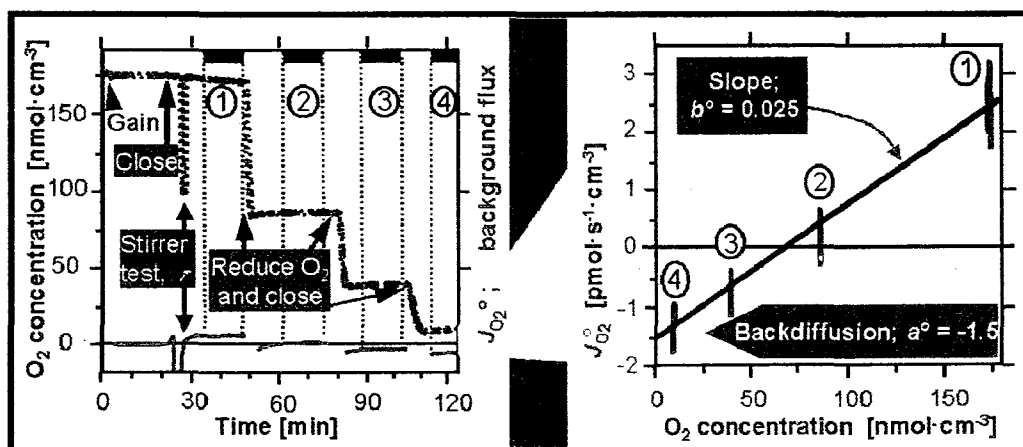
Illustration 7: Instrumental background is automatically performed by the DatLab software. Background measurements provide a control for instrument function (Gnaiger, E. 2004).

CHEMICAL BACKGROUND TEST

Chemical background checks are performed to measure chemical auto-oxidation with the substrates used for determination of the activity of cytochrome c oxidase. Chambers are closed after mitochondrial medium (MiR05) is added to with no biological sample. Once oxygen flux is stabilized, cytochrome c, ascorbate, and TMPD are injected into the chamber. Marks are labeled soon after the initial overshoot of flux and set to regular intervals until the critical oxygen concentration of 40-50µM is attained. Chemical background test results are displayed in DatLab excel file.

STATISTICAL ANALYSIS

Values are presented as means ± SE. Comparisons of oxygen consumption between methods were evaluated by a paired T-test. The level of significance was set at $p < 0.05$.

CHAPTER 4: RESULTS

Effect of Cryopreservation on State 3 Respiration on Freeze-Thaw and Fresh Greenland Samples

ADP-Stimulated state 3 respiration

Saponin-permeabilized mitochondria from freeze-thawed Greenland samples were induced into state 3 respiration with ADP titration in the presence of substrates malate + glutamate and succinate. Table 4.1 summarizes the oxygen consumption rates (pmol/mg/sec) for state 3 in freeze-thaw and fresh Greenland muscle samples. State 3 ADP-driven respiration rates for complex I substrates malate + glutamate and complex I + II with further addition of succinate were significantly lower than that of fresh Greenland muscle tissue (Figure 4.2). State 3 for complex I in the presence of malate + glutamate was lower for freeze-thaw Greenland (10.04 ± 4.23) compared to fresh Greenland samples (24.15 ± 9.51), $p < 0.05$. Similarly, state 3 respiration rates with succinate (figure 4.3) for freeze-thaw Greenland samples was also lower (26.85 ± 9.12) compared to that for fresh Greenland samples (42.62 ± 16.59), $p < 0.05$. These indicate that cryopreservation of muscle tissue significantly reduced mitochondrial respiration with complex I substrates and complex I + II substrates. The percent reduction of state 3 with complex I substrates and complex I+II substrates is seen in figure 4.4; state 3 respiration with substrates malate + glutamate (GM3) was significantly lower (41%) in freeze-thaw Greenland samples than fresh Greenland samples. State 3 respiration with complex I+II substrates and succinate was also significantly lower (63%) in freeze-thaw Greenland samples than fresh Greenland samples.

Table 4.1: State 3 Respiration with various substrates for Freeze-Thaw Greenland and Fresh
Greenland samples

Samples	Freeze-Thaw Greenland Samples			Fresh Greenland Samples		
Samples	GS3	GS3c	GM3	GS3	GS3c	GM3
S# 6VL	31.02	38.72	8.05	43.4	34.2	22.6
S#5Del	23.61	44.72	7.72	44.4	32.4	26.2
S#13VL	10.91	23.55	1.85	68.1	41.5	34
S#6Del	27.65	49.34	8.18	41.7	31.6	25.8
S# 4Del	46.33	69.50	16.44	19.4	14.8	11.6
S#9Del	33.24	55.61	15.7	35.1	29.5	17.9
S#14VL	14.25	35.85	8.88	26.7	12.7	18.6
S#13Del	20.35	56.70	8.68	35.0	17.0	26.4
S#3 Del	21.81	28.33	10.45	28.8	18.9	16
S#12Del	26.16	35.81	8.78	32.4	25.7	14
S#1 Del	21.97	58.53	7.88	51.3	36.6	25.9
S# 1VL	29.85	55.85	6.91	74.7	54.3	44.9
S#8 Del	35.88	55.52	15.48	31.1	25.7	16.8
S#15VL	33.00	71.71	15.64	64.6	40.4	37.6
Mean	26.86*	48.56*	10.05*	42.62	29.66	24.16
S.D.	9.12	14.58	4.24	16.59	11.63	9.51
S.E	2.44	3.90	1.13	4.43	3.11	2.54

State 3 respiration with addition of substrates malate + glutamate GM3; state 3 respiration with additional
titration of substrate succinate, GS3; state 3 respiration in the presence of cytochrome c, GS3c. Oxygen
consumption is in pmol/mg/sec. *Significantly different from fresh Greenland samples ($p<0.05$)

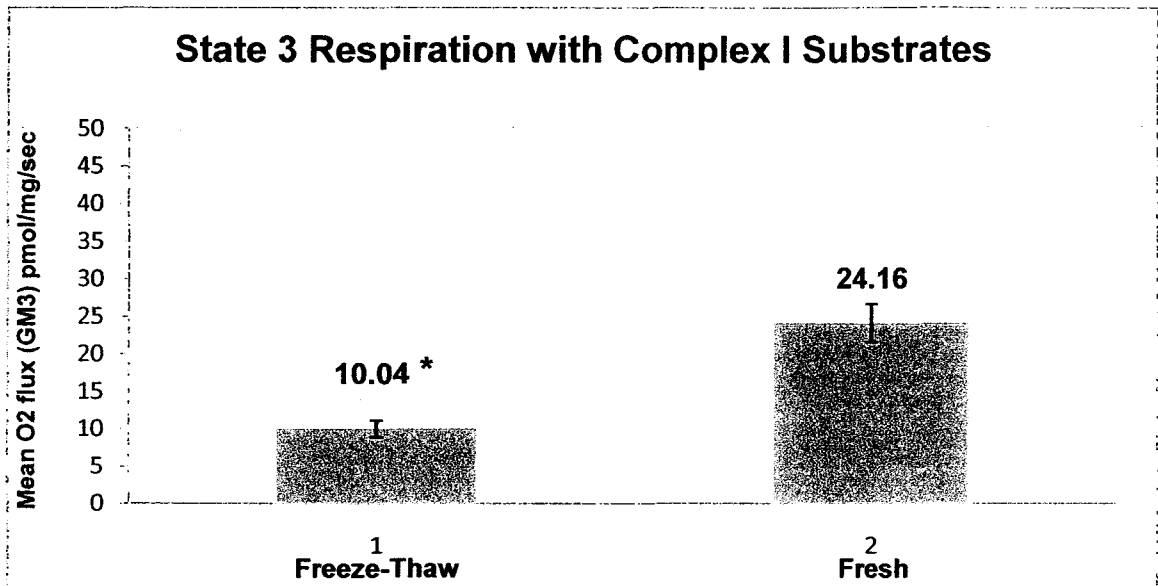Figure 4.2 : State 3 respiration with addition of complex I substrates malate + glutamate, GM3.
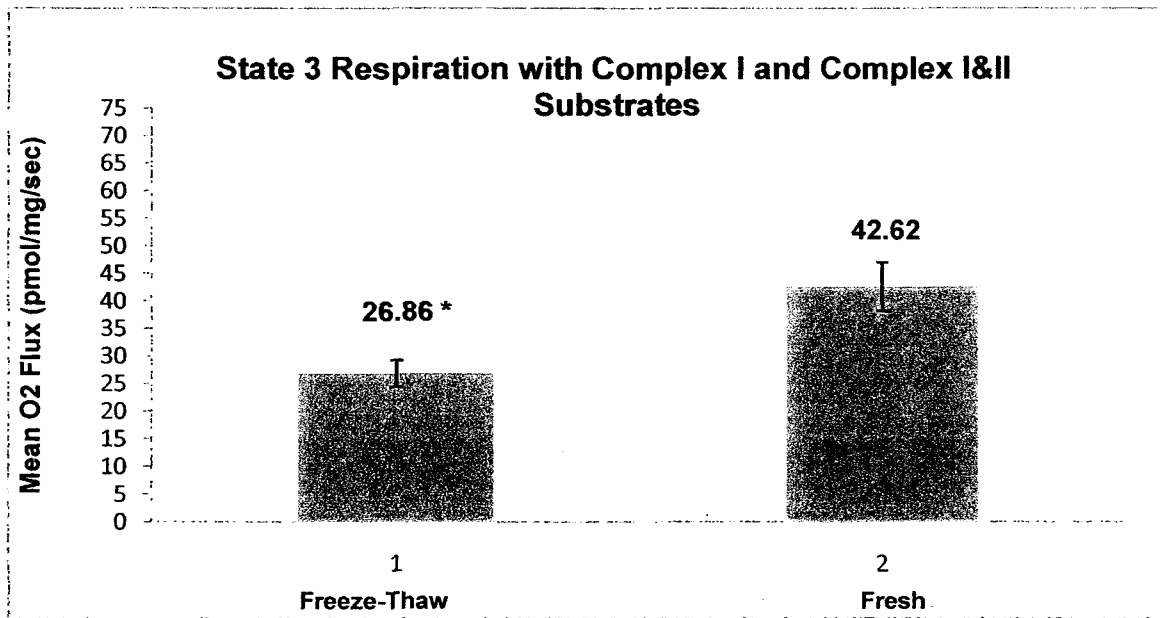*Significantly different from fresh Greenland samples (t-value = 0.0006; p<0.05)

Figure 4.3: State 3 respiration with complex I+II substrates upon addition of succinate (GS3), in freeze-thaw and fresh Greenland samples
* Significantly different from fresh Greenland samples (t-value = 0.01; p<0.05)

% Reduction in State 3

Mean Ratio of O2 Flux

0.42 — GM3 Thawed / GM3 Fresh (Bar 1)
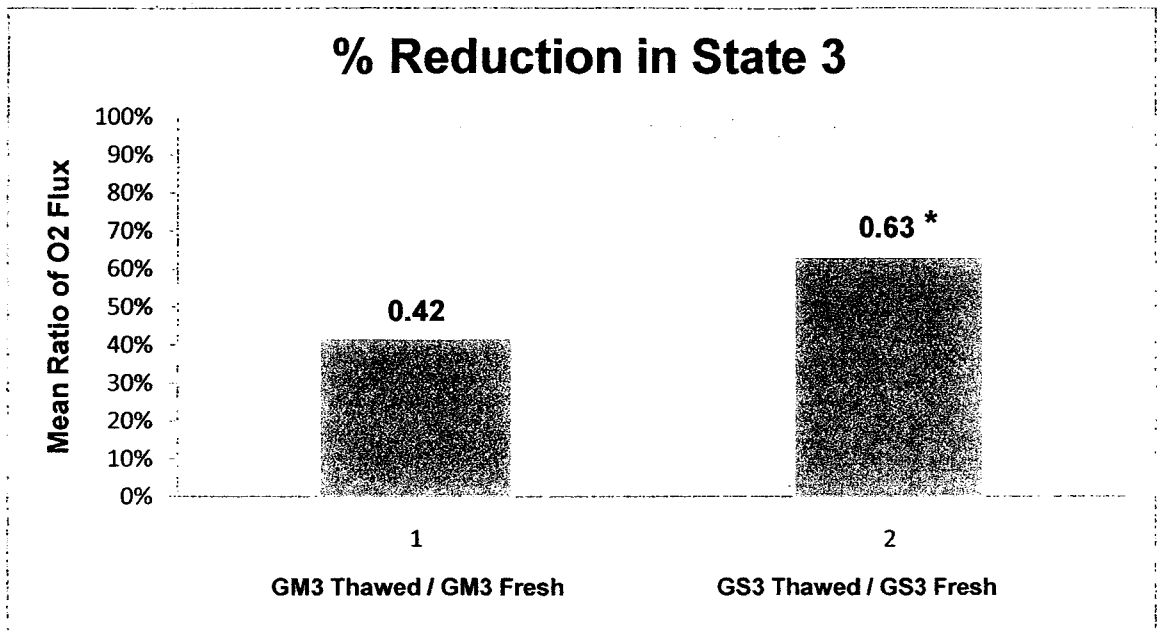
0.63 * — GS3 Thawed / GS3 Fresh (Bar 2)

Figure 4.4: State 3 percent reduction of oxygen consumption in freeze-thaw and fresh Greenland samples. Bar 1 is state GM3 of the freeze-thaw Greenland samples over the GM3 state of fresh Greenland samples; Bar 2 is the same but state GS3. * Significantly different from fresh muscle tissue (t-value = 0.002; $p < 0.05$)

Figure 4.5 (below) shows the ratio of state 3 respiration with substrates malate + glutamate (GM3) and with succinate (GS3). In the freeze-thaw Greenland samples, state 3 respiration with succinate is 2.67 times higher than state 3 with malate + glutamate (Bar 1) which is significantly different from fresh Greenland muscles samples (1.76) shown as bar 2 on figure 4.5.

The magnitude of relative (%) reduction in state 3 respiration with complex I+II substrates was larger than that observed for complex I substrates alone. This is due to the fact that convergent input of electrons through complex I+II are not simply additive but synkinetic or convergent and thus result in an O2 flux rate that is generally larger than the additive sum of each complex due to the stimulation of the intermediate Q-junction that functions as an intermediate electron transfer complex between complex I and 2.

Ratio of State 3 Respiration with Complex I&II and Complex I Substrates
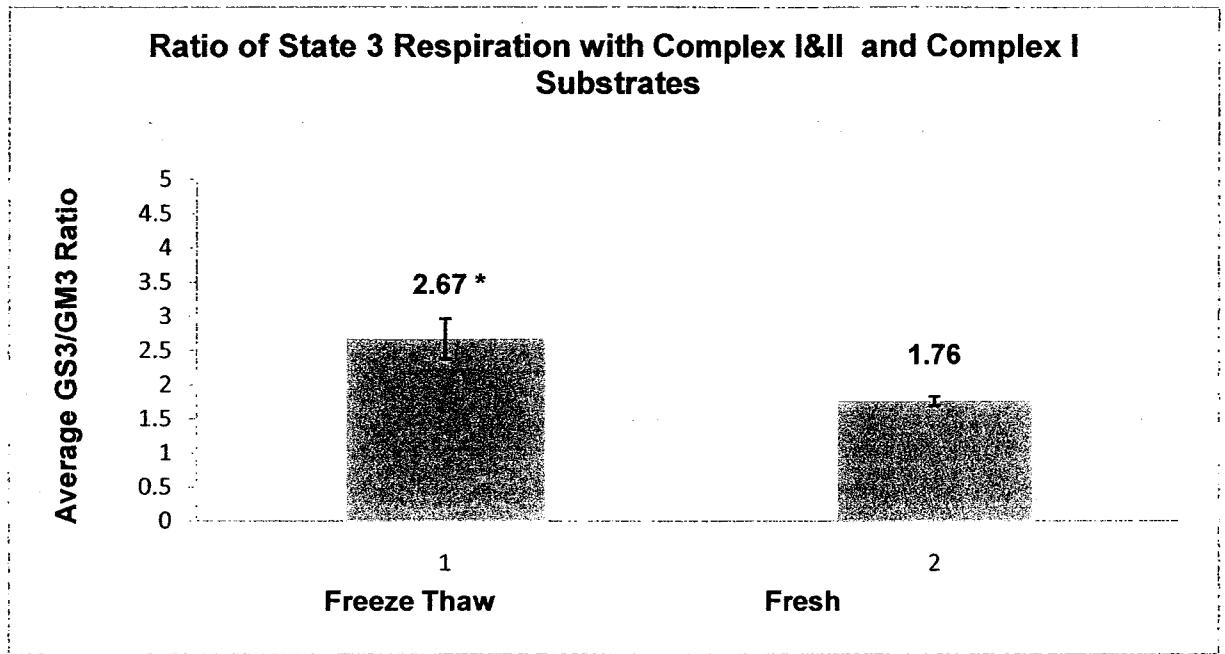
Figure 4.5: Ratio of State 3 respiration with complex I + II substrates (GS3) over state 3 respiration with complex I substrates (GM3) for freeze-thaw Greenland (Bar 1) and fresh Greenland samples (Bar 2).
 * Significantly different from fresh muscle tissue (p<0.05)

Effect of Cryopreservation on Cytochrome c State 3 Respiration in Freeze-Thaw and

Fresh Greenland Samples

Figure 4.6 shows a significant increase in state 3 respiration upon the addition of cytochrome c in freeze-thaw Greenland samples (21.69 ± 14.58) compared to fresh Greenland samples (12.96 ± 7.07) indicating that cryopreservation may cause cytochrome c loss and a potential disruption of the inner mitochondrial membrane. However, with cytochrome c addition by titration as an electron donor to cytochrome c oxidase (COX) which is also known as complex 5 of the respiratory chain and the final electron donor to molecular oxygen, state 3 flux rate with complex I + II, was in fact similar to the value of fresh tissue, (48.54 for freeze-thaw Greenland samples compared

to 55.57 for fresh Greenland samples, figure 4.7). This indicates that some part of the

freezing or thawing process contributes to a loss of mitochondrial state 3 respiration.

The Effect of Cytochrome C
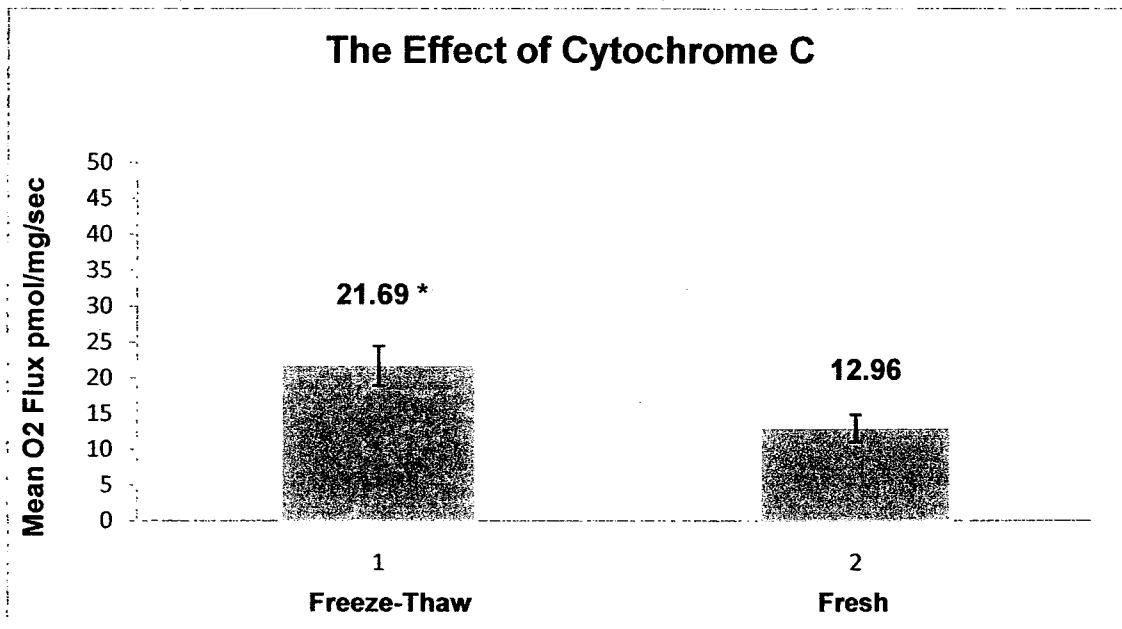
Figure 4.6: Cytochrome C effect in freeze-thaw and fresh Greenland samples (GS3c-GS3)
* Significantly different from fresh muscle tissue (t- value = 0.006; p<0.05

Cytochrome C Effect on Oxygen Consumption
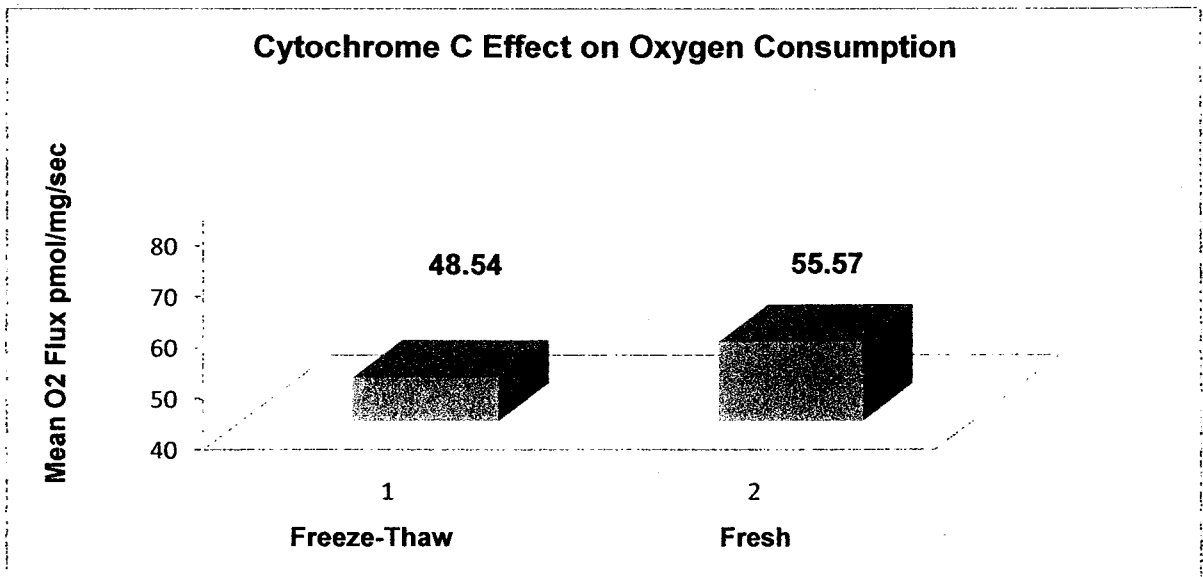
Figure 4.7: State 3 Cytochrome c effect on oxygen consumption of Freeze-thaw and fresh Greenland samples. The addition of cytochrome c to frozen thawed Greenland and fresh Greenland samples results in similar state 3 O2 flux with complex I+II, indicating relative intactness of the major respiratory complexes I – IV between sampling procedures.

Effect of DMSO on Mitochondrial State 3 Respiration in Fresh Rat Soleus Tissue

Means and standard deviations of the sample's descriptive statistics are shown in Table 4.8. Oxygen consumption of samples were originally in pmol/mL/sec, but since each sample weighed different amounts, oxygen consumption of varying states were calculated to obtain comparable values (pmol/mL/sec * 2.0mL / mg tissue = pmol/mg/sec). The soleus muscle from Wister Rats was used as control experiments whereby sections of the same muscle tissue were used for fresh control samples and DMSO samples. The table shows the different oxygen consumption values at state 3 with complex I+II substrates with addition of succinate for the control samples and DMSO samples; the average oxygen consumption at state GS3 for control samples 38.69 ± 11.76 and 34.35 ± 8.62for DMSO samples indicating cryoprotective agent DMSO yields comparable state 3 respiration flux to that of fresh tissue. Figure 4.9 shows that state 3 (ADP-driven) respiration rates for complex I substrates with malate and glutamate and complex I and II with addition of succinate were not significantly lower than that of fresh muscle tissue (34.35 and 38.69 respectively). After the addition of cytochrome c the values of oxygen consumption of control and DMSO samples were 39.17 ± 21.21 and 42.03 ± 12.61 respectively. This indicates that the cryopreservation medium DMSO may have caused some cytochrome c loss and potentially disrupted the inner mitochondrial membrane (figure 5.0). However, given this consideration, the state 3 flux rates are quite comparable.

Table 4.8: Oxygen Consumption at State 3 for Fresh Control Samples and DMSO

Samples

| Sample | Oxygen Consumption pmol / mg/ sec | | | |
	GS3 CTRL	GS3c CTRL	GS3 DMSO	GS3cDMSO
2008-01-17	42.87	46.43	46.96	56.97
2008-01-18	44.58	31.79	33.82	42.64
2008-01-22	26.40	28.22	30.03	29.59
2008-01-23	26.40	26.84	42.48	41.56
2008-03-01a	56.70	22.64	26.83	26.35
2008-03-01b	35.17	79.12	25.95	55.09
Mean	**38.69**	**39.17**	**34.35**	**42.03**
Std. Dev	11.76	21.21	8.62	12.61
SE	4.80	8.66	3.52	5.15

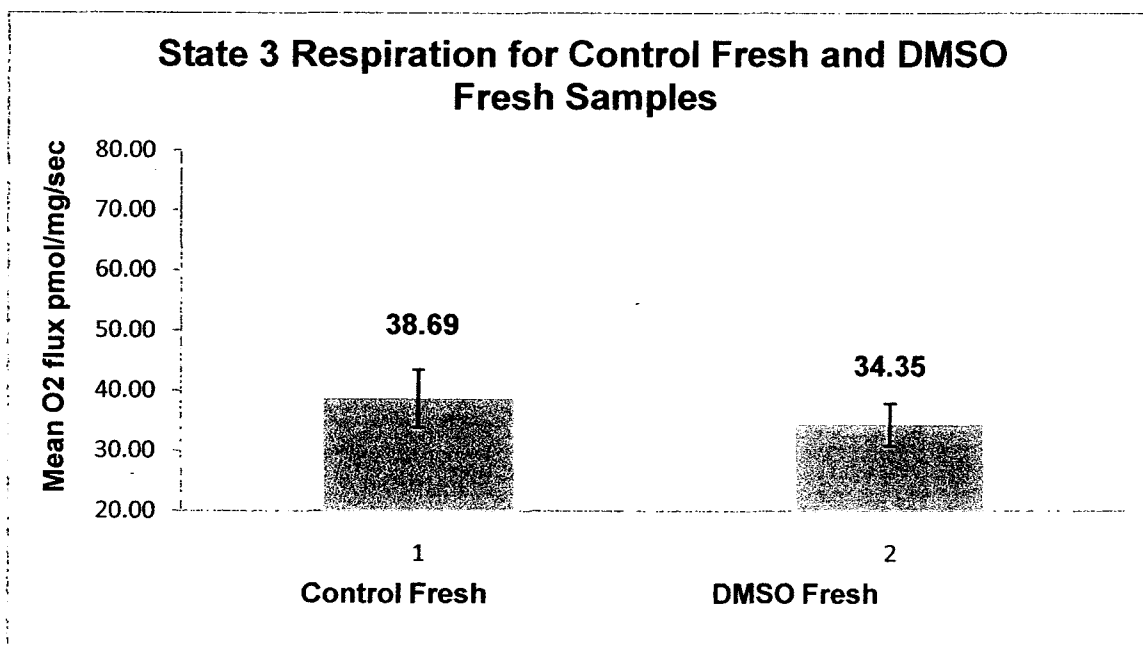Figure 4.9: State 3 respiration rates for complex I substrates malate and glutamate and complex I+ II with substrate succinate, showing no independent effect of the cryopreservation medium DMSO (t-value: 0.53)
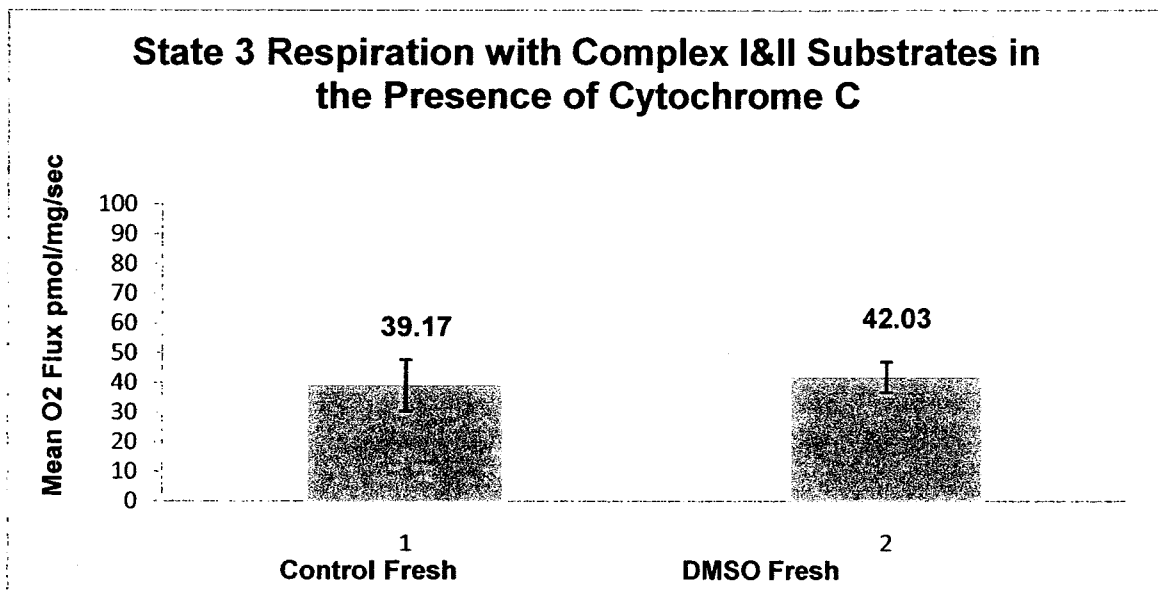
State 3 Respiration with Complex I&II Substrates in the Presence of Cytochrome C

Figure 5.0: State 3 respiration with complex I+II substrates and addition of cytochrome c substrate (t-value: 0.64).

CHAPTER 5: DISCUSSION

The present study examined the functional capacity of mitochondria in cryopreserved muscle biopsy samples and compared oxygen flux rates to freshly harvested muscle from the same biopsy sample. The purpose of the study is to better understand if the process of cryopreservation has an effect on mitochondrial ADP driven respiration rates. Addition of complex I substrates malate + glutamate and complex I+II substrates with addition of succinate on permeabilized mitochondria enables state 3 respiratory rates to be analyzed evaluating the electron transport system and its efficiency of electron cycling to the final acceptor water and the production of ATP. Another goal of this investigation was to determine the effect of cryoagent, DMSO on mitochondrial respiration and compare to fresh animal muscle tissue.

Cryopreservation on ADP-Stimulated Respiration

The results of study show that cryopreserved muscle showed robust coupled state 3 mitochondrial respiration comparable to fresh muscle. However, state 3 ADP-driven respiration rates for complex I substrates with malate and glutamate and complex I and II with addition of succinate were lower than that of fresh muscle tissue. This is in contrast to similar studies where rates of ADP-stimulated respiration of permeabilized fresh fibers with pyruvate + malate after subsequent succinate addition were similar to those for freeze-thaw DMSO muscle fibers (Kuznetsov et al, 2003). Control experiments show no difference between DMSO fresh tissue and fresh tissue alone, indicating that DMSO does not affect mitochondrial respiration rates. Thus, some part of the cryopreservation process reduced mitochondrial respiration with complex I and complex I+II substrates.

The relative reduction in state 3 respiration with complex I+II substrates was larger than that observed for complex I substrate alone. This may be due to the orientation of complex I and II and their roles in the electron transport system (ETS). Electrons pass from complex I to carrier coenzyme Q that is embedded in the membrane. From coenzyme Q electrons are passed to complex III to complete cellular respiration. Complex II, succinate dehydrogenase, is a separate starting point and is not part of the NADH-complex I pathway (Figure 6.0 below). Therefore, electrons from two separate entry points in the ETS will have to channel through the coenzyme Q junction before passing through to complex III. Thus, high O_2 flux rate is indicative of a stimulated Q-junction from convergent input of electrons through complex I+II (Figure 6.0 below). A consequence of having separate pathways for entry of electrons is that an ETS inhibitor can affect one part of the pathway without interfering with the other part. Respiration can still occur depending on choice of substrate.
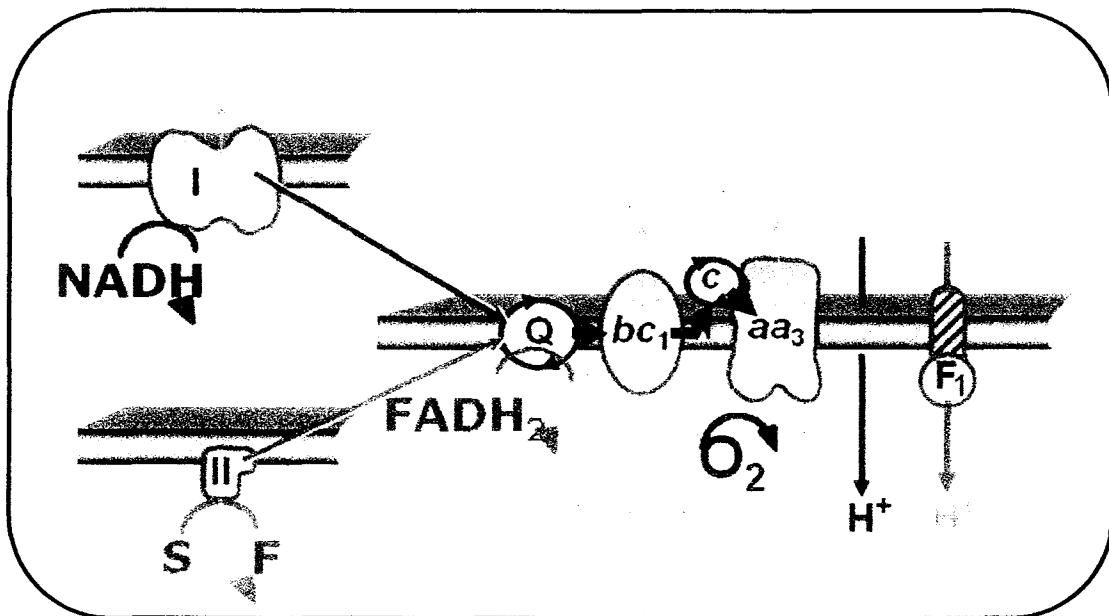

Illustration 8: Convergent input of electrons from complex I and II exerts an additive effect and results in an O2 flux rate that is generally larger than each individual complex.

Cytochrome C Effect from Cryopreservation

The significant increase in state 3 respiration upon addition of cytochrome c indicates that a depletion of a substantial part of the endogenous cytochrome c and a possible rupture of the outer mitochondrial membrane during the freeze-thawing with DMSO. However, addition of cytochrome c by titration as an electron donor to cytochrome c oxidase, state 3 flux rates with complex I+II was similar to the flux rate of fresh tissue. Given that respiratory flux is similar in freeze-thawed and fresh tissue with addition of cytochrome c, then cryopreservation did not necessarily disrupt the collective activity of the major inner mitochondrial membrane, but only induced a significantly greater cytochrome c loss. Cytochrome c acts as a floating electron transfer complex, which is stabilized by the inner mitochondrial transmembrane protein Cardiolipin, which is known to be particulately delicate in well established short term interventions such as cardiac and liver transplantation. Thus, the permeabilization of muscle tissues are not likely to be detrimental or deleterious to the integrity of mitochondria in the present study, since the control experiments in fresh tissue usually show consistently an approximate 10% reduction effect on state 3 respiration. This suggests that the combined reduction of state 3 respiration between freeze thawed and fresh tissue is due primarily to cytochrome c loss during freeze-thaw procedures that disrupt the mitochondrial membranes. Moreover, we know that DMSO has somewhat of a protective role in tissues, therefore, it is not the cryo-agent used but rather the freezing and storing of tissues that result in unknown damage to the mitochondria and that DMSO prevents most but not all damage. This is similar to Kuznetsov (2003) study in which they report that freezing mitochondria without DMSO destroys their structural integrity and functional ability and significantly higher cytochrome c levels are maintained in mitochondria cryopreserved with addition of DMSO evident by respirometric measurements.

Cytochrome c to COX · Cyt c transfers electrons to the COX enzyme Complex... But is not bound and is mobile
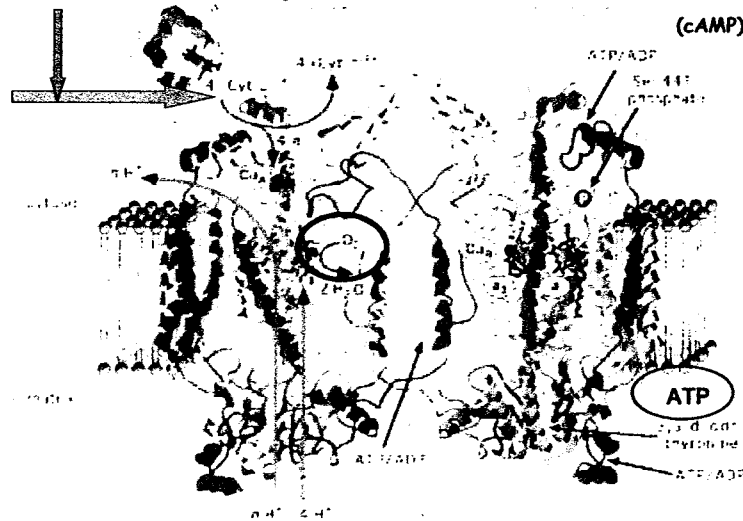
(cAMP)

ATP

Illustration 9: Cytochrome C molecule imbedded in lipid bilayer. Bold circle inset shows the binding site and final acceptor, water and formation of ATP.

Taken together, mitochondria of freeze-thaw muscle fibers demonstrate good response to various substrates of complex I and complex I+II of the electron transport system. The data indicates that a cytochrome c effect from cryopreservation; however addition of cytochrome c as a substrate in respirometric analyses shows comparable oxygen flux rates to fresh muscle tissue. Data presented suggests that an optimal cryopreservation protocol would likely preserve functional mitochondria to be used for routine studies in mitochondrial physiology and clinical applications. Some limitations of the study include a small sample size; the collection and analysis of human biopsies from a previous study were not conducted with current experiments and therefore data may be misinterpreted, not recorded or missing; the control experiments were performed using animal biopsies and may show slight variation compared to human biopsies; no standard cryopreservation and/or freezing and thawing protocol is available as the literature seems to be lacking in the area of cryopreservation of mitochondria.

FUTURE APPLICATIONS

Advances in cryopreservation technology have led to methods that allow low-temperature maintenance of muscle tissue without affecting the structural and functional integrity of isolated mitochondria. Successful optimization of cryopreservation protocols will permit long term preservation of muscle biopsies and reduce time limitations when performing basic mitochondrial analysis in metabolic and bioenergetic studies. Cryopreservation methods will allow the possibility to ship samples to a variety of geographical locations, allow the opportunity to create a database consisting of various samples with well defined pathologies and integrate various research centers for additional analysis and comparative studies. Moreover, cryopreservation of mitochondria will ensure long term stability but will not correct any mitochondrial defects already present in tissues allowing better diagnosis and broader screening of mitochondrial abnormalities in various diseases. Furthermore, the technique will allow for long term storage of muscle tissue and may serve as controls in diagnostic procedures. In summary, cryopreservation will significantly expand the range of molecular and metabolic studies that can be performed on human and animal tissue without the constraint of mitochondrial longevity *ex vivo*, increase the time frame needed for various functional analyses and ensure reproducible results and continuity in interdisciplinary and inter-laboratory studies.

CONCLUSION

In summary, the findings from the current study suggest that cryopreservation of mitochondria for batch storage and subsequent use in substrate-linked oxygen uptake studies remains unsatisfactory at the present. However, the data herein indicate that it is more likely a cytochrome c effect from cryopreservation, and that apart from this effect the functional integrity of mitochondria remains intact. Further investigation is warranted to determine what effect cryopreservation has on the different components involved in mitochondrial respiration, especially on stabilizing cytochrome c. Furthermore, future inquiries are needed to quantitate the specific cryoprotective qualities of several different cryoprotective agents, specify the underlying mechanisms of the freeze-thaw phases, determine tissue specificity, and develop a "gold" standard by which the method can be used and optimized for various tissues. Nevertheless, the method offers the potential opportunity to create a database of various samples with well-defined pathologies accessible for comparative studies or studies to be performed in various geographical locations. Furthermore, the technique will be fundamental in the diagnosis and screening of mitochondrial injuries and pathologies in future clinical studies

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