Isolation of Functional Mitochondria from Rat Skeletal Muscle Using *The PCT Shredder* with an Optional Pressure Cycling Technology (PCT) Processing Step

Introduction

Isolation of intact mitochondria from human and animal tissue is crucial for studies in aging, diabetes and cancer. High quality functional mitochondrial isolates are also important for drug screening studies [1]. Mitochondria isolation from solid tissue is usually performed by labor-intensive homogenizer-based methods [2] that require extensive operator experience. To facilitate efficient and reproducible mitochondria preparation, we have developed a semi-automated method to release mitochondria from solid fibrous tissues, such as muscle, using *The PCT Shredder* for tissue homogenization. This method can be combined with a subsequent treatment using pressure cycling technology (PCT) to further increase yield, if desired.

The PCT Shredder

The PCT Shredder is designed to quickly and gently grind fibrous samples such as plant leaves and animal muscle tissue. Since shredding is performed in a disposable sample container (Figure 1), the likelihood of cross contamination with other samples is very low. In addition, shredding for 10-20 seconds generates relatively little heat and thus does not increase protein denaturation or damage delicate organelles.

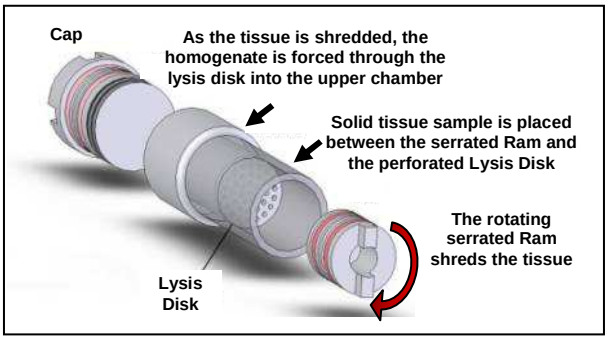

Figure 1. The PCT Shredder FT500-S Shredder PULSE Tube. As the tissue is disrupted, the Shredder Ram of the PULSE Tube forces the homogenate through the holes of the Lysis Disk and into the upper compartment, significantly reducing the risk of sample over-homogenization and mitochondrial damage.

Pressure Cycling Technology (PCT) - optional

PCT destabilizes molecular interactions by rapidly and repeatedly raising and lowering pressure in the reaction vessel from ambient to high pressures (up to 45,000 psi [310 MPa, 3000 Atm]). High hydrostatic pressure acts preferentially on the more compressible constituents of the sample, leading to destabilization of molecular interactions, but not of covalent bonds. At lower pressures, e.g. 10,000 - 20,000 psi, PCT can lyse most cells and release their intracellular contents, including intact organelles. This method has been shown to be relatively gentle, and ~~it~~ has already been used to isolate mitochondria from cell culture [3].

Methods

Mitochondria-enriched fractions were prepared from freshly harvested adult rat gastrocnemius muscle. Tissue was rapidly excised and placed into ice-cold Muscle Mitochondria Isolation Buffer (MMIB) (120mM KCl, 20mM HEPES, 5mM $MgCl_2$, 1mM EGTA, pH adjusted to 7.4 with KOH) [4]. The samples were kept on ice, or at 4˚C, for all subsequent steps. The tissue (1.5-1.8 g) was roughly minced and transferred to MMIB supplemented with 0.25 mg/mL bacterial proteinase type XXIV (Sigma). The tissue was further minced while incubating for 5 minutes at 4˚C in the enzyme solution. The tissue was washed twice in ice-cold MMIB, transferred to eight FT500-S PULSE Tubes (~0.2 g per tube), and shredded with 0.5 mL MMIB for 10 seconds using *The PCT Shredder*. The metal Shredder stand was pre-chilled to compensate for any heat that might have been generated during shredding.

Control tissue samples were transferred to a 50 mL glass homogenizer with a Teflon pestle and homogenized gently by hand. The homogenate was transferred to a centrifuge tube and the homogenizer was rinsed with additional MMIB buffer to remove remaining tissue. All subsequent steps were carried out as described below except the control was not subjected to *The PCT Shredder* or PCT treatment.

Samples were either processed directly for mitochondria purification at this point, or were subjected to pressure cycling for additional mitochondria extraction.

For PCT treatment, the volume of each shredded sample was brought up to 1.4 mL per PULSE Tube with additional MMIB. PCT treatment with a Barocycler was performed at 10,000, 20,000 or 30,000 psi for 5 cycles at 4˚C. Each cycle consisted of 20 seconds at high pressure and 5 seconds at atmospheric pressure.

Tissue homogenates from each of the eight PULSE Tubes were pooled and centrifuged for 8 minutes at 1000 x g. The supernatant was centrifuged for 8

minutes at 14,000 x g to pellet the mitochondria-enriched fraction. After centrifugation, the mitochondrial pellet consisted of two layers: the darker bottom layer composed of intact mitochondria, and the pale top layer containing damaged mitochondria (note: this layer was also always present in the controls). To separate the layers, the bottom of the inverted tube was gently tapped on the bench several times, causing the top layer to slide down the side of the tube while the lower layer remained attached. The pale material was then aspirated and discarded. The dark pellet was suspended in fresh MMIB using 5-10 gentle strokes in a 1 or 2 mL glass homogenizer with a Teflon pestle. The pooled mitochondrial suspension was transferred to a 1.5 mL microfuge tube and centrifuged a second time at 14,000 x g. The final pellet was suspended by gently homogenizing the pellet in 30 µL of MMIB using a small plastic pestle. The final volume of the samples was 70-120 µL.

Mitochondrial respiration was measured using an Oroboros Oxygraph-2k system. The rates of respiration at state 2 (V2), state 3 (V3) and state 4 (V4) were determined, and a respiratory control ratio (RCR) was calculated as the ratio V3/V4 (RCR 3/4) or V3/V2 (RCR 3/2).

Results and Discussion

Here we describe a novel method for extraction of functional mitochondria from skeletal muscle tissue using *The PCT Shredder* with the option of subsequent PCT treatment for increased yield.

No significant difference was observed in the respiratory control ratios (RCRs) of mitochondria extracted by the standard manual homogenizer method compared to *The PCT Shredder*. Respiration assays of mitochondria extracted with and without pressure cycling confirm that mitochondria exposed to hydrostatic pressure of 10,000 psi exhibit normal respiration kinetics (Figure 2, Lower Panel).

As expected, mitochondria exposed to 30,000 psi are severely damaged, as indicated by the very low RCR values. It is likely that such high pressure results in isolation of fragments, rather than intact mitochondria. However, the mitochondria exposed to 20,000 psi appear almost as robust as controls, indicating that a wide range of pressures is compatible with viability.

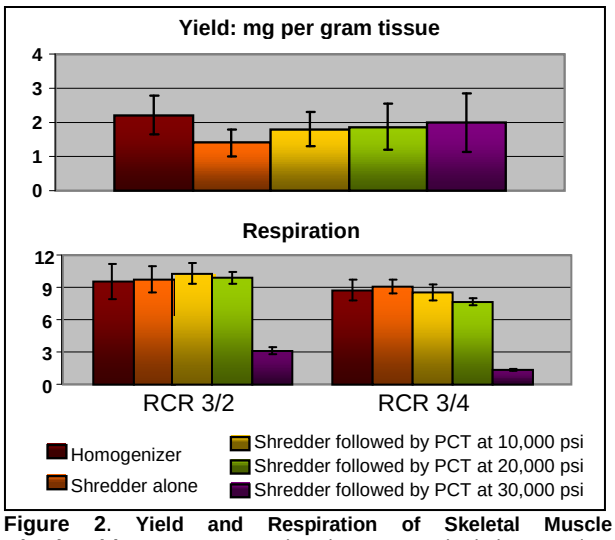

Figure 2. Yield and Respiration of Skeletal Muscle Mitochondria were extracted using a standard homogenizer method or *The PCT Shredder* with or without subsequent pressure cycling at 10,000, 20,000 or 30,000 psi. **Upper Panel:** More mitochondria are released by PCT after disruption with *The PCT Shredder* than by *The PCT Shredder* alone, with yields that approach the control method (Control, n=15; Shredder alone, n=19; 10,000 psi, n=18; 20,000 psi, n=3; 30,000 psi, n=2). Final protein yield of the mitochondria-enriched preparations was measured by BCA assay. **Lower Panel:** Respiratory control ratios (RCRs) are a measure of mitochondrial function and intactness. RCRs of mitochondria prepared using *The PCT Shredder* are comparable to the homogenizer control. Mitochondria exposed to 30,000 psi (n=2) are abnormal, while those treated at 10,000 psi (n=7) and 20,000 psi (n=3) are comparable to controls (homogenizer: n=5, Shredder alone: n=5).

Protein assays of the mitochondria-enriched preparations demonstrate that sample processing using PCT after initial disruption with *The PCT Shredder* releases more mitochondria than using *The PCT Shredder* alone, and that this yield approaches that obtained using the more traditional and labor-intensive manual homogenizer method (Figure 2, Upper Panel). The results also demonstrate that a pressure of 10,000 psi is sufficient to increase yield and that little additional yield is recovered at higher pressures.

As judged by 2D gel electrophoresis, the protein profiles of control samples isolated by manual homogenization are essentially equivalent to the test samples prepared by *The PCT Shredder* followed by PCT at 10,000 psi (Figure 3). Western blots (Figure 4) show that the mitochondria preparations are enriched in markers for all three mitochondrial compartments, further confirming that the mitochondria are intact.

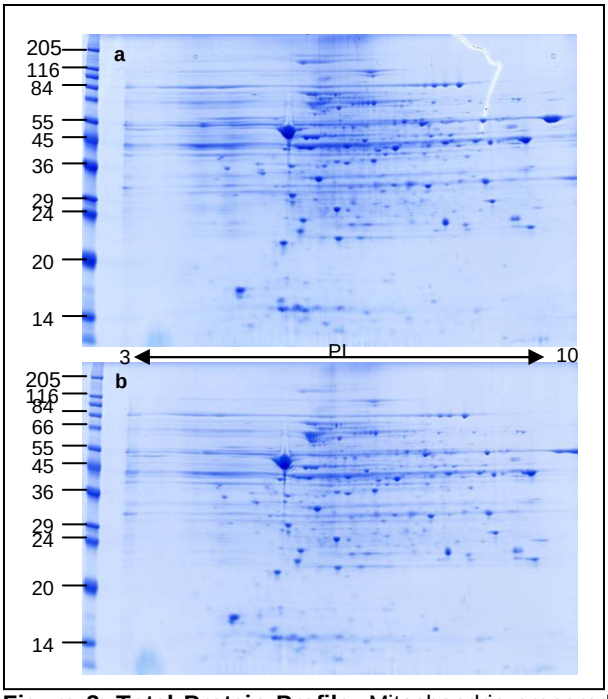

Figure 3. Total Protein Profile Mitochondria prepared using *The PCT Shredder* followed by pressure cycling at 10,000 psi (a) and by the control homogenizer method (b) were essentially indistinguishable (200 µg/gel).

Conclusion

Traditional manual homogenization methods for isolation of functional mitochondria from tissues rely heavily on operator training, experience and skill. Without considerable training, common mistakes, such as tissue over-homogenization, can result in damaged mitochondria, and thus might cause highly variable results.

Here we described a reproducible and convenient method for isolation of intact and functional mitochondria from fresh skeletal muscle tissue using *The PCT Shredder*. During shredding, the design of the FT500-S PULSE Tube forced the homogenate through the holes of the Lysis Disk and into the upper compartment. This simple but effective design significantly reduced the likelihood of sample over-homogenization. In addition, we demonstrated that overall yield of mitochondria can be increased if initial processing in *The PCT Shredder* is followed by a brief PCT treatment at 10,000 psi. The mitochondria isolated using this novel method are intact and functional, and can be used for proteomic as well as functional studies. This *PCT Shredder*-based method, with or without subsequent PCT treatment, is simple, easy to learn, and eliminates many of the problems of traditional manual homogenization preparation methods for mitochondria.

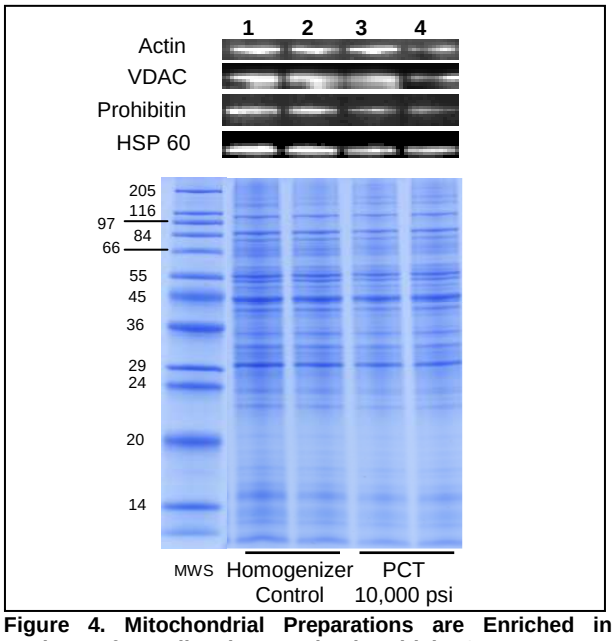

Figure 4. Mitochondrial Preparations are Enriched in Markers for All Three Mitochondrial Compartments Coomassie blue-stained SDS-PAGE gels and Western blots of mitochondrial samples isolated by homogenizer are essentially equivalent to the samples prepared using *The PCT Shredder* with pressure cycling at 10,000 psi. (VDAC, outer mitochondria membrane; Prohibitin, inner mitochondria membrane; HSP60, mitochondrial matrix).

References

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