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BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

RAPID ISOLATION AND PURIFICATION OF MITOCHONDRIA FOR TRANSPLANTATION USING TISSUE DISSOCIATION AND DIFFERENTIAL FILTRATION

by

JANINE PREBLE

B.A., Saint Anselm College, 2010

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2014

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Approved by

First Reader ____

Gwynneth Offner, Ph.D.
Associate Professor of Medicine, Director M.A. Medical Sciences Program

Second Reader ____

James McCully, Ph.D.
Cardiothoracic Surgery Director, Beth Israel Deaconess Medical Center

ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. McCully and Dr. Pacak for their help in conducting this research.

This study was supported by National Heart, Lung, and Blood Institute Grant HL-103542, and The Anesthesia Research Distinguished Trailblazer Award to CAP.

RAPID ISOLATION AND PURIFICATION OF MITOCHONDRIA FOR TRANSPLANTATION USING TISSUE DISSOCIATION AND DIFFERNTIAL FILTRATION

JANINE PREBLE

ABSTRACT

Researchers have identified several methods for treating acute myocardial infarction (AMI) patients affected by ischemia and reperfusion injury. Some of these therapies include thrombolysis, balloon angioplasty, and coronary arterial bypass graft (CAGB). This lab has previously demonstrated that transplantation of mitochondria into the ischemic zone of a rabbit heart during reperfusion significantly improved recovery as compared to current techniques. In order for this therapy to be translated into the clinic a rapid isolation method for producing highly pure and functional mitochondria will be required.

Previously described mitochondrial isolation methods using differential centrifugation and/or Ficoll gradient centrifugation require 60 to 100 minutes to complete. Herein, a method for rapid isolation of mitochondria from mammalian tissue biopsies is described. In this protocol, manual homogenization is replaced

with the tissue dissociator's standardized homogenization cycle. This allows for uniform and consistent homogenization of tissue that is not easily achieved with manual homogenization. Following tissue dissociation, the homogenate is filtered through nylon mesh filters which eliminates repetitive centrifugation steps. Mitochondrial isolation time is less than 30 minutes compared to 60-100 minutes using alternative methods. This isolation protocol yields approximately 2×10^{10} viable and respiration competent mitochondria from 0.18 ± 0.04 g (wet weight) tissue sample.

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ABBREVIATIONS

AMI……………………………………………………..…….Acute Myocardial Infarction

ATP…………………………………………………….…Adenosine Tri Phosphate

BCA……………………………………………………….……Bicinchoninic Acid

BSA…………………………………………………..……Bovine Serum Albumin

CAGB………………………………..................Coronary Arterial Bypass Graft

DNA………………………………………………………………Deoxyribonucleic Acid

mPTP……………………………………Mitochondrial Permeability Transition Pore

NIH…………………………………………………………National Institutes of Health

OXPHOS…………………………………………………Oxidative Phosphorylation

PBS………………………………………………………Phosphate Buffered Saline

PCI…………………………………………… Percutaneous Coronary Intervention

RCI……………………………………………...………Respiratory Control Index

ROS……………………………………………………….Reactive Oxygen Species

TEM………………………………………………..Transmission Electron Microscopy

INTRODUCTION

History of Mitochondria

Mitochondria were first identified back in the 18th century[1]. Since this initial discovery, scientists have worked to gain a greater understanding of mitochondrial form and function. Cytologists discovered the ultrastructure of the mitochondria. Biochemists identified mitochondria as the location for cellular respiration. Molecular biologists discovered that mitochondria have their own deoxyribonucleic acid (DNA). And, physiologists uncovered the mechanisms involved in mitochondrial functions which include adenosine triphosphate (ATP) production, apoptosis and calcium signaling [1].

Mitochondria are found in every cell of the body except red blood cells and are responsible for producing more than 90% of the body's energy[2-6]. When mitochondria do not function properly, energy production is significantly reduced which can lead to cell injury, cell death, and sometimes system failure[2]. Consequently, mitochondria have recently become an important focus for researchers exploring the role of mitochondrial dysfunction in disease. These studies have been performed using mitochondria isolated from numerous tissues and cells derived from both healthy samples as well as disease models.

Mitochondria and Disease

Mitochondrial mutations often result in oxidative phosphorylation (OXPHOS) dysfunction. Because OXPHOS is vital for cell survival, these mutations result in cellular dysfunction and in some cases cell death[7]. These mutations are thought to play a role in several diseases. Organs like the brain and heart suffer immensely from mitochondrial mutations because they rely heavily on oxidative function[5]. Researchers have recently been exploring the role of mitochondrial dysfunction in neurological disorders including Parkinson's, Huntington's and Alzheimer's disease[8,9]. In addition, the role of mitochondrial dysfunction in cancer is being explored[10-12]. Other researchers are investigating the detrimental effects mitochondrial dysfunction can have on the heart. This lab and others have explored the role of mitochondrial damage in the heart during ischemia and reperfusion[13-17].

Mitochondrial Damage Caused by Ischemia and Reperfusion

Reperfusion is a major cause of myocardial cell damage following AMI. Reperfusion induces a number of cellular abnormalities including a large influx of calcium, reduced ATP production, an increase in reactive oxygen species (ROS), and a loss of membrane phospholipids[18]. Due to inflammation, leucocyte migration, and cellular swelling, hemorrhage is common in the myocardium after reperfusion[19]. In the most current research, particular attention has been placed

on the damage suffered by mitochondria. Mitochondrial insults that result from reperfusion include a change in volume, a change in oxidative phosphorylation, and a change in calcium content[16]. Additionally, ROS contribute to mitochondrial injury by inducing the opening of the mitochondrial permeability transition pore (mPTP). The opening of this pore leads to cell death and contributes to infarct size in patients suffering from AMI[18]. These mitochondrial impairments are thought to be a major reason many AMI patients have a poor recovery[16].

Clinical Treatments

Researchers have identified many methods for treating AMI patients affected by reperfusion injury. Some of these therapies include thrombolysis, balloon angioplasty, and CAGB[19]. One ischemic post conditioning therapy entails interrupting reperfusion with a slightly inflated angioplasty balloon that obstructs the coronary artery. This therapy has had success in reducing the size of the infarct. This success, however, is limited to patients fitting certain criteria[20]. A different study suggested that primary percutaneous coronary intervention (PCI) was the best method to reduce reperfusion injury and improve patient recovery[21]. Although many of these therapies have been useful in reducing injury caused by myocardial reperfusion, researchers acknowledge that there are still many obstacles that need to be overcome including the large cost for developing new

therapies, and the transition of the utilization of treatments used in animal models to humans[22].

Despite these difficulties, investigators are continuing to propose novel techniques because current AMI treatments have not eliminated all patient complications. Some of these more recent techniques involve transplantation of pharmacologic therapeutic agents into the infarcted zone of the myocardium in an attempt to regenerate damaged tissue[16]. However, once again the success is limited. One particular approach being pursued by this lab is transplantation of healthy mitochondria into the myocardium just prior to reperfusion[17]. In order for investigators to fully explore this technique's therapeutic potential in humans, a clinically oriented mitochondrial isolation procedure will be required.

Isolation of Mitochondria

The earliest published accounts of mitochondrial isolation date to the 1940s[1,23-25]. The first documented attempt demonstrated mitochondrial isolation by grinding liver tissue in a mortar followed by centrifugation in a salt solution at low speed[1,23]. Later, other groups expanded upon the original procedure and demonstrated tissue fractionation based on differential centrifugation[1,24-25]. These early methods formed the basis of current techniques which often incorporate homogenization, and/or differential centrifugation[26-32]. The number of homogenization and centrifugation steps varies among protocols. These

repetitive steps increase the time for mitochondrial isolation and ultimately reduce viability. In addition, manual homogenization can cause mitochondrial damage and inconsistent results if not properly controlled[27,33]. Thus, a more standardized and rapid mitochondrial isolation method would greatly benefit researchers exploring the potential of mitochondrial therapy in the clinic.

Objectives

To greater meet the requirements for clinical utilization a rapid mitochondrial isolation procedure has been developed that utilizes standardized tissue dissociation followed by differential filtration. Recently, homogenization and differential centrifugation was used to isolate mitochondria for transplantation into myocardial tissue[16,17]. This lengthy isolation procedure required approximately 90 minutes and the clinical applicability of this method was therefore limited. To allow for acute therapeutic use in clinical and surgical treatment a rapid mitochondrial isolation procedure has been developed that can be performed in less than 30 minutes.

The major benefits of this protocol are that standardized tissue dissociation allows for uniform and consistent homogenization of tissue that is not easily achieved with manual homogenization. In addition, the use of differential filtration in place of differential centrifugation eliminates time-

consuming and repetitive centrifugation steps allowing for more rapid isolation of highly purified, viable and respiration competent mitochondria.

It is hypothesized that the ability to isolate viable and respiration competent mitochondria in less than 30 minutes will allow for clinical applicability. This isolation protocol has potential for use in coronary arterial bypass grafting surgery (CABG) and other therapeutic procedures.

METHODS

Preparation of Solutions

Several stock solutions were prepared including: 1 M K-HEPES (pH 7.2), 0.5 M K-EGTA (pH 8), 1 M KH_2PO_4, and 1 M $MgCl_2$.

10X Phosphate Buffered Saline (PBS) was prepared by weighing 80g of NaCl, 2g of KCl, 14.4g of Na_2HPO_4, and 2.4g of KH_2PO_4 into 1 L double distilled H_2O (pH 7.4).

1X PBS was prepared by transferring 100 mL of 10X PBS into 900 mL of double distilled H_2O.

Homogenizing buffer (pH 7.2) containing 300 mM sucrose, 10 mM K-HEPES, and 1 mM K-EGTA was prepared. The homogenizing buffer was stored at 4 ^{o}C.

Respiration buffer containing 250 mM sucrose, 2 mM $KH_2PO_{4,}$ 10 mM $MgCl_{2,}$ 20 mM K^+-HEPES buffer (pH 7.2) and 0.5 mM K^+-EGTA (pH 8.0) was prepared. The respiration buffer was stored at 4 ^{o}C.

Subtilisin A stock solution was prepared by weighing 4 mg of Subtilisin A into a 1.5 mL microfuge tube which was stored at -20 ^{o}C until use. Immediately prior to the mitochondrial isolation, Subtilisin A was dissolved in 1 mL of homogenizing buffer. Bovine Serum Albumin (BSA) stock solution was prepared

by weighing 20 mg of BSA into a 1.5 mL microfuge tube which was stored at -20 ^{o}C until use. Immediately prior to the mitochondrial isolation, BSA was dissolved in 1 mL of homogenizing buffer.

Animals

All experimentation was approved by the Institutional Animal Care and Use Committee at Harvard Medical School and conformed to the National Institutes of Health (NIH) guidelines regulating the care and use of laboratory animals (NIH Publication No. 5377-3, 1996). All research was performed in accordance with the *American Physiological Society's Guiding Principles in the Care and Use of Animals*.

Mitochondrial Isolation

Liver and skeletal muscle was excised from a female Lewis rat and each was stored in a 50 mL conical tube filled with 1X PBS at 4 ^{o}C. Tissue samples were removed to a pre-chilled petri dish and trimmed of any fat and connective tissue using sterilized forceps and scissors. The tissue was washed in ice cold homogenizing buffer 3 X.

Next, six mitochondrial samples were prepared from each tissue using a 6mm biopsy punch (Miltex, York, PA). Each sample consisted of two 6mm biopsy punches. The samples were transferred to a gentleMACS dissociation C tube

(Miltenyl Bitoec, Auburn, CA) containing 5 mL of ice cold homogenizing buffer. Samples were then homogenized via the gentleMacs tissue dissociator's (Miltenyl Biotec, Auburn, CA) standardized 60 second cycle. The dissociation C tube was removed to an ice bucket and 250 µL of Subtilisin A stock solution was added to the homogenate and mixed by inversion. The C tube was incubated on ice for ten minutes.

A 40 µm filter (BD, Franklin Lakes, NJ) was placed onto a 50 mL conical tube and pre-wet with homogenizing buffer. The buffer was discarded from the tube and the homogenate was filtered into a new 50 mL conical tube. At this point 250 uL of BSA stock solution was added to the filtrate and the homogenate was mixed by inversion. Next, a 40 µm filter was placed onto a 50 mL conical tube and pre-wet with homogenizing buffer. The buffer was discarded from the tube and the homogenate was filtered into the 50 mL conical tube. This step was repeated using a 10 µm filter (pluriSelect, San Diego, CA).

The filtrate was then transferred to two pre-chilled 1.5 mL eppendorf tubes and centrifuged at 9000 x g for ten minutes at 4 ^{o}C. The supernatant was removed and the pellets were combined and re-suspended in about 1 mL of ice-cold respiration buffer. An outline of the procedural steps in the isolation of mitochondria using tissue dissociation and differential filtration is shown in Figure 1. Total procedural time was less than 30 minutes.

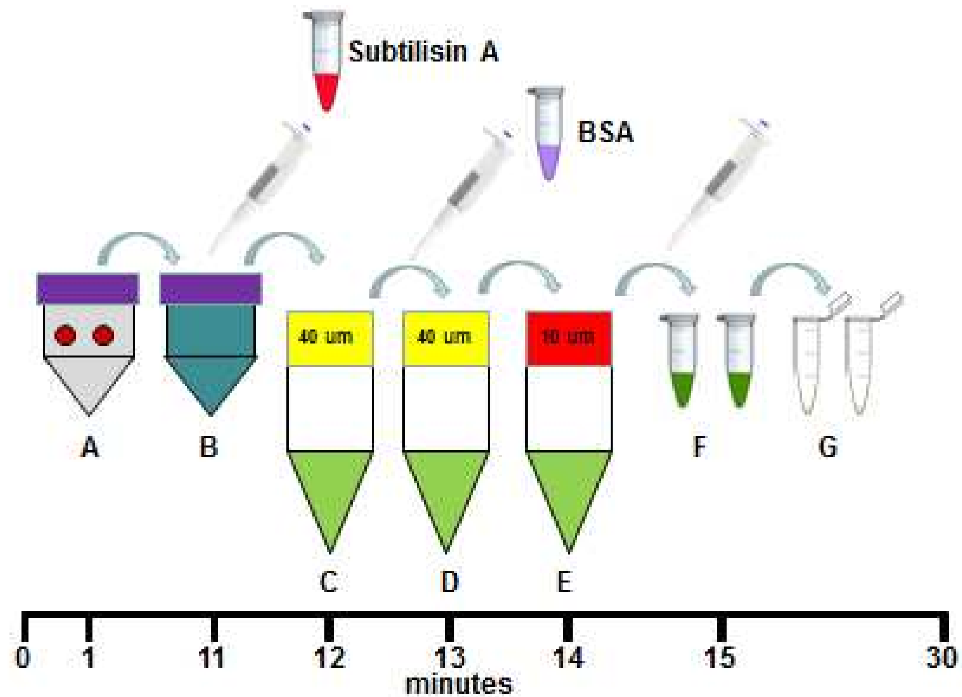

Figure 1 –Schema for the isolation of mitochondria using tissue dissociation and differential filtration. A: Transfer two 6 mm biopsy sample punches to 5 mL of Homogenizing Buffer in a dissociation C tube and homogenize the samples using the tissue dissociator's 1 minute homogenization program. B: Add 250 µL Subtilisin A stock solution to the homogenate in the dissociation C tube and incubate on ice for 10 minutes. C: Filter the homogenate through a pre-wetted 40 µm mesh filter in a 50 mL conical centrifuge tube on ice and then add 250 µL of BSA stock solution to the filtrate. D: Re-filter the filtrate through a new pre-wetted 40 µm mesh filter in a 50 mL conical centrifuge on ice. E: Re-filter the filtrate through a new pre-wetted 10 µm mesh filter in a 50 mL conical centrifuge tube on ice. F: Transfer the filtrate to 1.5 mL microfuge tubes and centrifuge at 9000 x g for 10 minutes at 40C. G: Remove the supernatant and re-suspend and combine the mitochondrial pellets in 1 mL Respiration Buffer. Total procedure time is less than 30 minutes.

ATP Luminescence Assay

In order to determine the metabolic activity of isolated mitochondria, an ATP luminescence assay was performed using the ATPlite Luminescence 1000 assay system (Perkin Elmer, Billerica, MA). All reagents and standards were supplied in the assay kit. The assay was performed according to the manufacturer's instructions. A summary of the procedure is as follows: Kit reagents were equilibrated to room temperature. A 10 mM ATP stock solution was prepared by dissolving a lyophilized ATP pellet in 1,170 µL of double distilled water. The ATP standard stock solutions and prepared mitochondrial samples were stored on ice. 5 mL of substrate buffer solution was added to a vial of lyophilized substrate solution. This solution was mixed gently and placed in the dark. Next, 100 µL of Respiration Buffer was added to all wells of a black, opaque bottom, 96 well plate (VWR). Then, 10 µL of mitochondria from each of the six prepared samples was added to the appropriate wells. Note: Samples were plated in triplicate. The top row of the plate was left empty for the standards and 10 uL of Respiration Buffer was added to the last three wells for the negative control as indicated on the plate map (Figure 2). Next, 50 µL of mammalian cell lysis solution was added to all wells, including standards and controls. The plate was incubated at 37 ˚C for 5 minutes on an orbital shaker (Heidolf, Germany) at 125 rpm. During the incubation ATP standards in concentrations of 0.1 mM, 0.05 mM, 0.01 mM, 0.005 mM, 0.001 mM, and 0.0001 mM ATP were prepared from

the 10 mM ATP stock solution. Standards were stored on ice. Following the incubation, 10 uL of ATP standards were added to corresponding wells as indicated on the plate map. Note: Standards were performed in duplicate (Figure 2). Then, 50 μL of the reconstituted substrate solution was added to each well. Again, the plate was incubated at 37 ˚C on the orbital shaker for 5 minutes at 125 rpm.

The luminescence of each well was recorded using Gen5 1.11 (BioTek) software on a computer linked with a Synergy H4 multi-mode microplate reader (BioTek, Winooski, VT). Settings were adjusted as follows: Read type: Endpoint, Integration Time: 0:01.00 MM:SS.ss, Filter Sets: 1, Emission: Hole, Optics Position: Top, Sensitivity: 100, Top Probe Vertical Offset: 1.00 mm. When the plate was ready to be analyzed, it was placed into the tray with well A1 in the upper left corner. A standard curve was calculated from which the unknown mitochondrial ATP content was determined. Note: Higher values correlate with increased ATP levels and higher metabolic activity.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.1 mM ATP standard (10 µL)	0.1 mM ATP standard (10 µL)	0.05 mM ATP standard (10 µL)	0.05 mM ATP standard (10 µL)	0.01 mM ATP standard (10 µL)	0.01 mM ATP standard (10 µL)	0.005 mM ATP standard (10 µL)	0.005 mM ATP standard (10 µL)	0.001 mM ATP standard (10 µL)	0.001 mM ATP standard (10 µL)	0.0001 mM ATP standard (10 µL)	0.0001 mM ATP standard (10 µL)
B	Mito Sample 1 (10 µL)	Mito Sample 1 (10 µL)	Mito Sample 1 (10 µL)	Mito Sample 2 (10 µL)	Mito Sample 2 (10 µL)	Mito Sample 2 (10 µL)	Mito Sample 3 (10 µL)	Mito Sample 3 (10 µL)	Mito Sample 3 (10 µL)	Mito Sample 4 (10 µL)	Mito Sample 4 (10 µL)	Mito Sample 4 (10 µL)
C	Mito Sample 5 (10 µL)	Mito Sample 5 (10 µL)	Mito Sample 5 (10 µL)	Mito Sample 6 (10 µL)	Mito Sample 6 (10 µL)	Mito Sample 6 (10 µL)	Respiration Buffer (10 µL)	Respiration Buffer (10 µL)	Respiration Buffer (10 µL)			
D												
E												
F												
G												
H												
I												

Figure 2- Plate map for ATP assay. This plate map illustrates how to set up standards (A1-A12), mitochondria samples (B1-C6), and negative controls (C7-C9) for the ATP assay. During the assay, 100 µL of Respiration Buffer, 50 µL of mammalian cell lysis solution and 50 µL of reconstituted substrate solution are added to all wells (A1-C9).

Assessment of Mitochondrial Yield

Mitochondria preparations were quantified using a Bicinchoninic Acid (BCA) protein assay as described by Smith *et al*[34]. Mitochondria preparations were counted with the Beckman Coulter Counter 4 (Beckman Coulter, Danvers, MA). To allow for comparison mitochondrial number was also determined by hemocytometer (Fisher Scientific, Pittsburg, PA).

Assessment of Mitochondrial Viability

Mitochondria were stained with MitoTracker Red CMXros (Invitrogen, Carlsbad, CA) as previously described[16,17]. Mitochondrial purity was determined by transmission electron microscopy (TEM) as previously described [16,17]. Mitochondrial respiration was assessed using a Clark type electrode as previously described[16,17].

RESULTS

Tissue samples obtained using a 6 mm biopsy punch weighed 0.18 ± 0.04 g (wet weight). The number of mitochondria isolated as determined by particle size counting was $2.4 \times 10^{10} \pm 0.1 \times 10^{10}$ mitochondria for skeletal muscle and $2.75 \times 10^{10} \pm 0.1 \times 10^{10}$ mitochondria for liver preparations (Figure 3A). Mitochondrial number was underestimated as determined by hemocytometer as $0.11 \times 10^{10} \pm 0.04 \times 10^{10}$ mitochondria for skeletal muscle and $0.34 \times 10^{10} \pm 0.09 \times 10^{10}$ mitochondria for liver preparations (Figure 3A).

Mitochondrial diameter as determined by size based particle counter is shown in Figure 3B. The representative tracing shows the isolated mitochondria are localized under one peak with mean diameter of 0.38 ± 0.17 μm in agreement with previous reports[25].

Mitochondrial protein/ g (wet weight) starting tissue as determined by BCA assay was 4.8 ± 2.9 mg/g (wet weight) and 7.3 ± 3.5 mg/g (wet weight) for skeletal muscle and liver samples respectively (Figure 3C).

Mitochondrial purity was determined by TEM and is shown in Figure 3D. Mitochondria are shown to be electron dense with less than 0.01% being fractured or damaged. Contamination by non-mitochondrial particles was less than 0.001%.

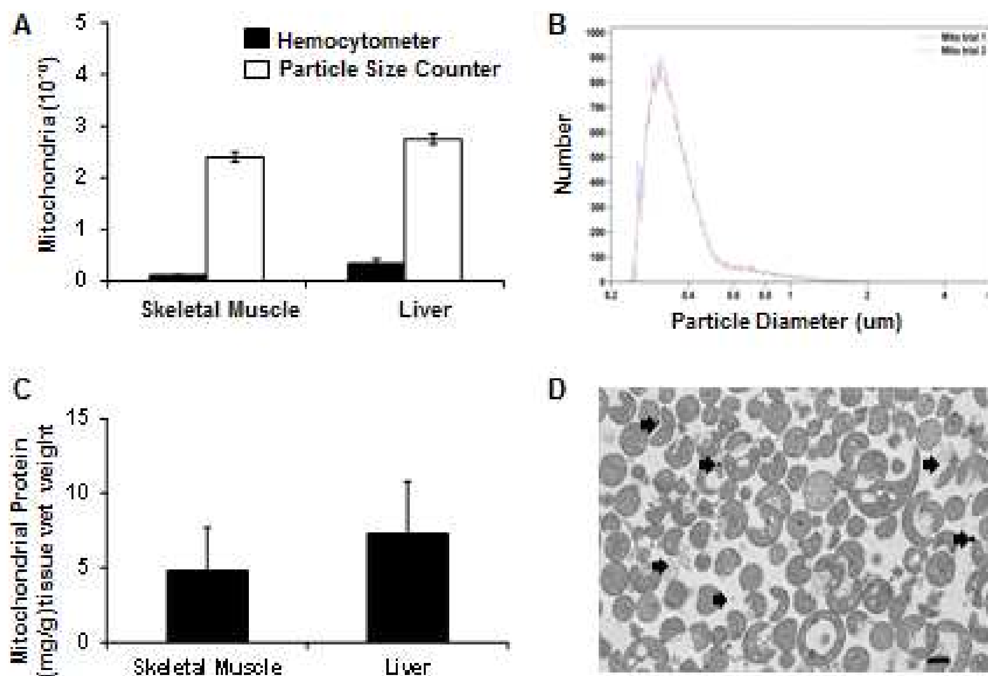

Figure 3 – Mitochondrial yield and purity. A: Hemocytometer and particle size counter mitochondria number isolated from 0.18 ± 0.04 g tissue (wet weight) for skeletal muscle and liver. B: Mitochondrial size distribution as detected by particle size counter. C: Mitochondrial protein mg/g tissue wet weight for skeletal muscle and liver. D: Transmission electron microscopy image of isolated mitochondria. Scale bar is 100 nm. Arrows indicate possible contamination by non mitochondrial particles and damaged mitochondria.

Mitochondrial viability was determined by MitoTracker Red as previously described[16,17]. Results indicate that the isolated mitochondria are intact and able to maintain membrane potential (Figure 4A-C).

ATP was determined using a luminescent assay kit. A plate map for the ATP assay is shown in Figure 2. ATP standards were plated in duplicate. Mitochondrial samples and negative controls were plated in triplicate. ATP content was 10.67± 4.38 nmol/mg mitochondrial protein and 14.83 ± 4.36 nmol/mg mitochondrial protein for skeletal muscle and liver samples respectively (Figure 4D).

Mitochondrial oxygen consumption rate was 178 ± 17 nM O_2/min/mg mitochondrial protein for skeletal muscle and 176 ± 23 nM O_2/min/mg mitochondrial protein for liver preparations. Respiratory control index (RCI) values were 2.45 ± 0.34 and 2.67 ± 0.17 for skeletal muscle and liver sample preparations respectively (Figure 4E). These results are similar to those reported in previous studies using manual homogenization and differential centrifugation to isolate mitochondria[16-17].

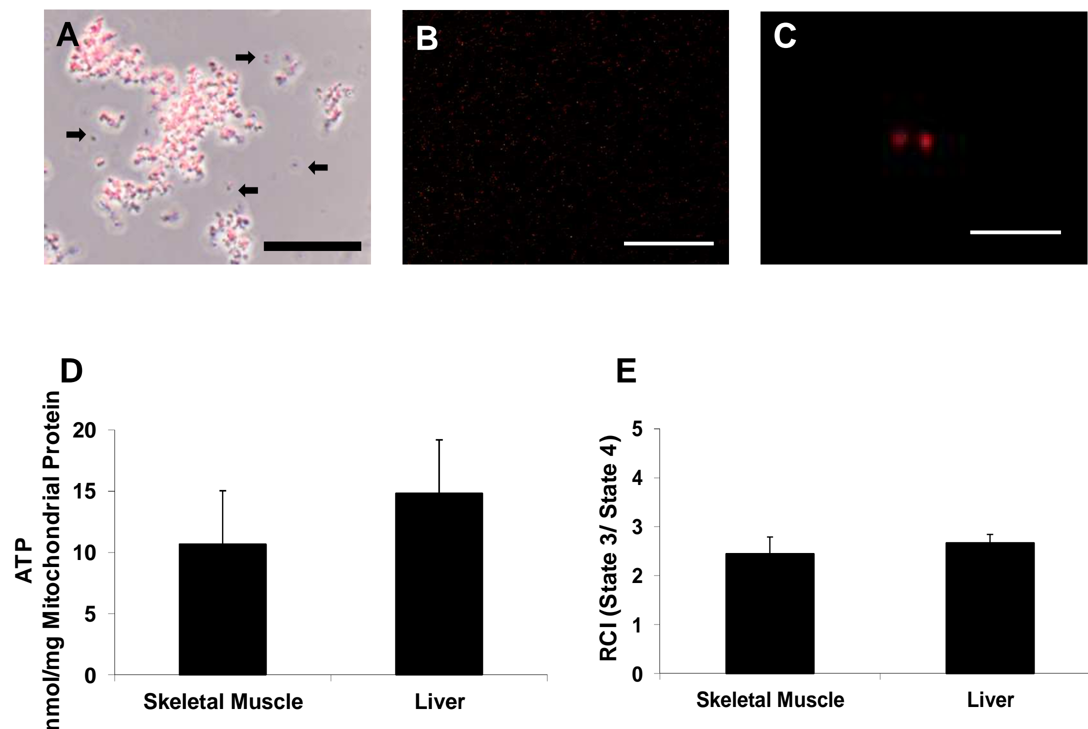

Figure 4 – Mitochondrial viability. Representative photomicrographs of isolated mitochondria A: under phase contrast illumination and B and C: under fluorescence, with mitochondria labeled with MitoTracker Red CMXRos. Scale bars are 25 µm (A,B) and 5 µm (C). These images indicate that mitochondria maintained membrane potential. D: ATP content nmol/mg mitochondrial protein as determined by ATP assay and E: RCI (state 3 / state 4) as determined by Clark electrode.

DISCUSSION

To successfully isolate mitochondria using this protocol it is essential to keep all solutions and tissue samples on ice to preserve mitochondrial viability. Even when maintained on ice, isolated mitochondria will exhibit a decrease in functional activity over time[35]. It is recommended that all solutions and additions be pre-prepared. Subtilisin A is pre-weighed and stored in 4 mg aliquots in 1.5 mL microfuge tubes and stored at -20 ^{o}C. Similarly BSA is pre-weighed and stored in 20 mg aliquots in 1.5 mL microfuge tubes that can be stored at -20 ^{o}C . Just prior to use the tubes are removed from -20 ^{o}C and dissolved in 1 mL of homogenizing buffer for use in the mitochondrial isolation procedure.

Two biopsy punches from mammalian tissue obtained using a 6 mm biopsy punch provide sufficient mitochondria for use in clinical and surgical procedures for therapeutic interventions[16-17]. In past studies, tissue samples of a similar size provided enough mitochondria to improve recovery in an ischemic rabbit heart[16-17]. To estimate mitochondrial number this lab has used two methods, hemocytometer and particle size counting. The use of particle size counting is recommended. The Beckman Coulter Counter 4 uses electrical impedance to measure the volume of particles passing through an aperture of a defined size. The particle size counter is costly but provides accurate and reliable estimates and is user independent. Mitochondrial number can be measured more cheaply using a hemocytometer. This lab's studies have demonstrated that this

method provides variable estimates that are approximately one order of magnitude less than that obtained using a particle size counter. It has also been noted that counts obtained by hemocytometer are highly dependent on the user. To ensure consistent estimates all counts using a hemocytometer should be performed by one person.

One advantage of this mitochondrial isolation technique is that extra filtration steps are utilized to ensure the isolated mitochondria are of high quality and purity. Digital images of mitochondria preparations that were filtered through two 40 μm filters only and preparations that were filtered through two 40 μm filters and one 10 μm filter were taken (Figure 5). All mitochondria preparations appeared relatively clean but preparations filtered an additional time through a 10 μm filter were considerably cleaner. Although filtration is highly effective it can be difficult to filter the homogenate through the filters because cellular debris can clog up the pores. It is helpful to slowly pipette the homogenate through the first filter into a new conical tube. If the filter becomes blocked with cellular debris, change the filter. For the next filtration simply pour the filtrate through a new 40 μm filter into a new conical tube.

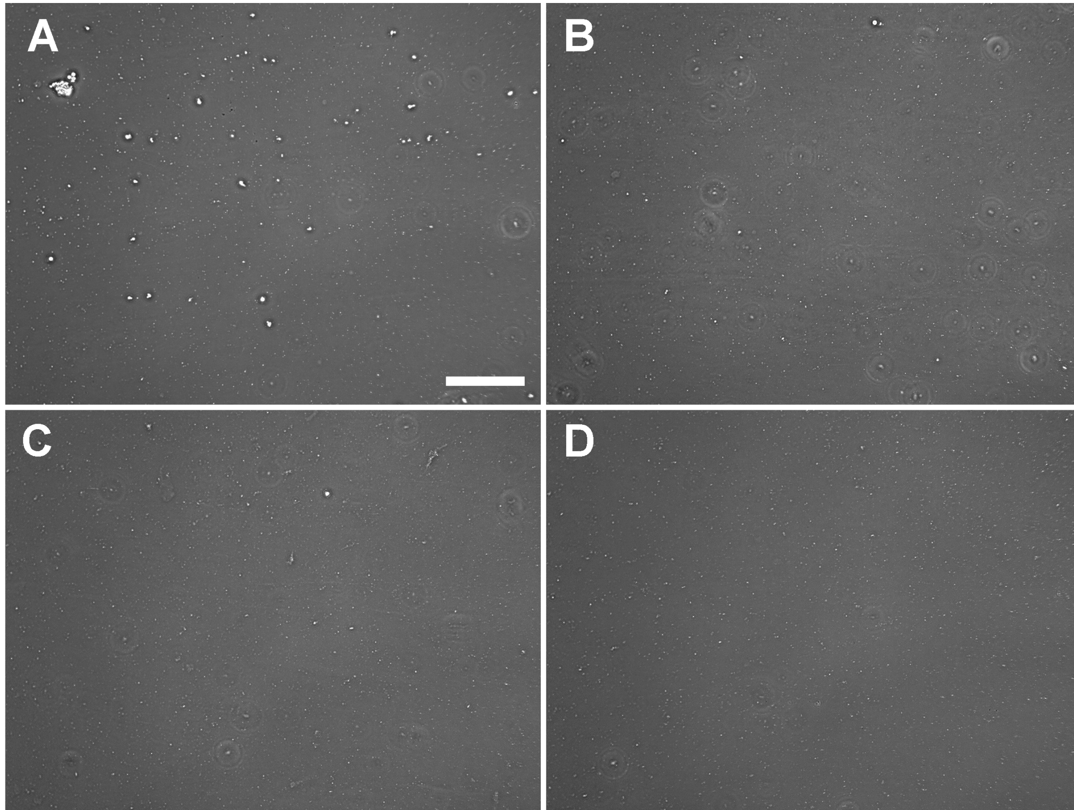

Figure 5- Digital Microscope 20x phase images of mitochondria preparations. A) Liver mitochondria preparation filtered through two 40 µm filters. B) Liver mitochondria preparation filtered through two 40 µm filters plus one 10 µm filter. C) Skeletal muscle mitochondria preparation filtered through two 40 µm filters. D) Skeletal muscle mitochondria preparation filtered through two 40 µm filters plus one 10 µm filter. Scale bar = 50 µm.

This lab has found ATP assay kits to be useful for determining mitochondrial function. The kit supplies all necessary reagents and provides a simple and fast method for determining the metabolic activity of isolated mitochondria. The ATP assay provides similar results as those obtained using a Clark type electrode and therefore is compatible with previous data analysis[36-37].

This method for rapid isolation of mitochondria using tissue dissociation and differential filtration provides an isolation time frame compatible for clinical and surgical therapeutic intervention[16-17]. A major advantage of this mitochondrial isolation protocol is that it allows for isolation of a high yield of viable, respiration competent mitochondria free of contamination in less than 30 minutes. Differential filtration in place of differential centrifugation significantly reduces procedure time. Other protocols incorporate several centrifugation steps with overall isolation time being 60 minutes to 100 minutes[16-17,30-33]. Another advantage of this protocol is that tissue homogenization is standardized. The commercial tissue dissociator provides a standardized cycle and yields consistent and reproducible results. This is in contrast to manual homogenization that is subject to user variability and inconsistency.

Because tissue dissociation is achieved in a closed system, the sample preparation has a better chance of remaining sterile than a sample prepared using manual homogenization. Sterilizing all solutions prior to performing the

isolation and using only sterilized materials, equipment, tools, *etc*. makes this procedure even more clinically relevant.

Transplanted mitochondria have great potential to improve AMI patient recovery by providing cardioprotection and enhancing post ischemic function. Transplanted mitochondria are taken up by cells as early as two hours post transplantation[16-17]. Once inside the cell, transplanted mitochondria augment ATP levels and maintain viability[16-17]. Another significant finding of this lab is that transplanted mitochondria do not induce an immune response. Post mitochondrial injection, serum samples can be analyzed using ELISA, indirect immunofluorescence and a multiplex (42-plex) assay [16,17]. Transplantation of mitochondria does not induce inflammation, an auto-immune response or upregulation of chemokines/cytokines[16-17]. This finding makes the possibility of using transplanted mitochondria as a treatment for AMI patients even more probable.

In addition, transplantation of mitochondria into the ischemic zone of the myocardium results in increased ATP production, upregulation of proteins associated with mitochondrial complexes, and upregulation of cytokines[16-17]. Upregulated cytokines are those associated with angiogenesis, immunomodulation, arteriogenesis, prevention of apoptosis, progenitor cell

migration, and enhanced cell salvage. These cytokines improve post ischemic recovery by decreasing necrosis and apoptosis[16-17].

This technique can be used in the clinic alone or in conjunction with current therapies to improve patient outcomes. Although all prior experimentation was done in animals, this lab is hopeful that this treatment will have the same effects in humans.

LIST OF JOURNAL ABBREVIATIONS

Adv Exp Med Biol	Advances in Experimental Medicine and Biology
Am J Physiol Heart Circ Physiol	American Journal of Physiology - Heart and Circulatory Physiology
Anal Biochem	Analytical Biochemistry
Anat Rec	Anatomical Record
Ann N Y Acad Sci	Annals of the New York Academy of Sciences
Ann Thorac Surg	The Annals of Thoracic Surgery
Antioxid Redox Signal	Antioxidants & Redox Signaling
Biochem J.	The Biochemical Journal
Cell Death and Differentiation	Cell Death Differ
Curr Protoc Cell Biol	Current Protocols in Cell Biology
J Biol Chem	The Journal of Biological Chemistry
J Cell Biology	The Journal of Cell Biology
J Exp Med	The Journal of Experimental Medicine
J Pharmacol Exp Ther	The Journal of Pharmacology and Experimental Therapeutics
J Vis Exp	Journal of Visualized Experiments: JoVE
Klin Onkol	Klinick□ Onkologie
Methods Cell Biol	Methods in Cell Biology

Methods Enzymol	Methods in Enzymology
Methods Mol Biol	Methods in Molecular Biology
Nat Protoc	Nature Protocols
Nat Rev Cancer	Nature Reviews: Cancer.
Nat Rev Neurol	Nature Reviews: Neurology.
New Engl J Med	The New England Journal of Medicine

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