Effective Semi-Automated Extraction of Intact Mitochondria from Solid Tissues Using Gentle Mechanical Homogenization and Pressure Cycling Technology.

PBI Pressure BioSciences Inc.

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Abstract

Impaired mitochondrial function has been linked to many diseases, such as stroke, heart disease, cancer, Type II diabetes and Parkinson's disease. Mitochondria-enriched preparations are needed for proteomic and metabolomic studies that may provide crucial insights into tissue-specific mitochondrial function and dysfunction, and answer fundamental questions of cellular energetics and oxidative stress.

Extraction of mitochondria from whole tissue samples is typically performed using Potter-Elvehjem homogenizers or similar labor-intensive manual disruption methods [1] that require extensive operator experience, and often result in damage to fragile organelles and high sample-to-sample variability. Here we describe a semi-automated method (Figure 1) that uses a novel, gentle mechanical homogenizer (The PCT Shredder) and hydrostatic pressure to release intact mitochondria from fresh rat tissues with minimal hands-on time. Pressure Cycling Technology (PCT)-based tissue homogenization is conducted under controlled thermodynamic conditions (time, temperature and pressure) leading to more reproducible results. The quality of mitochondria preparations was characterized by electron microscopy, 2D PAGE, Western blotting and respiration assays. Our data demonstrate that mitochondria extracted by the PCT sample preparation system (PCT-SPS) are intact, functional, and exhibit a protein profile comparable to control samples isolated using a conventional Potter-Elvehjem homogenizer. The resulting mitochondria-enriched samples were also subjected to trypsin digestion followed by nanoLC-MS/MS analysis on an LTQ-Orbitrap. Proteomic profiles of mitochondria samples prepared using the novel extraction technique were compared to those extracted using a conventional manual method to demonstrate the purity of mitochondrial preparations extracted using the novel PCT-SPS method.

Introduction

PCT destabilizes molecular interactions by rapidly and repeatedly raising and lowering pressure in the reaction vessel from levels of up to 45,000 psi to atmospheric. At pressures in the 10,000-20,000 psi range, PCT can be used to gently lyse cells and release intact organelles, such as mitochondria. This method has been shown to be relatively gentle, and has already been used to isolate mitochondria from cell culture [2].

Figure 1A. Workflow for Mitochondria Isolation

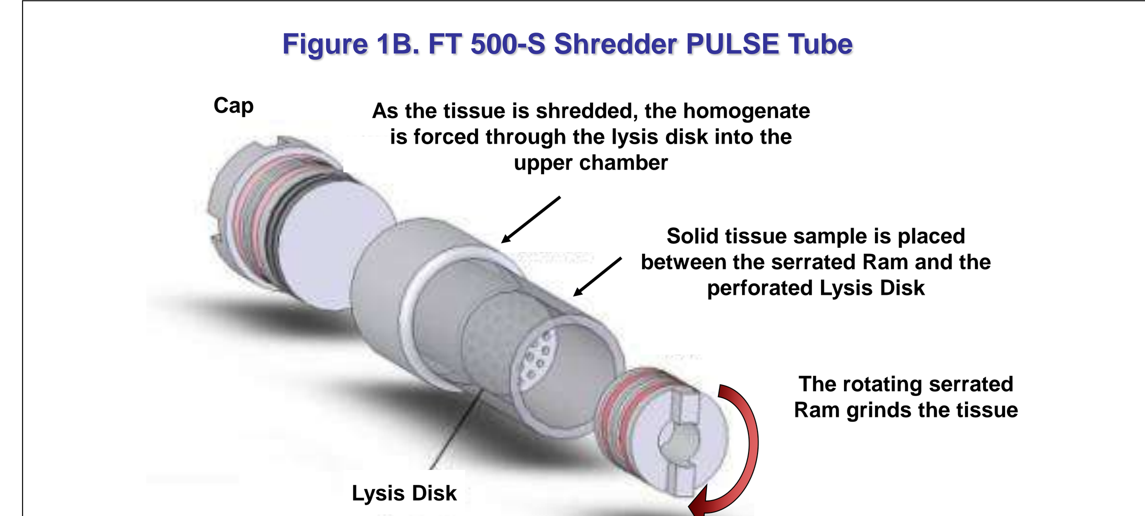

Figure 1B. FT 500-S Shredder PULSE Tube

Methods

Muscle mitochondria-enriched fractions were prepared from fresh rat gastrocnemius muscle per manufacturer's instructions using the PBI Mitochondria Isolation Kit: Rat Muscle. Lung mitochondria-enriched fractions were prepared from frozen/thawed rat lung tissue as described in the PBI Mitochondria Isolation Kit: Rat Lung. All necessary reagents were provided with the kits. Samples were shredded for 10 seconds in FT 500-S Shredder PULSE Tubes, and were either pressurized at 10,000, 20,000 or 30,000 psi for 5 cycles at 4˚C, or were not subjected to pressurization ("Shredder alone"). Mitochondria were extracted from fresh rat kidney tissue without shredding, by pressure cycling at 10,000 psi followed by differential centrifugation [3], as is shown in Figure 1A

Control samples were processed by hand in a glass/Teflon homogenizer. In order to generate consistent controls and eliminate user-to-user variability, all control samples produced by manual homogenization were prepared by the same experienced individual.

For all methods, differential centrifugation was carried out at 4˚C by first spinning the whole tissue homogenate at 1,000g for 8 minutes to pellet tissue debris, intact cells and nuclei. The supernatant was then centrifuged at 14,000g for 8 minutes to pellet the mitochondria-enriched fraction, which was subsequently washed several times to reduce carryover of soluble cytosolic proteins.

Respiration was measured by an Oroboros Oxygraph-2k system.

For MS analysis, mitochondria-enriched samples were lysed by sonication. The lysates were reduced and alkylated prior to digestion. PCT-enhanced trypsin digestion was performed at 20,000 psi at 37˚C (300 cycles, 25 sec at high pressure, 10 sec. at atmospheric pressure per cycle). Each sample was digested in triplicate. Samples were subjected to nanoLC-MS/MS analysis on an LTQ-Orbitrap. Only proteins with an identification confidence level of ≥90% were considered in this report.

Results

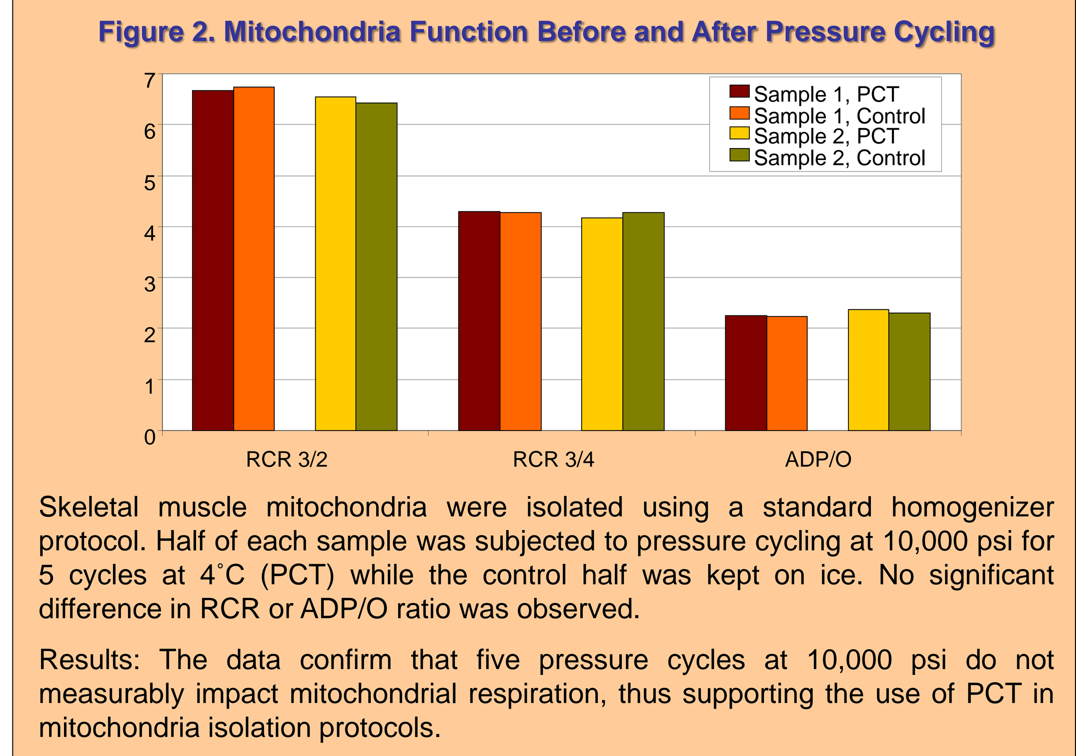

Figure 2. Mitochondria Function Before and After Pressure Cycling

Skeletal muscle mitochondria were isolated using a standard homogenizer protocol. Half of each sample was subjected to pressure cycling at 10,000 psi for 5 cycles at 4˚C (PCT) while the control half was kept on ice. No significant difference in RCR or ADP/O ratio was observed.

Results: The data confirm that five pressure cycles at 10,000 psi do not measurably impact mitochondrial respiration, thus supporting the use of PCT in mitochondria isolation protocols.

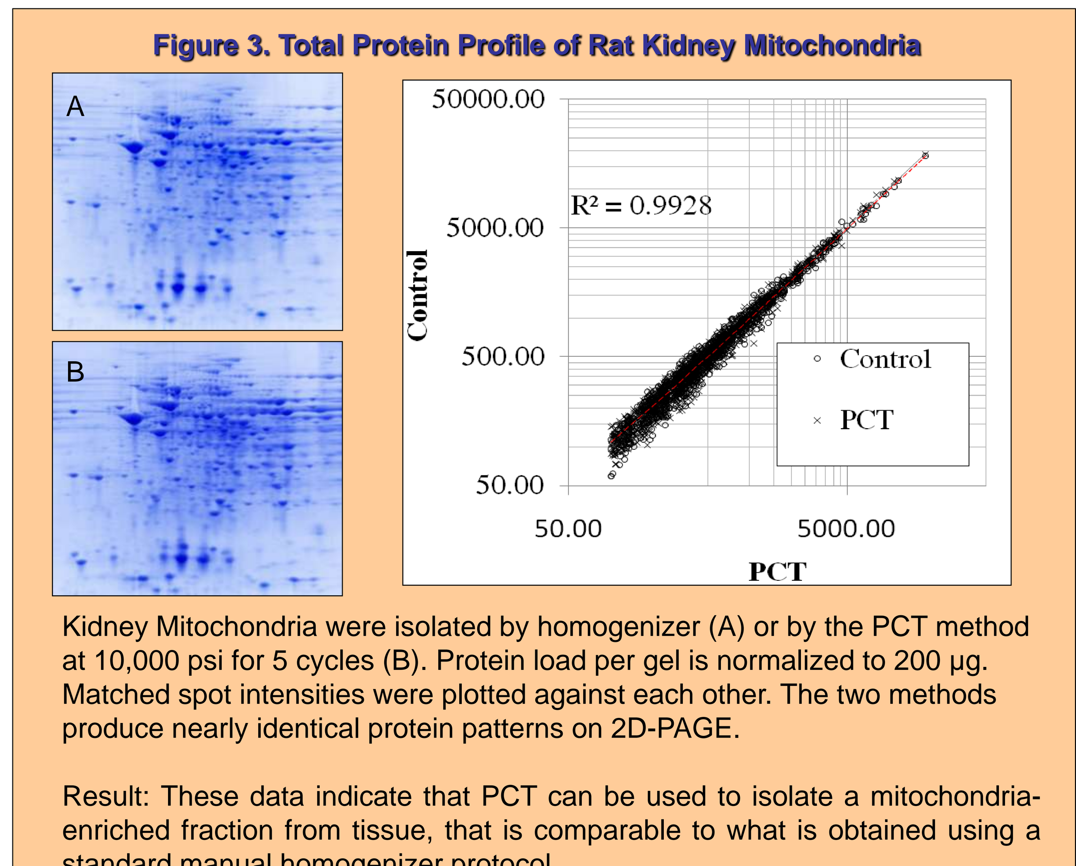

Figure 3. Total Protein Profile of Rat Kidney Mitochondria

Kidney Mitochondria were isolated by homogenizer (A) or by the PCT method at 10,000 psi for 5 cycles (B). Protein load per gel is normalized to 200 µg. Matched spot intensities were plotted against each other. The two methods produce nearly identical protein patterns on 2D-PAGE.

Result: These data indicate that PCT can be used to isolate a mitochondria-enriched fraction from tissue, that is comparable to what is obtained using a standard manual homogenizer protocol.

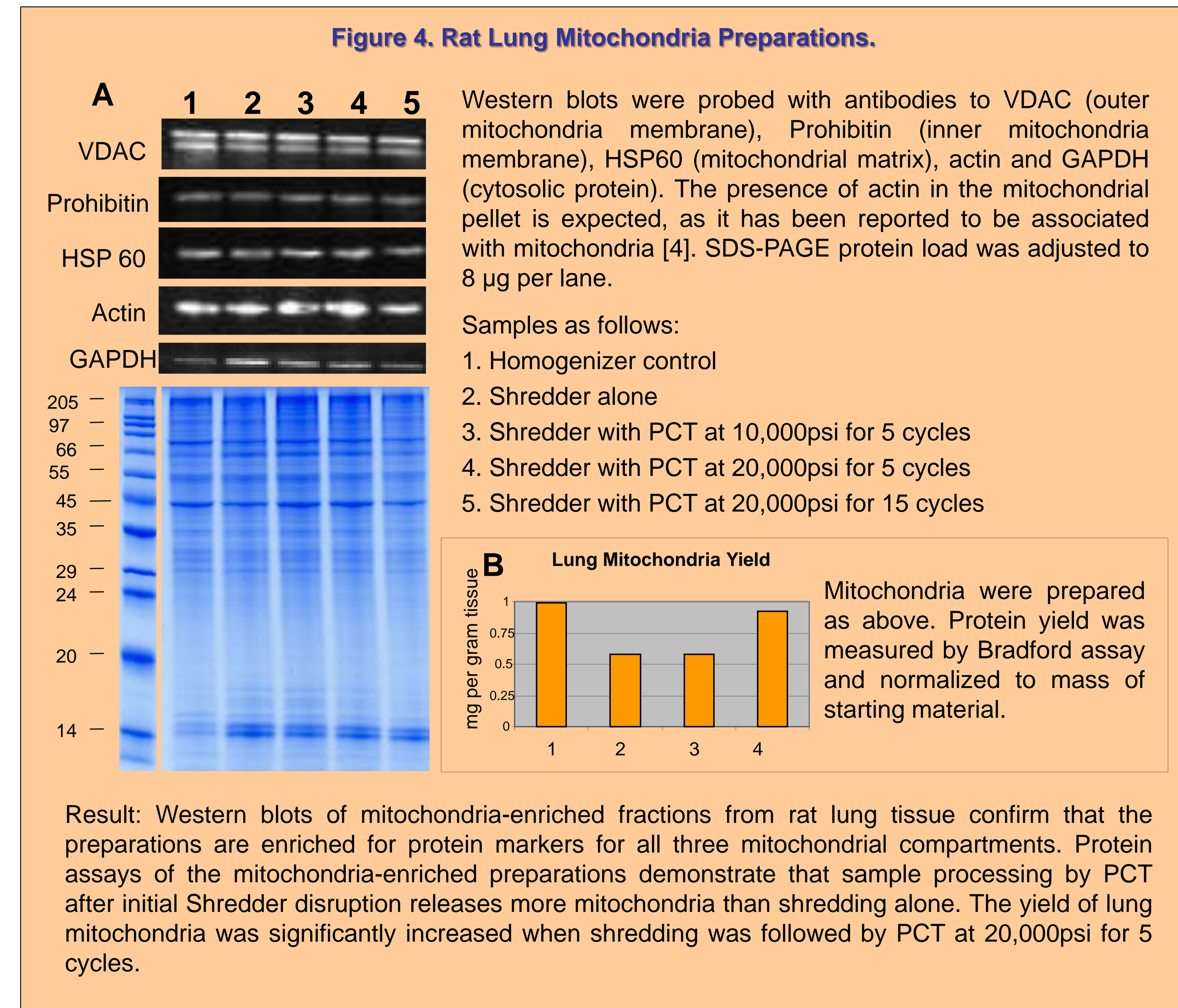

Figure 4. Rat Lung Mitochondria Preparations.

Western blots were probed with antibodies to VDAC (outer mitochondria membrane), Prohibitin (inner mitochondria membrane), HSP60 (mitochondrial matrix), actin and GAPDH (cytosolic protein). The presence of actin in the mitochondrial pellet is expected, as it has been reported to be associated with mitochondria [4]. SDS-PAGE protein load was adjusted to 8 µg per lane.

Samples as follows:

1. Homogenizer control
2. Shredder alone
3. Shredder with PCT at 10,000psi for 5 cycles
4. Shredder with PCT at 20,000psi for 5 cycles
5. Shredder with PCT at 20,000psi for 15 cycles

Mitochondria were prepared as above. Protein yield was measured by Bradford assay and normalized to mass of starting material.

Result: Western blots of mitochondria-enriched fractions from rat lung tissue confirm that the preparations are enriched for protein markers for all three mitochondrial compartments. Protein assays of the mitochondria-enriched preparations demonstrate that sample processing by PCT after initial Shredder disruption releases more mitochondria than shredding alone. The yield of lung mitochondria was significantly increased when shredding was followed by PCT at 20,000psi for 5 cycles.

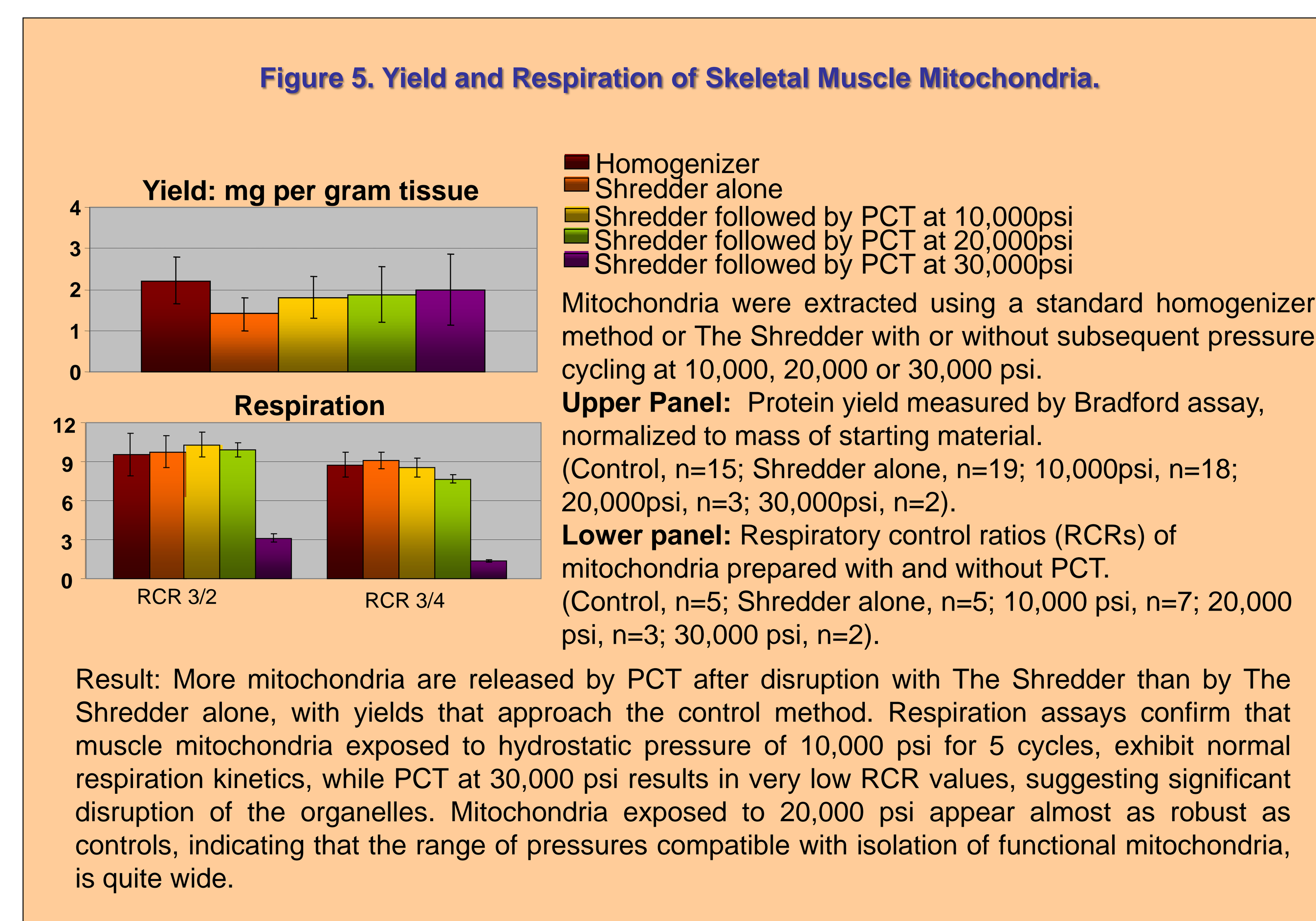

Figure 5. Yield and Respiration of Skeletal Muscle Mitochondria.

Mitochondria were extracted using a standard homogenizer method or The Shredder with or without subsequent pressure cycling at 10,000, 20,000 or 30,000 psi.

Upper Panel: Protein yield measured by Bradford assay, normalized to mass of starting material.
(Control, n=15; Shredder alone, n=19; 10,000psi, n=18; 20,000psi, n=3; 30,000psi, n=2).

Lower panel: Respiratory control ratios (RCRs) of mitochondria prepared with and without PCT.
(Control, n=5; Shredder alone, n=5; 10,000 psi, n=7; 20,000 psi, n=3; 30,000 psi, n=2).

Result: More mitochondria are released by PCT after disruption with The Shredder than by The Shredder alone, with yields that approach the control method. Respiration assays confirm that muscle mitochondria exposed to hydrostatic pressure of 10,000 psi for 5 cycles, exhibit normal respiration kinetics, while PCT at 30,000 psi results in very low RCR values, suggesting significant disruption of the organelles. Mitochondria exposed to 20,000 psi appear almost as robust as controls, indicating that the range of pressures compatible with isolation of functional mitochondria, is quite wide.

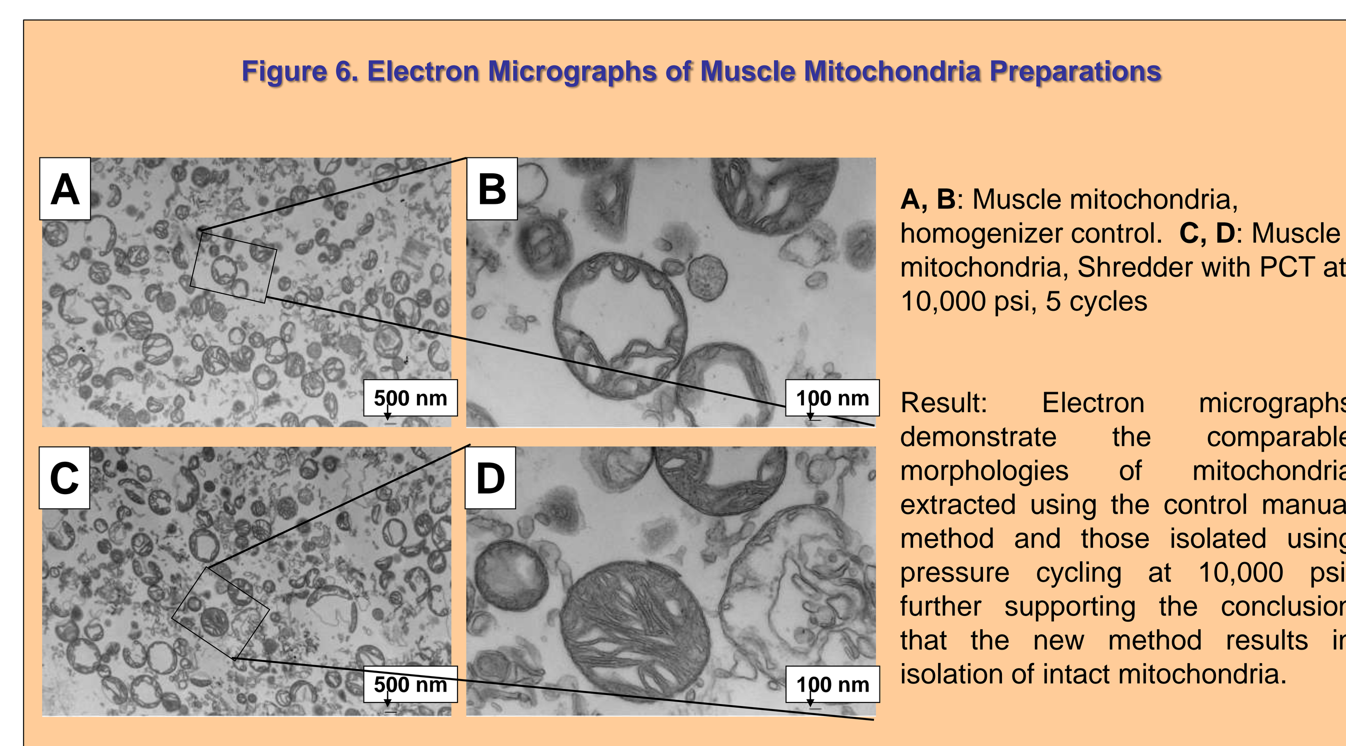

Figure 6. Electron Micrographs of Muscle Mitochondria Preparations

A, B: Muscle mitochondria, homogenizer control. **C, D**: Muscle mitochondria, Shredder with PCT at 10,000 psi, 5 cycles

Result: Electron micrographs demonstrate the comparable morphologies of mitochondria extracted using the control manual method and those isolated using pressure cycling at 10,000 psi, further supporting the conclusion that the new method results in isolation of intact mitochondria.

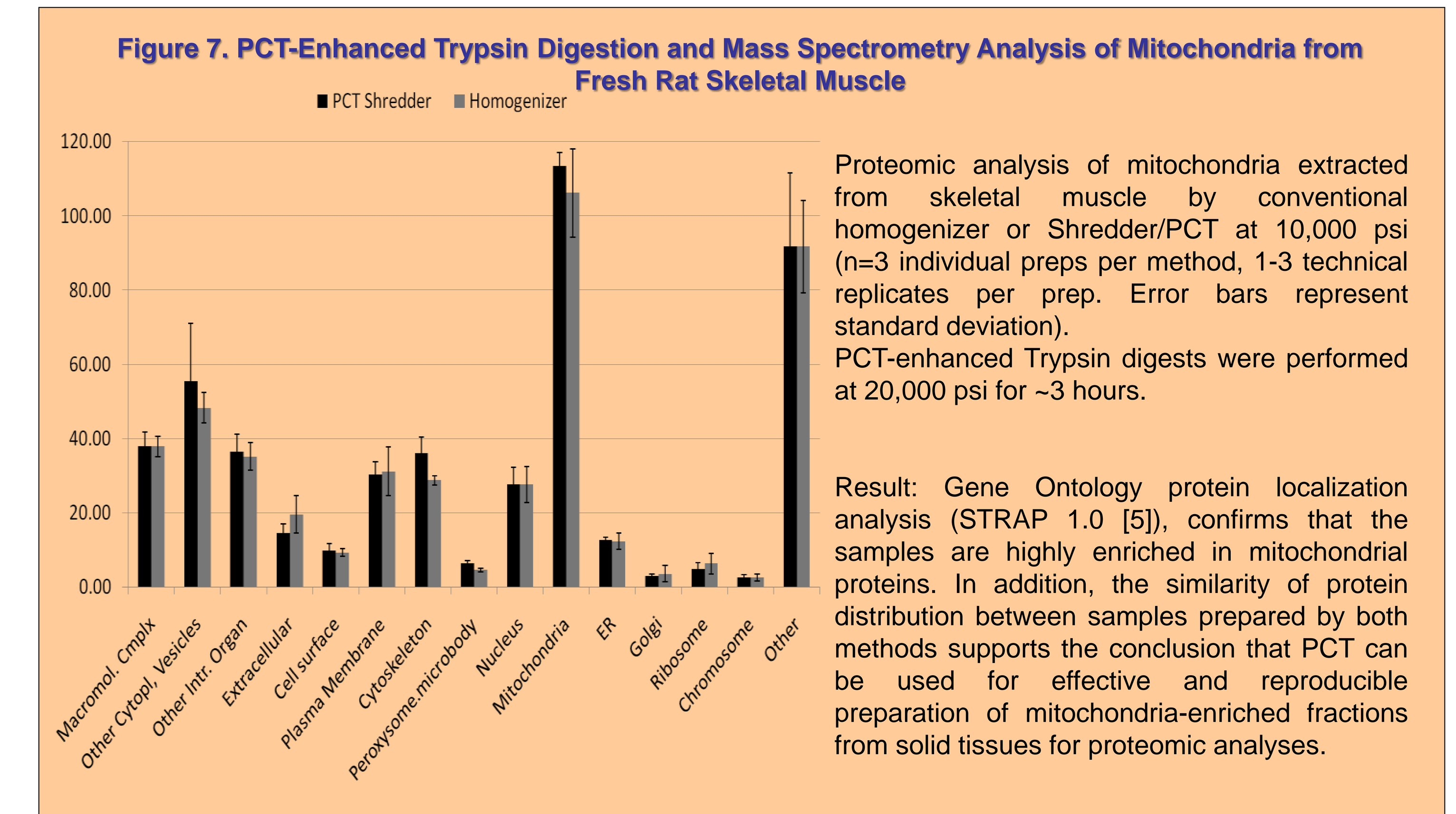

Figure 7. PCT-Enhanced Trypsin Digestion and Mass Spectrometry Analysis of Mitochondria from Fresh Rat Skeletal Muscle

Proteomic analysis of mitochondria extracted from skeletal muscle by conventional homogenizer or Shredder/PCT at 10,000 psi (n=3 individual preps per method, 1-3 technical replicates per prep. Error bars represent standard deviation).

PCT-enhanced Trypsin digests were performed at 20,000 psi for ~3 hours.

Result: Gene Ontology protein localization analysis (STRAP 1.0 [5]), confirms that the samples are highly enriched in mitochondrial proteins. In addition, the similarity of protein distribution between samples prepared by both methods supports the conclusion that PCT can be used for effective and reproducible preparation of mitochondria-enriched fractions from solid tissues for proteomic analyses.

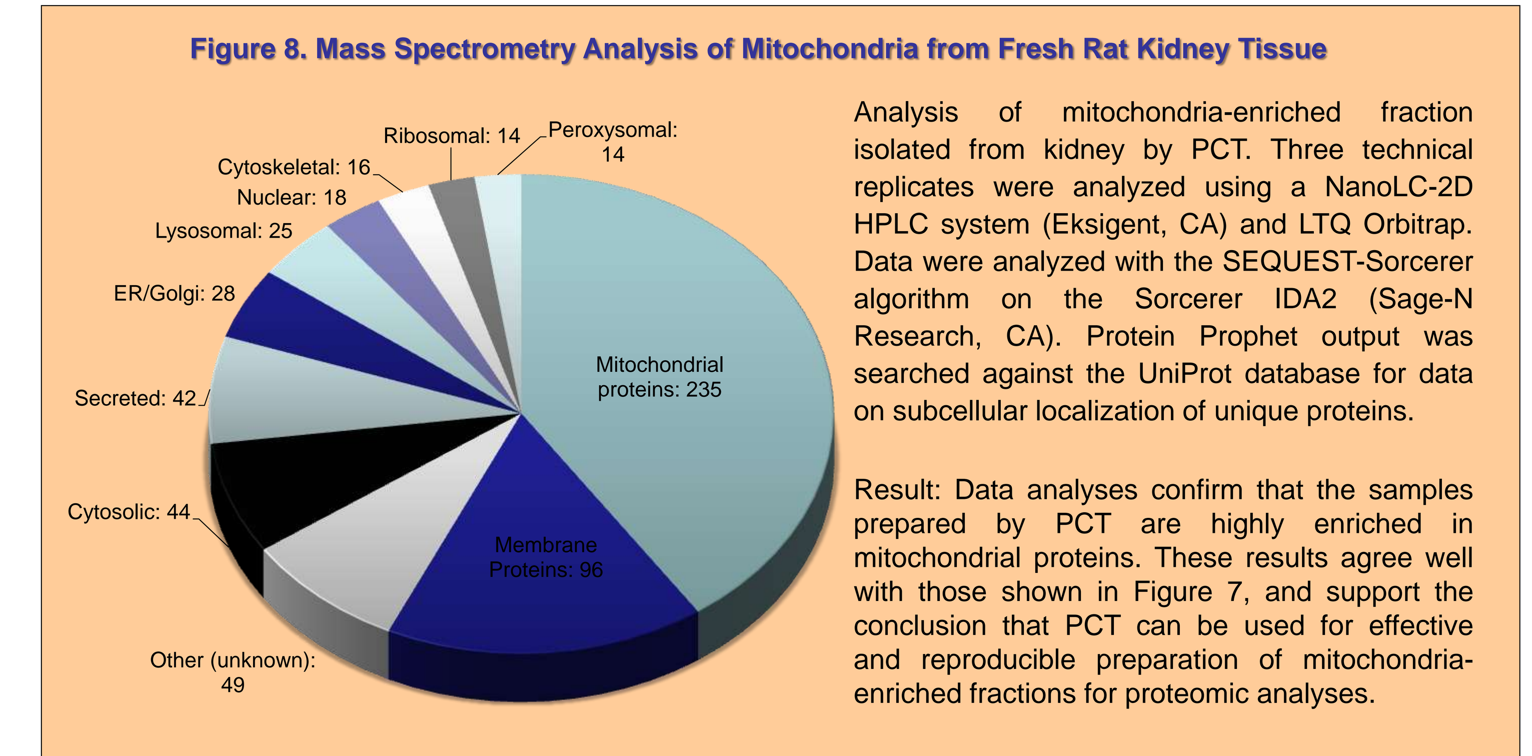

Figure 8. Mass Spectrometry Analysis of Mitochondria from Fresh Rat Kidney Tissue

Analysis of mitochondria-enriched fraction isolated from kidney by PCT. Three technical replicates were analyzed using a NanoLC-2D HPLC system (Eksigent, CA) and LTQ Orbitrap. Data were analyzed with the SEQUEST-Sorcerer algorithm on the Sorcerer IDA2 (Sage-N Research, CA). Protein Prophet output was searched against the UniProt database for data on subcellular localization of unique proteins.

Result: Data analyses confirm that the samples prepared by PCT are highly enriched in mitochondrial proteins. These results agree well with those shown in Figure 7, and support the conclusion that PCT can be used for effective and reproducible preparation of mitochondria-enriched fractions for proteomic analyses.

Conclusions

We demonstrate convenient extraction of intact mitochondria from solid tissues, using the new PBI Mitochondria Isolation kits. These kits utilize the Shredder for initial tissue disruption, followed by pressure cycling-enhanced cell lysis to release intact mitochondria. Intact and functional mitochondria were also isolated from freshly harvested rat kidneys, using a similar PCT-based protocol.

Traditional manual methods for isolation of intact mitochondria from solid tissues rely heavily on operator training, experience and skill. Considerable training is required in order to avoid common mistakes, such as tissue over-homogenization, that can result in damaged mitochondria and highly variable results. The new method described here allows for reproducible and convenient isolation of intact mitochondria-enriched preparations that are comparable to those obtained by more traditional, labor intensive manual homogenization. During initial tissue shredding, the design of the FT 500-S PULSE Tube forces the homogenate through the holes of the Lysis Disk into the upper compartment (Figure 1B). This simple design significantly reduces the likelihood of sample over-homogenization. Our results demonstrate that overall yield of mitochondria can be increased if initial tissue disruption by the Shredder method is followed by a brief PCT treatment at 10,000 psi (for muscle mitochondria) or 20,000 psi (for lung mitochondria). Since PCT-based cell lysis is conducted under controlled thermodynamic conditions (time, temperature and pressure), it is expected to yield more reproducible results than manual methods. Our data demonstrate that this isolation method results in significant enrichment in mitochondrial proteins for proteomic applications. In addition, the mitochondria isolated using the PCT sample preparation system are intact, functional, and exhibit an overall protein profile comparable to controls isolated using a conventional manual method. Further analysis is under way to determine if there are subtle differences in protein composition or quantity between methods.

References

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