TITLE:

Measuring mitochondrial electron transfer complexes in previously frozen cardiac tissue from the offspring of sow: A model to assess exercise induced mitochondrial bioenergetics changes.

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SUMMARY :

Preparation of mitochondria enriched samples from previously frozen archived solid tissues allowed the investigators to perform both functional and analytical assessments of mitochondria in various experimental modalities. This study demonstrated (i) how to prepare mitochondria enriched preparations from frozen heart tissue and (2) perform analytical assessments of mitochondria

ABSTRACT:

Measuring the mitochondrial electron transfer complex (ETC) profile from previously frozen heart tissue samples from offspring born to an exercised sow provided descriptive data about exercise induced mitochondrial biochemical changes in heart tissue from the offspring born to the exercised sow. The hypothesis that was proposed and tested was that regular maternal exercise of a sow during pregnancy would increase the mitochondrial efficiency of offspring heart bioenergetics. This hypothesis was tested by isolating mitochondria using a mild-isolation procedure so that mitochondrial ETC, and supercomplex profiles were assessed. The procedure described here allowed for the processing of previously frozen archived heart tissues, and eliminated the necessity of fresh mitochondria preparation for the assessment of mitochondrial ETC complexes, supercomplexes, and ETC complex activity profiles. This protocol described the optimal ETC protein complex measurement in multiplexed antibody-based immunoblotting, and super complex assessment using blue-native gel electrophoresis.

INTRODUCTION:

The goal of this protocol was to provide detailed steps to obtain mitochondria enriched preparation from previously frozen heart tissue with a new technology of low energy mechanical disruption of tissue that improves tissue lysis and extraction of mitochondria. With this method, improved extraction efficiency without generating high shear stress or high temperature and short homogenization time (10-12 s) becomes achievable [1].

To obtain mitochondria from archived frozen tissue is a valuable asset to perform both functional [2] and biochemical studies [3] otherwise not easily repeatable under the exact experimental conditions. A classic Potter-Elvehjem teflon pestle glass homogenizer or Dounce homogenizer has been used and still is being used in research laboratories to homogenize soft tissues such as liver, kidney, and brain; however, homogenizing hard tissues like muscle and heart require more homogenization time, enzyme treatment, high speed homogenization, and extensive user experience. This is disadvantageous for extraction of intact organelles such as mitochondria from hard tissue such as muscle and heart. The method described in this protocol is used to obtain high yield mitochondria enriched preparation to analyze mitochondrial electron transport chain (ETC) protein complexes and their supercomplex formation in heart tissues harvested from offspring born to exercised and sedentary sow, flash frozen in liquid nitrogen, and stored at -80^{0}C for future use. This method allows the user to isolate mitochondria enriched preparation from previously frozen archived tissues.

External nanomaterial exposure to pregnant rodents can negatively affect cardiac function and mitochondrial respiration and bioenergetics on progeny during the gestation [4].
Nevertheless, aerobic exercise-induced positive changes in fetal myocyte bioenergetics during pregnancy is yet to be documented. However, emerging studies provide evidence that maternal aerobic exercise during pregnancy has a positive influence on fetal cardiac function [5]. In order to provide further evidence, an analysis of the longitudinal effects of maternal exercise on off spring cardiac mitochondrial respiratory chain complexes (i.e., Complex I through Complex V) during pregnancy was performed

This study has significant health relevance since the results may suggest that maternal exercise improves the efficiency of energy production in the cardiac mitochondria of the offspring. In this study, sows (a female pig)were used as an animal model for two reasons: **(i)** cardiac physiology is similar to human [6] and **(ii)** heart tissue harvest from offspring from different time points is feasible under an institutional IACUC approval. The proposed study aims to answer many of the fundamental questions linking maternal exercise, and its potential positive effects on the cellular and biochemical makeup of the offspring's heart tissue. This approach requires gentle, yet effective mitochondria isolation techniques from previously frozen cardiac tissue obtained from the lengthy and costly longitudinal studies that addressed the issues of the bioenergetic changes within fetal cardiac myocytes during the pregnancy. The method described in this study allows utilizing large sums of previously frozen archived tissue for mitochondria enriched preparation for both analytical and functional studies. This study will also help fill the knowledge gap in this field by providing preliminary data, which could lead to

future studies determining the effects of maternal exercise on heart health in utero and beyond.

PROTOCOL:

Frozen offspring heart tissues were received from Dr. Sean Newcomer along with the institutional IACUC approval letter. The heart tissues were obtained from a long-term longitudinal study, flash frozen in liquid nitrogen and stored in -80^{0}C for future use. All protocols concerning the processing of offspring heart tissue followed the guidelines of Kansas City University IBC and IACUC committees.

1. **Preparation of buffers and reagents**

   Note: Prepare all samples as per the manufacturer's guidelines. Use ultra-purified water or equivalent in all recipes and protocol steps. Wear personal protection equipment (lab gloats, facemask, gloves, and googles/face shield) during this procedure. Buffer volumes are good for processing six tissue samples.

1. Prepare 1.5 liter tissue washing buffer by combining 154.035 g sucrose (0.3 M final), 3.904 g HEPES (10 mM final), 0.111g EDTA (0.2mM final). Add ingredients to 1.0 liter of water while on the stir plate, adjust the pH to 7.2 with dilute HCl, and make up to final volume to 1.5 liters.

   Note: Approximately 200 mL of washing buffer is needed per sample (50-300 mg). Six tissue samples can be processed in a day. The remaining washing buffer will be used to make an isolation buffer.

2. Prepare the isolation buffer from wash buffer previously described in step-1 above by adding 210 mg BSA in 210 mL wash buffer. Adjust the pH to 7.4 with dilute NaOH solution.

   Note: Approximately 35 mL isolation buffer is needed per tissue sample; therefore, 210 mL of isolation buffer is required for six samples. Prepare this buffer by adding fresh BSA solution (1mg/mL final) on the day of preparation.

   <u>Alternative approach</u>: Bovine serum albumin can be substituted by less expensive acid-free analogues such as ovalbumin.

   Note: BSA is used to isolate mitochondria as it improves the mitochondrial properties by removing natural uncouplers, like free fatty acids. BSA has been found to have antioxidant activity by increasing oxidation rate of substrates, especially succinate, in brain mitochondria [7]

3. Prepare 150 mL of resuspension buffer by combining 0.011 g EDTA (Final concentration of 0.2 mM EDTA or use 150 µL of 200 mM stock EDTA solution for 150 mL resuspension

buffer), 12.836 g sucrose (0.25 M sucrose final concentration), and 0.236 g Tris (10mM Tris-HCl final). Adjust the pH to 7.8 with 0.1 N NaOH. Add 60 µL of the protease inhibitor cocktail just before use.

4. Prepare the trypsin solution by dissolving 7.5 mg trypsin in 3.0 mL of 1 mM HCl (2.5 mg trypsin/mL final). This amount is sufficient for one sample.

   Note: The trypsin solution should be prepared based on the number of the tissue samples planned to be process.

5. Prepare the trypsin inhibitor solution from soybean by combining 13.0 mg trypsin inhibitor in 20 mL of isolation buffer containing BSA (0.65 mg/mL trypsin inhibitor final).

   Note: The trypsin inhibitor solution should be freshly prepared.

6. Prepare the ammonium persulfate reagent by combining 0.1 g ammonium persulfate in 1 mL of water (10% ammonium persulfate final).

   Note: The ammonium persulfate reagent should be either freshly prepared or a large volume can be aliquoted and kept in -20^{0}C freezer.

7. Prepare the aminocaproic acid solution (ACA) by combining 0.131 g of 6-amino caproic acid in 1.0 mL, and store in 4^{0}C (1M 6-aminocaproic acid final).

8. Prepare the Coomassie brilliant blue dye solution by dissolving 1 g of Coomassie G-250 in 0.5 mL water, and 0.5 mL of 1 M of aminocaproic acid (10% dye solution final concentration)

9. Prepare 5% Digitonin for BN-PAGE. Calculate approximately how much digitonin is needed in the sample preparation plus some extra volume to offset the pipetting errors (overestimate by 10 mg). For 15 mg: First, dilute 15 mg of digitonin in 100% DMSO 30 µL. Vortex to help the digitonin get into solution. Next, add the remaining 90% ddH2O (270 µL). Gently heat to get into solution (vortex), and then cool on ice.

10. Prepare the native PAGE anode buffer. Add 50 mL 20 x native PAGE running buffer to 950 mL water to 1L. Prepare fresh for immediate use. This can be used as a 1x running buffer.

11. Prepare a dark blue cathode buffer. Dissolve 0.0160 g of Coomassie brilliant blue G-250 in 80 mL native PAGE anode buffer; mix well.

Note: Prepare fresh for immediate use. Only 60 mL may be needed, but make an extra 20 mL in the event of any leaks.

12. Prepare the light blue cathode buffer. Add 20 mL of dark blue cathode buffer into 180 mL of native PAGE anode buffer and mix well.

    Note: This buffer can only be used once.

13. Prepare 5% Coomassie G-250 as a sample additive. Dilute the 200 µL 10% Coomassie stock already made (step-8) with 200 µL 1M ACA and store at the 4^0C.

14. Prepare the 4x native PAGE sample buffer by combining 40% glycerol, 0.04% Ponceau S, 25mM Tris-HCl (pH 6.8), 192mM glycine in 10 mL final volume, aliquot them, at -20^0C in the freezer.

2. Mitochondria isolation from frozen heart tissue

Note: The mitochondria isolation procedure is preferably performed in a 4^0C cold room; however, in case of unavailability of the cold room, a large size ice bath would work as well.

1. Take out previously frozen heart tissue samples from the -80^0C C freezer.

   Note: A maximum of four tissue samples may be comfortably processed for mitochondria isolation in a given day.

2. Weigh the tube containing the heart tissue sample and record the weight.

   Prepare three beakers (each 50 mL) containing 40 mL of **washing buffer**. Cool each beaker in the ice-salt bath with equal amounts of ice and salt mixed for 3 to 5 min before proceeding to the next step. Put magnetic stirring bars in each beaker and set the stirrer for medium speed.

   Note: Do not over-freeze the beakers and use immediately just after clouds of ice crystals start to appear.

3. Put frozen heart tissues directly into the ice-cold solution in the first beaker. Wait approximately 5 minutes while stirring and applying slight pressure to the tissue using tweezers to get out as much blood as possible.

4. Weigh the empty tube and subtract from the original weight (step-2 above) to obtain a net weight of the heart tissue.

5. Take the tissue out with forceps, and squeeze the heart with a clean paper towel of filter paper to remove blood. Then, place the tissue into the second beaker. Wait approximately 5 minutes while stirring to remove an excess blood.

6. Gently dry the tissue on a paper towel. Remove all fat, clotted blood, auricles and fasciae (white tissue). Then, pool the ventricular tissue.

7. Shred the tissue samples by using the shredder by following steps 7.1 through 7.9

7.1. Place the heart tissue (~50-300 mg) into the tissue shredder chamber from the ram

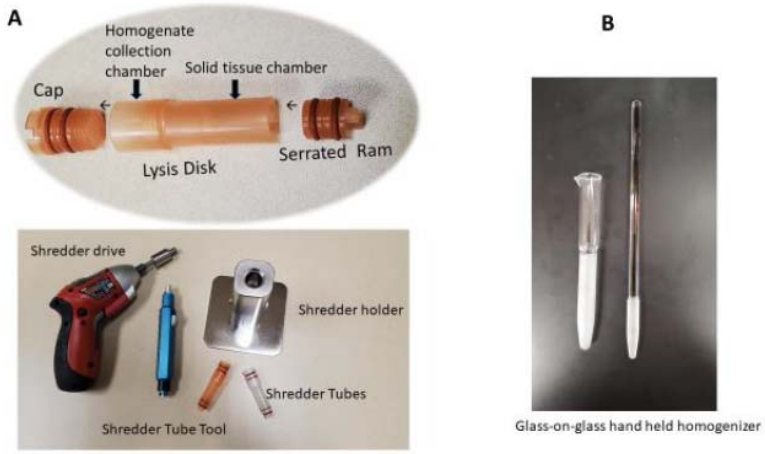


FIGURE-1: Tissue shredder chamber (tube) for use with the shredder.
(A). Tissue homogenization in the shredder tube requires about 10-12 s per tissue sample. Tissue homogenization is completed once the homogenized tissue pass through the perforated disk into the upper chamber. **(B).** A glass-on glass hand-held homogenizer

side (Figure 1-A)

7.2. Add ~0.2-0.8 mL of washing buffer to the cap side of the tube.

Note: The total volume of the sample and the washing buffer combined during the shredding should not exceed 0.7-0.8 mL

7.3. Snugly cap the shredder chamber with the shredder cap, using the tube tool.

Note: Do not over tighten the shredder cap

7.4. Place the shredder chamber into the shredder holder unit with the ram side dawn. The ram will engage a fitting in the shredder holder.
7.5. Insert the bit of the shredder driver, let the bit engages the shredder cap. Make sure that the bit is snugly locked into place.

> **Note:** Always use the fully charged shredder driver.

7.6. Manually apply pressure downward to the shredder drive and shredder chamber until the pressure indicator on the shredder holder reaches a setting of approximately "2" on the shredder holder.

Note: The setting can be higher level if shredding tissue is more fibrous Run the shredder driver for approximately 10-12 seconds by pressing the trigger of the shredder driver, while maintaining the setting at "**2**"

Check to see if all or most of the solid mass of the tissue has been shredded through the lysis disk and passed into the upper chamber of the shredder chamber by looking at the tube.

> **Note:** It may be necessary to shred the tissue sample for a longer period. Simply return the tube to the shredder holder and continue the process.

> **Note:** Most kinds of tissue samples require only 10-12 seconds of shredding. While all shredding will produce some heat due to friction, this short processing time will not significantly heat the sample

After the sample has been shredded, lift the shredder chamber out of the holder unit and use the tube tool to remove the shredder cap and place it on a clean surface for later use.

> **Note:** The shredder cap should be considered contaminated with sample extract, and depending on the sample type, may be contaminated with infectious materials. We recommend not using the same shredder cap for any other tissue samples to prevent cross-contamination.

8. Transfer the shredded heart tissue into the third beaker with 40 mL of washing buffer and stir bar and let the tissue be stirred for 5 minutes at a medium speed while on the stir plate.

9. Filter the suspension through a nylon mesh monofilament (74-micron pore size) and discard the filtrate. Wash the shredded tissue on the filter three times with 10 mL of washing buffer.

> **Note:** Make sure that the nylon mesh monofilament is pre-wetted with water.

Transfer the washed and shredded tissue into a 50 mL beaker with 20 mL of washing buffer, and place the beaker in the ice bath on the magnetic stirrer. Add 0.5 mL of trypsin solution

while constantly stirring for 15 minutes. Approximately half way through the incubation (at about 7.5 minutes), transfer the tissue suspension into a loosely fitted hand held glass-on-glass homogenizer that will hold 0-15 mL of volume (Figure 1-B). Gently homogenize with 4-to-5 strokes to disperse the suspension without producing froth. Place the homogenate into a 50 mL beaker and gently mix on a stirring plate for 15 minutes at 4^0C.

10. After 15 minutes of incubation, add 10 mL of the isolation buffer that contains the trypsin inhibitor into the beaker containing the homogenate and incubate for 1 min at 4^oC while gently stirring

11. Filter the suspension again through a nylon filter and save the filtrate in a 50 mL conical tube.

12. Collect the particulate fraction left on the nylon filter and place in the glass-on glass homogenizer. Add 15-20 mL of the washing buffer and gently homogenize by hand for 25-30 seconds.

    Note: The filtrate flows very slowly at this step. To increase filtrate flow, place the funnel tip touching the inside of the conical tube

13. Combine the homogenate with the filtrate, balance the tubes, and centrifuge at 600 x g for 15 minutes 4^0C.

14. Filter the resulting supernatant into a new conical tube using a nylon filter to avoid contamination by the pellet. Run the centrifuge again at 8,500 x g for 15 minutes at 4^0C.

    Note: Use a plastic transfer pipet to remove supernatant and sacrifice 0.2 mL of supernatant from the top of the fluffy pellet.

15. Discard the supernatant and rinse the pellet 2-3 times with 1 mL of the isolation buffer. Each time be sure to discard the fluffy white outer rim layer.

    Note: Wash the pellet by adding isolation buffer using a pipette with 1 mL tip, or a new transfer pipet. Add the buffer to the side of the tube not directly over the pellet. Pouring down the side of the tube will prevent breaking the pellet as well as prevent the contamination of the supernatant. Next, aspirate the buffer with a transfer pipet and repeat the process two more times.

16. Add 5 mL of the isolation buffer to each tube; break up the pellet using a pipette with 1 mL tip, or a new transfer pipet.

17. Dilute the suspension with 20 mL of additional isolation buffer and run the centrifuge again at 8,500 x g for 15 minutes at 4^0C.

18. Discard the supernatant and resuspend the pellet in 5 mL resuspending buffer then add 15 mL more of the buffer for a total of 20 mL, and centrifuge at 8,500 X g for 15 min. at 4^0C Discard the supernatant. The resulting brown pellet contains washed intact mitochondria.

19. Resuspend the mitochondria enriched pellet in 250 µL of resuspending buffer including protease inhibitors, aliquot in 25 µL, quick freeze in liquid nitrogen, and store in -80^{0}C deep freezer for several years.

    Note: Spare 5 µL of mitochondria enriched preparation for the protein analysis assay.

    Note: For a BN-PAGE study, aliquot the resuspended pellet in 50 µg of mitochondrial protein per tube. Centrifuge at 8,500 X g for 15 min at 4°C. Carefully aspirate the supernatant and store the mitochondrial pellet (50 µg protein / aliquot) in a -80°C deep freezer for several years.

3. Mitochondrial electron transfer complex (ETC) assessment

Individual mitochondrial ETC proteins (i.e., Complex-I,II,II,IV,V) can be assessed by standard immunoblotting assay. Due to the variety of samples (four time points (48 h, 3m,6m, 9m), two experimental conditions (exercised and sedentary), and a number of immunoprobing antibodies (five antibodies), a multiplexed immunoblotting is recommended.

<u>Multiplexed Immunoblotting</u>

1. Resolve 50 µg of mitochondria enriched protein sample /well in 4-20% gradient poly acrylamide gel electrophoresis (100V, 90 min).
2. Transfer proteins onto PVDF membrane (75 V; 60 min)
3. Block the membrane in a blocking buffer solution for a minimum 1hr at 4^{0}C.

   Note: Blocking time can be extended for up to overnight at 4^{0}C. SEA BLOCK blocking buffer contains non-mammalian ingredients (i.e., fish proteins) that are less likely to have specific binding interactions with antibodies as well as other mammalian proteins present in typical methods.
4. Probe the membrane with the antibody cocktail including anti-Complex-I,II,III,IV, V antibodies and incubate overnight on an orbital shaker at 4^{0}C
5. Next day, wash the membrane, and probe with IR-tagged secondary antibody for 2 h on an orbital shaker at RT
6. Wash five times with 1 X TBS-T, and once with 1X TBS

   Note: For the last wash, do not include detergent in the TBS buffer.
7. Image the blot in an Image analyzer.

4. The mitochondria supercomplex assessment by Blue Native Poly Acrylamide Gel Electrophoresis (BN_PAGE)

A supercomplex profile of ETC can also be assessed by employing BN-PAGE technique

1. Prepare the sample buffer cocktail for 20 µl per 50 µg mitochondria-enriched pellet protein (7 µl water + 5 µl 4 X native poly acrylamide gel electrophoresis sample loading buffer + 8 µl 5% Digitonin + 2µl Coomassie G-250).

Note: The user may make a stock sample buffer cocktail depending on number of the samples available (50 µg protein/sample). The user should choose appropriate digitonin amounts based on the protein concentration of the mitochondria–enriched pellet (Table-1) [8].

Heat the digitonin stock at 95°C for 3 min, mix, and cool on ice, before making the master mix.
Add the Coomassie G-250 sample additive just before loading the samples into the gel.

2. Add 20 µl of the sample buffer cocktail in a tube that contains 50 µg of mitochondrial protein pellet. Solubilize/resuspend the pellet by gently mixing the pellet with a pipet without generating froth.
3. Incubate the mixture on ice for 20 minutes to achieve maximum solubilization of the mitochondria- enriched pellet. From time to time, gently flick the tubes to enhance solubilization.

4. While waiting for incubation, prepare the native PAGE anode buffer/1x running buffer and the dark blue colored cathode buffer.

5. Centrifuge the solubilized mitochondria enriched pellet at 20,000 x g for 10 min at 4^0C.

6. After centrifugation, collect 15ul of the supernatant into new tubes placed on ice.

7. Add the appropriate amount of 5% Coomassie G-250 reagent to the supernatant obtained in step-6 (refer to **Table-1)**

   Note: The 5% Coomassie G-250 volume should be such that the final concentration of the dye is ¼ the detergent concentration. This step should be done just before loading the samples into the gel.

Table-1 : Sample Buffer Cocktail Preparation with two differenet detergent/protein ratio

Protein	Digitonin/protein ratio (g/g)		4 X Sample Buffer µL)		5% Digitonin (µL)		Water (µL)	Final volume (µL)		5% Coomassie G-250 sample additive (µL)	
50 ug	8		5		8		7	20		2	
50 ug	4		5		4		11	20		1	

Table-1: To achieve maximum solubilization of membrane proteins from the mitochondrial suspension, the digitonin / mitochondrial suspension protein concentration should be adjusted to between 4-8 g digitonin / g protein. In the heart tissue, 400 µg digitonin for 50 µg of mitochondrial suspension proteins (i.e., 8 g digitonin / g mitochondria protein) were used in order to maximize to solubilize the super complexes from the mitochondria suspension.

8. Pour a 3% - 8% gradient gel, remove the comb, and wash out wells of the gel with 1x running buffer three times.

   Note: The gel best polymerizes if poured a day before

9. Set up the electrophoresis system and place the gel in the apparatus.

10. Fill the sample wells with the dark blue colored cathode buffer.

11. Load 10 µl of the unstained protein standard as a native molecular weight marker on the first and last well of the gel.

12. Load the entire amount of sample supernatant + Coomassie sample additive prepared in step-7 above into the wells using a flat-head gel-loading tips.

13. Carefully fill the mini cell buffer dam with approximately 60 mL of dark blue colored cathode buffer without disturbing the samples in the wells, and then fill the buffer tank with the native PAGE 1x running buffer.

14. Turn on the power supply and electrophorese the samples at 150 V for approximately 30 min.
15. Remove the dark blue color cathode buffer from the buffer dam (inner chamber) with a transfer pipet, or aspirate the sample wells. Fill the inner chamber with 150-200 mL of light blue colored cathode buffer and run the electrophoresis at 250 V for 60 min.

    Note: The electrophoresis time may be determined by when the dye front migrates just 0.5 centimeter above the bottom of the gel cassette.

16. Remove the gel from the cassette and place it in a clean plastic container. Wash the gel with good quality distilled water for 15 minutes on a rocker/orbital shaker.

    Note: To remove the gradient gel from the cassette, handle the cassette from the bottom, which is stronger and therefore chances of breakage are minimized.

17. Pour off water, immerse the gel in a sufficient amount of Coomassie GelCode Blue stain reagent, and incubate for 1 hour on an orbital/rocker at room temperature.

Note: Mix the GelCode Blue stain reagent solution before use by gently swirling the bottle several times.

18. Decant the dye solution, add distilled water, rinse several times with water, and put on the orbital/rocker for destaining.

    Note: The user may insert a piece of sponge (~3 cm X 0.5 cm) into the gel destaining container and leave the gel for overnight destaining. The sponge adsorbs the dye particles without requiring multiple water change.

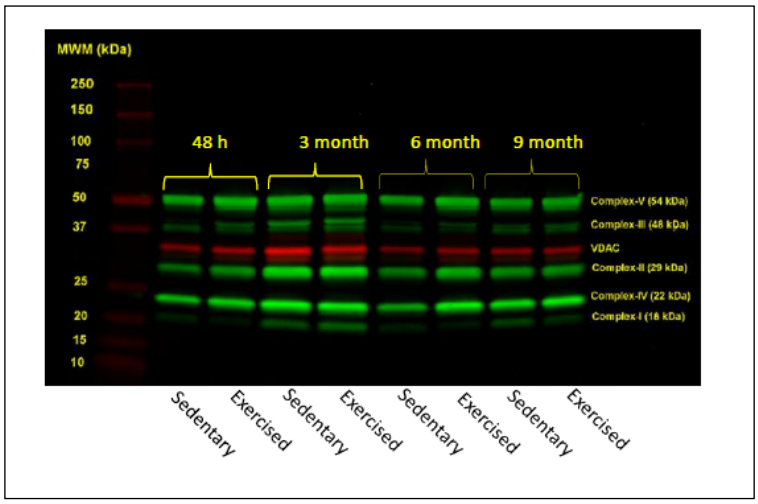
19. Analyze the gel in an image analyzer.

5. Mitochondria supercomplex assessment by immunoblot blot analysis

1. Perform a new set of BN-PAGE without staining the gel at the end of electrophoresis
2. Follow the same procedure in **step-3** (mitochondrial electron transfer complex (ETC) assessment) to complete the immunoblotting.

REPRESENTATIVE RESULTS:

Following the protocol, a good yield of mitochondria enriched protein mixture from heart tissue was prepared. Approximately 15 mg/mL of mitochondria enriched protein mixture was obtained from average of 1.2 g frozen heart tissue harvested from the offspring of the sow. Observations indicated that that less than 0.5 g of frozen heart tissue did not yield a sufficient amount of mitochondrial enriched protein mixture to carry out a BN-PAGE assay. The amount of mitochondrial preparation was sufficient to perform **(i)** a standard immunoblot analysis for assessing the individual electron transfer complexes (ETC) (i.e., Complex- I,II,III,IV, and Complex-V) , **(ii)** a mitochondrial supercomplex assessment by BN-PAGE and immunoblot analysis, and **(iii)** limited enzymatic assays for the select complex. The cocktail of antibodies raised against to individual ETC protein complexes provides a technical feasibility that each antibody will recognize its own target protein in one single immunoblot assay.



A mitochondria enriched preparation from the left ventricle of the heart, obtained from the offspring of the sow was prepared. The offspring were born to two groups of sow; (1) one group was exercised during pregnancy; and (2) one group was sedentary. The heart tissues of both groups were harvested at different time points after the birth (48 h, 3 m, 6m, and 9m). Whether maternal exercise has a positive impact on mitochondrial ETC of the offspring during gestation was the hypothesis that was tested in this study. Four heart tissues were comfortably processed in any given day due the lengthy mitochondria enriched isolation process.

FIGURE-2 Immunoblot profile of mitochondrial ETC complexes from offspring heart tissue. The mitochondrial enriched protein mixture was resolved in a 4-20% gradient gel under reducing conditions. Proteins were immunoprobed by a commercial antibody cocktail, and anti-VDAC antibody was used for loading control (red color band). The cocktail of antibodies were raised against bovine heart Complex-I (antigen NDUFB8), bovine heart Complex-II (antigen C-II30 (FeS), bovine heart Complex II,III (antigen C-III-Core 2), human Complex IV, subunit II (antigen C-IV-II), and bovine heart complex-V (antigen C-V-α) The secondary antibody was labeled with an infrared tag, and image was analyzed by an image analyzing software. Individual Complex profiles across the age groups were provided in Supplemental Figure 1 (a, b,c,d,e).

Figure-2 represents a typical ETC protein profile. Antibody cocktails allowed for immunoblotting in a multiplex format. Each antibody recognized its own target protein, providing an acceptable resolution.

The protein band intensities were digitally analyzed. Tissues obtained from the offspring of exercised sow presented relatively reduced levels in some of the ETC complexes (Suppl. Figures-1a-e). The mitochondria enriched preparation must be resolved in a gradient gel (4-20%) as the immunoprobing was performed in a multiplexing format. This approach will eliminate the necessity of running several fixed percent of SDS/PAGE gels, and run-to-run variability.

Mitochondrial proteins were resolved in a 3-8 % gradient BN- PAGE (Figure-3A) and immunoprobed with antibody cocktails. In both exercised and sedentary groups, multiple supercomplex formation was observed (Figure-3B). A different gradient gel format (3-8%) was prepared due to the large size of the supercomplexes that can be resolved in BN-PAGE.

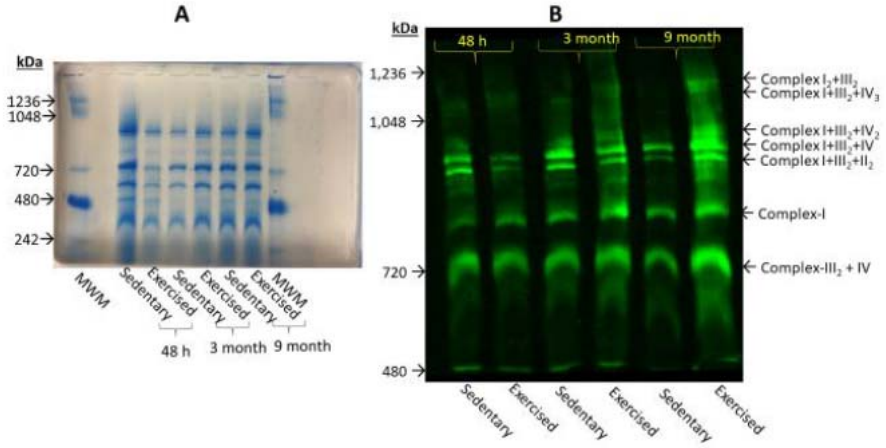


FIGURE-3 Mitochondrial supercomplex profile by Blue-Native Polyacrylamide gel electrophoresis (BN-PAGE). (A). Mitochondrial enriched protein mixtures were resolved in 3-8% BN-PAGE, and stained with a Coomassie blue reagent (Gel Code, Blue Stain Reagent). **(B).** Mitochondrial enriched protein mixtures were resolved in a 3-8% BN-PAGE, transferred onto a PVDF membrane, and immunoprobed with the antibody cocktails for supercomplexes. In both assays, the protein load was 150 µg/well. A six month data collection point was not included due to an insufficient protein yield during the preparation. The most prominent supercomplex increase observed was at nine months in the exercised group as compared to the sedentary group.

A mitochondria enriched preparation described in this protocol was used for measuring mitochondrial ETC complex activities. Figure-4 represents data recorded for Complex I-II activity assessment. This assay was performed according to the established protocol [3].

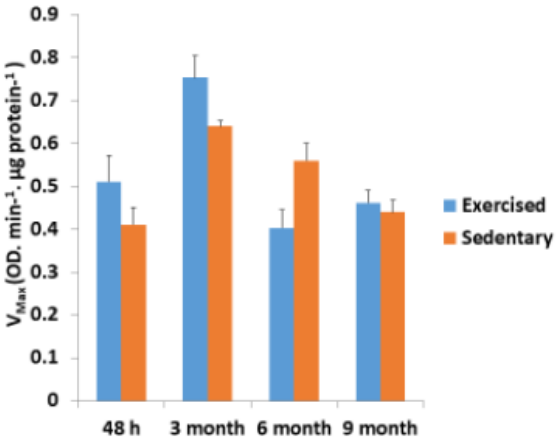


FIGURE-4 Complex I-II activity measurements. A mitochondria enriched protein mixtures obtained from different age group (48 hours, 3 months, 6 months, and 9 months) were analyzed for the Complex I-II activity using a kinetic assay. The exercised group showed an increased Complex I-II activity as compare to that of sedentary group. The difference between the two groups was not statistically significant according to a paired t-test ($P>0.05$). The error bars depicted in the figure represent standard error of the mean (SEM) for n=4.

DISCUSSION:

The critical steps for this protocol are indicated here. **(i)** First, tissue homogenization should be carefully handled so that excessive sheer effects will not be applied during the tissue homogenization process. A tissue shredder should be used , a part of pressure cycling technology (PCT) for initial tissue homogenization[9]. This step will reduce the excessive stroke cycle of glass-on-glass homogenizer (Figure-1B) that may further destroy already fragile mitochondria due to frozen tissue conditions. **(ii)**Second, the frozen tissue weight will be important in order to obtain sufficient amounts of the mitochondria enriched preparation. The recommended starting tissue amount will be greater than 0.8 g, depending on the source of the tissue. The heart tissue is very rich in mitochondria[10, 11]; therefore, the recommended amount will be sufficient to obtain a mitochondria enriched preparation. (iii)Third, all procedures should be performed in a cold room. If cold room will not be available, large sized ice-bath will be sufficient.

The feasibility of obtaining mitochondria enriched preparations from previously frozen heart tissue has been demonstrated by this study. This protocol will be essential for providing access to archived frozen tissue from which mitochondria can be isolated, and from which mitochondrial ETC enzyme complexes can be assessed. A recent study demonstrated that mitochondrial respiration from previously frozen tissues and cells could be measurable as well[2]. Because of this advancement, investigators will be able to utilize previously collected bio samples for further analyses.

To assess the profile of individual ETC and supercomplexes is made easier by performing a classical immunoblotting in a multiplexing format that utilizes multiple antibodies during the immunoblotting procedure on the same PVDH membrane. It is critical for the user to determine the amount of the protein to be loaded into gels for SDS-PAGE and BN-PAGE The resuspension volume should be as minimal as possible in order to obtain high protein concentrations so that user will not have a problem in sample loading volume. The protein amount to be loaded in BN-PAGE depends on the yield of the mitochondria enriched preparation. A 150 µg of mitochondria enriched protein mixture was loaded per well in BN-PAGE due to the small amount of original heart tissue. The 50-150 µg mitochondria enriched pellet preparation would be sufficient for 1 mm thick-gel. The user may prefer a 1.5-millimeter thick gel for sample loading however, the

resolution of protein bands may not be of an acceptable quality. The less mitochondrial protein resuspension per well was not tested, because antibody cocktails used in immunoblot assays were predetermined in their concentration; however, users can make their own antibody cocktail to increase the signal/noise rate.

A fully polymerized gradient gel (3-8 %) for BN-PAGE gels is recommended. 3-8% gradient gels have not been commercially available; therefore, homemade gradient (3-8%) standard mini gels (9x6 cm) can be used. Pouring the gel 24 hours before, and allowing the gel to polymerize overnight in RT will provide an acceptable gel formation. If a user needs better separation of supercomplexes, the authors recommend the use of to use larger gel formats (either midi gel 13.5 X 6.5 cm or long gel 28 X 16 cm)

The l application of this protocol will allow investigators to isolate mitochondria and analyze the profile of ETC and supercomplexes in previously frozen archived tissues. All national and regional biospecimen depositories keep the bio samples in ultra-freezing conditions (i.e. -80^{0}C). The large quantity of tissues are being archived by institutions and drug companies for further studies and reagent sharing purposes. The National Institute of Health (NIH) mandates that any NIH supported projects are required to share the reagents produced from the supported projects. These biorepositories are very valuable sources for obtaining frozen archived tissues for obtaining preliminary data for grant applications. To analyze enzyme activities of supercomplexes was not performed in this study; however, protocols are available to perform such assays[8] .

Using the described methods in this paper, individual ETC complexes and supercomplexes of mitochondria enriched preparations obtained from the heart tissue of the offspring of sows were analyzed. Offspring born to exercised sows presented low levels of ETC during the first six months of their life, and eventually leveling up by the nine months (Table-2).

ETC Complex	48 h	3 m	6 m	9 m
Complex-I	↓	↓	↓	No change
Complex-II	↓	↓	No change	No change
Complex-III	↓	No change	No change	↓
Complex-IV	↑	↑	↑	No change
Complex-V	↓	↓	↓	No change

Table-2: Mitochondrial electron transport chain protein complexes profile. The table summarizes the profile of mitochondrial ETC complex levels in the exercised group of sows. All complexes, except Complex-IV, showed a decreasing pattern across the age groups.

This observation may suggest that offspring born to the exercised sows during the pregnancy indicated fewer, but more efficient ETC complexes in their heart tissue mitochondria (Figure-4). However, supercomplex formation in nine months exercised group has been found to be elevated. Exercise may somehow affect the rearrangement of supercomplexes in conditions of increased energy demand [12]. In addition, Complex-V (ATP synthase) has exhibited a low profile in the first six months after birth. This response may be due to the probability of epigenetically modified ETC protein complexes. The small sample size (n=4) for this study was not sufficient to arrive at such a conclusion; however, patterns of low ETC complex protein levels, high Complex I-II

activity, and elevated supercomplex formation may suggest that bioenergetics in heart tissue of the progeny of exercised sows are influenced by exercise. Fewer but more efficiently working mitochondrial ETC complexes, and the formation of increased Supercomplexes are an interesting phenomenon to be investigated. This project is still in progress.

In summary, a simple and straightforward mitochondria enriched sample preparation from previously frozen archived heart tissue was provided in this study. Mitochondrial ETC complexes can be analyzed by standard immunoblotting with multiplexed antibody cocktails. BN- PAGE followed by immunoblotting can analyze supercomplexes presence. This preparation can also allow users to assess mitochondrial ETC complex activities.

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