## Human heart tissue mitochondria isolation: experience from patients with tetralogy of Fallot

**Jefferson Kasan Hidayat**<sup>1,2*,</sup> Aulia Widyaningrum<sup>3</sup>, Nadila Amanda Zuliani<sup>3</sup>, Franciscus Dhyanagiri Suyatna<sup>4</sup>, Ratna Farida Soenarto<sup>1</sup>, Mulyadi Muhammad Djer<sup>5</sup>

### ABSTRACT

**Background:** Mitochondria have crucial roles in human cardiomyocyte metabolism. The isolation of mitochondria from various cells and tissues has been described in previous studies. However, the isolation of human heart muscle is rarely performed because of the ethical problems inherent in obtaining fresh human heart muscle samples. Resected infundibular hypertrophy muscle in tetralogy of Fallot surgery provides a fresh sample source without causing ethical issues. This study aimed to isolate mitochondria from patients with tetralogy of Fallot.

**Methods:** The optimization of mitochondria isolation protocol in this *in vitro* study was performed to facilitate our study of ischemia and reperfusion injury. The samples of human heart tissue used in this study were resected infundibulum muscle collected during surgery to correct tetralogy of Fallot. Subjects were recruited from Cipto Mangunkusumo Hospital and Jakarta Heart Center Hospital between July 2021 and November 2022. Tissue was collected before aortic cross-clamp applied, after aortic cross-clamping applied and after aortic cross-clamping off. After collected tissue was trimmed and cleaned, mechanical disruption followed by enzymatic disruption using trypsin was performed. Homogenization was then performed with a sonicator. Mitochondria pellet was further obtained by differential fractionation of homogenate.

**Results:** Mean infundibular tissue weight obtained from the three periods mentioned was 64.92–109.7 mg. Our protein yield was 7.65–8.38 mg/ml. Mean JC-1 was 9945–12430 FLU/mgP. The median value of SDH activity shown by our mitochondria was 17.45–18.12 nmol/min/L.

**Conclusion:** The fractionation method allowed us to successfully isolate mitochondria from human heart tissue with intact membrane integrity.

**Keywords:** human heart tissue, lower-middle-income country, mitochondria isolation, tetralogy of Fallot.


### INTRODUCTION

Mitochondria is necessary for many essential physiological functions that allow a cell to survive. They synthesize most of the required adenosine triphosphate (ATP; 30 kg/day) and participate in ion transport; redox; oxidative phosphorylation; steroid, heme, and amino acid synthesis; and cell apoptosis.

The energy requirement of the heart muscle that pumps blood throughout our life is principally provided by mitochondria. The critical importance of mitochondria as energy producer is reflected by the organelles occupying around 35% of the cardiomyocytes' total volume.

Frezza et al. and Hogeboom et al. described a mitochondria isolation protocol based on differential centrifugation in the 1940s. They reported the existence of "large granules" collected from rat liver tissue that were hypothesized to be mitochondria. Notably, Hogeboom et al. made several discoveries in mitochondrial research, which led to mitochondrial isolation in various cells from different plant, animal, and human samples. Their findings facilitated the discovery and comprehension of energy-conservation mechanisms, the identification of mitochondrial DNA, the importation of mitochondrial precursor proteins, apoptosis, the transportation of metabolites and ions, and the dynamic behavior of mitochondria. Their observations also enhanced the understanding of many pathological processes, including neurodegeneration, neuronal morphogenesis and plasticity, infertility, and ischemic–reperfusion injury.

In this study, we aimed to isolate mitochondria from resected human infundibular muscle tissue obtained during tetralogy of Fallot correction surgery. The fresh infundibular muscle tissue collected during the operation is considered a waste product of the procedure. Before doing the study, we optimized the protocol using 17 heart tissues obtained from 17 subjects.

### METHODS

#### Materials

**Samples**

In this study, we used heart tissues from tetralogy of Fallot patients aged 1–6 years who underwent corrective surgery. Subjects were recruited from Cipto Mangunkusumo Hospital and Jakarta Heart Center Hospital between July 2021 and November 2022. Heart tissues were...
resected three times, including before ischemia (before cross-clamping), after ischemia (after cross-clamping), and after reperfusion (≥5 min after the cross-clamp was removed).

Reagents
We purchased 5× extraction buffer A (cat. no. E2778; Sigma-Aldrich, St. Louis, MO, USA), 5× storage buffer (cat. no. S9689; Sigma-Aldrich), albumin solution (cat. no. A0474; Sigma-Aldrich), trypsin (cat. no. T9201; Sigma-Aldrich), ultrapure water, 5,5,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolyldiazoylcarbocyanine iodide (JC-1) stain (cat. no. J4519; Sigma-Aldrich), 5× JC-1 assay buffer (cat. no. J4394; Sigma-Aldrich), succinate dehydrogenase (SDH) assay buffer (cat. no. MAK197A; Sigma-Aldrich), SDH substrate mix (cat. no. MAK197B; Sigma-Aldrich), SDH probe (cat. no. MAK197C; Sigma-Aldrich), 2,6-dichloroindophenol (DCIP) standard (cat. no. MAK197D; Sigma-Aldrich), SDH positive control (cat. no. MAK197E; Sigma-Aldrich), dimethyl sulfoxide (DMSO). The reconstituted stock was ready, it was diluted to a 50–500 nmol concentrations.

Preparation of reagents
Reagents for the mitochondrial isolation procedure
Extraction buffer A was an isotonic solution of 10 mM of N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; pH, 7.5), which contained 200 mM of mannitol, 70 mM of sucrose, and 1 mM of ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA). Ultrapure water was used throughout the procedure. The buffer was kept at 4°C before use. The concentrated buffer was refrozen at −20°C when not in use.

The storage buffer was 10 mM of HEPES (pH 7.4), which contained 250 mM of sucrose, 1 mM of ATP, 0.08 mM of ADP, 5 mM of sodium succinate, 2 mM of KH2PO4, and 1 mM of DTT and was diluted 5 x as described in the instruction brochure.

Albumin (a concentrated solution of 50 mg/mL in 10 mL) was prepared by diluting an aliquot of 10 mg/mL of albumin with a mixture of homogenate and buffer (see procedure). Trypsin was prepared by dissolving an aliquot of trypsin in 1× extraction buffer A at 0.25 mg/mL. The 1× extraction buffer contained 0.25 mg/mL of trypsin and was kept at 4°C before use.

Total protein concentration (TPC) (Bradford Assay) preparation
Protein assay dye reagent concentrate was diluted in a 1:4 ratio; for example, 1 mL of dye reagent was diluted with 4 mL of chilled ultrapure water. The diluted dye reagent could be kept at room temperature for 2 weeks.

The bovine serum albumin (BSA) standard was prepared by diluting 1 mg of BSA lyophilized powder with 1 mL of ultrapure water. Once the BSA standard stock was ready, it was diluted to a 50–500 μg/mL concentration range.

Preparation of reagent for JC-1 assay
JC-1 stain was prepared by dissolving the vial of concentrated JC-1 stain in 25 mL of dimethyl sulfoxide (DMSO). The reconstituted stain was kept at −20°C. An aliquot of JC-1 stain was diluted 5-fold with DMSO for further use in the JC-1 assay.

JC-1 assay buffer was a solution of 20 mM of 3-(N-morpholino) propane sulfonic acid (MOPS; pH, 7.5) that contained 110 mM of KCl, 10 mM of ATP, 10 mM of MgCl2, 10 mM of sodium succinate, and 1 mM of EGTA. The JC-1 buffer was prepared by diluting an aliquot of the buffer 5-fold with chilled ultrapure water. The diluted buffer was kept at 4°C before use, and the concentrated buffer could be kept at −20°C.

Reagents for SDH Assay
The SDH assay buffer was thawed to room temperature prior to utilization. The buffer was kept at −20°C. The SDH substrate mix was prepared by reconstituting the vial with 220 mL of ultrapure water and mixing by pipetting. The reconstituted solution was aliquoted and stored at −20°C. The substrate mix was kept on ice during the procedure. The SDH probe was thawed to room temperature before use. The SDH positive control was reconstituted with 100 mL of SDH assay buffer and mixed well by pipetting. The SDH positive control was aliquoted and kept at −80°C. The SDH positive control was kept on ice during the procedure. The DCIP standard was prepared for 0, 8, 16, 24, 32, and 40 nmol concentrations.

Procedure
Tissue preparation
The tissue sample was obtained from tissue resection before and after applying Saint Thomas’s cardioplegic solution. Each heart tissue sample was obtained from tetralogy of Fallot corrective surgery in a 15-ml Falcon tube filled with 2 mL of 1× phosphate-buffered saline pre-chilled at 4°C. The tube was kept in the ice box at 4°C, and the tissue sample was used for the isolation procedure ≥1 h after resection.

Heart mitochondrial isolation
During the isolation procedure, all reagents were kept in an ice box. The reagents were chilled at 4°C before the procedure. Resected tissues were trimmed off the remaining connective tissues, then weighed. The range of tissue weights used for this procedure was 50–200 mg. The tissue sample was washed with two volumes of extraction buffer A (MITOISO1 kit; Sigma-Aldrich) according to weight. The tissue sample was minced in a pre-chilled Petri dish on ice. The minced tissue was then transferred into a 2-mL centrifuge tube and incubated for 3 min with 10 volumes of extraction buffer A containing 0.25 mg/mL of trypsin. After incubation, the sample was spun down in a centrifuge for 10 s. Then, the supernatant was discarded, and the remaining pellet was incubated for 20 min with eight volumes of extraction buffer A containing 0.25 mg/mL of trypsin. After 20 min of incubation, albumin was added to the sample until the albumin concentration was 10 mg/mL. Homogenization was then performed with an ultrasonic cell disruptor for 30-
40 seconds with a brief pause every 10 times up and down movement to prevent overheating. The homogenate was further transferred into a 2-mL centrifuge tube and was centrifuged at 2500 rpm for 5 min. The supernatant was transferred into another 2-mL centrifuge tube and was further centrifuged at 10,800 rpm for 10 min. The second supernatant was discarded, and the resulting pellet was resolved with storage buffer (40 mL of storage buffer was added for every 100 mg of tissue weight).

TPC/Bradford assay
The TPC assay was determined using the Bradford method, based on Coomassie Brilliant Blue G-250 dye binding to the protein, and measured at 595 nm. The mitochondrial suspension (post-isolation procedure) was optimized by first measuring the optical density (OD) value before starting the assay. If the OD value was >1.8, the sample was diluted by 100x; If the OD value was >1.5, the sample was diluted by 50x; and so forth.

Ultrapure water was used for all dilutions. After dilution, 10 mL of standard and diluted samples were transferred into each well, and 200 mL of Bradford dye reagent was added to each well. The sample and dye reagent were homogenized by pipetting slowly or using a plate shaker. Samples were incubated at room temperature for ≥5 min but <1 h. Samples were then measured at 595 nm.

Cationic carbocyanine JC-1 dye uptake assay
The mitochondrial suspension sample with a known concentration value was diluted with 1× storage buffer into a 1-mg/mL concentration. The JC-1 assay mixture was prepared in a 2-mL centrifuge tube by mixing 1.9 mL of 1× JC-1 assay buffer, 40 mL of 1 mg/mL of the sample, 60 mL of 1× storage buffer, and 2 mL of JC-1 stain. The tube was inverted to homogenize the mixture, then incubated at room temperature in the dark for 7 min. The sample was next transferred into a cuvette or a dark microscope well, then assessed using a spectrophotometer with an excitation wavelength of 490 nm (slit, 5 nm) and emission wavelength of 590 nm (slit, 7.2 nm).

The JC-1 dye uptake assay result was calculated as follows according to the protocol provided by Sigma-Aldrich.9

\[
\text{FLU/mgP} = \frac{\text{[(FLU) \times df]} \times V}{(C \times \text{df})}
\]

Where FLU is fluorescence units, mgP is milligrams of protein, df is the dilution factor necessary to prepare 1 mg/mL of suspension (JC-1 uptake assay), ΔFL = FLU \_\text{sample} - FLU \_\text{blank}, C is the concentration (mgP/mL), and V is the volume of mitochondrial sample in milliliters.

SDH activity assay
The DCIP standard was prepared as explained in section 2.4. We added 5–50 mL of the sample into each well. If the sample volume was >50 mL, SDH assay buffer was added until the final volume of the sample and buffer in the well was 50 mL. The blank sample well was supplemented with 50 mL of SDH assay buffer, and added 20 mL of SDH positive control to the last well. The sample and positive control reaction mixture were prepared by mixing 46 mL of SDH assay buffer, 2 mL of SDH substrate mix, and 2 mL of SDH probe in each well. The reaction mixture for the blank sample was prepared by mixing 48 mL of SDH assay buffer and 2 mL of SDH probe. These reaction mixtures were added to the appropriate wells, not the DCIP standard wells. We pipetted or placed the microplate on the horizontal shaker to ensure the mixtures were well-homogenized. The microplate was incubated at 25°C in the spectrophotometer. The absorbance was measured at 600 nm at the initial time of reading. Incubation continued at 25°C, with a measurement taken at 600 nm every 5 min for 30 min. The final absorbance was measured when the most active sample had a value near or exceeding the end of the linear range of the standard curve/highest DCIP standard.

Sample preparation for TEM imaging
The sample was washed first by discarding supernatant and then adding PBS 1x into the pellet. Fixation was done by adding 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Post-fixation was followed by adding 2% osmium tetroxide and 2.5% K3Fe (CN)6 in distilled water. Serial dehydration of the sample was done with ethanol, followed by infiltration with propylene oxide and embedding in spur resin. Triple lead citrate was added prior to observation under TEM. The sample was observed using a JEOL JEM-1010 electron microscope at 80 kV, with 8000–12000 magnification.

Statistical analysis
Data distribution was analyzed with the Saphiro-wilk test. Data that follows normal distribution will be presented in mean with standard deviation. Data not following normal distribution will be presented in the median and range (min-max).

RESULT
Ten patients with Tetralogy of Fallot for mitochondrial isolation were examined. The tissue weights and mitochondrial isolate protein concentrations are depicted in Table 1. Mitochondrial isolation qualities were measured through JC-1 uptake and SDH activity assay.

Mitochondrial isolation and TPC measurement procedure
The expected macroscopic finding of the mitochondrial pellet in the storage buffer is shown in Figure 1. Mitochondrial isolation was performed on fresh tissue maintained at low temperatures. After mitochondrial isolation, the protein concentration was tested using the Bradford test. The results are presented in Table 1, Figure 2, and Figure 3. Mitochondrial isolation qualities were measured through JC-1 uptake and SDH activity assay.

JC-1 uptake assay
JC-1 assay was used to measure mitochondrial inner membrane potential. The results are presented in Table 2 and Figure 4. One missing data from the pre-ischemia sample was due to a light cover leak during incubation.

SDH activity assay
SDH activity assays were used to measure the purity of mitochondrial isolates through their enzyme activity. The results are presented in Table 3 below.
Table 1. Tissue weight and mitochondrial isolates vs. protein concentration

<table>
<thead>
<tr>
<th>Condition</th>
<th>Tissue weight (mg)a</th>
<th>Protein concentration (mg/mL)b</th>
<th>Total Protein (mg)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-ischemic (n=10)</td>
<td>64.92 ± 35.27</td>
<td>7.65 (2.14–36)</td>
<td>0.2412 (0.0968-0.6577)</td>
</tr>
<tr>
<td>Ischemic (n=10)</td>
<td>109.70 ± 56.96</td>
<td>8.38 (1.84–31)</td>
<td>0.3939 (0.1931-2.2163)</td>
</tr>
<tr>
<td>Post-reperfusion (n=10)</td>
<td>79.00 ± 44.95</td>
<td>8.19 (1.56–32.6)</td>
<td>0.5502 (0.1370-2.5650)</td>
</tr>
</tbody>
</table>

Note: a: Mean ± SD; b: Median (min-max)

Figure 1. Macroscopic visualization of expected mitochondria pellet.

Figure 2. Total protein of mitochondria isolates (median with range).

Figure 3. Mitochondria yield (median with range); total protein of mitochondria isolates (in mg) divided by tissue weight (in mg).

DISCUSSION

Mitochondria study has been an important issue in understanding the pathophysiology of many human diseases, particularly the heart. However, ethical considerations make living heart tissues difficult to obtain. Tetralogy of Fallot patients offers a way to conduct this kind of research through correction surgery in which resected non-functional infundibular muscle can be used as a tissue source.

The MITOISO1 kit from Sigma-Aldrich was used to ensure time- and cost-efficiency. The procedure used a minimal amount of tissue, i.e., a yield of 50–200 mg of tissue. The method of homogenization involved using a sonicator. Using a sonicator instead of the PTFE pestle and glass tube, as recommended by Graham et al., was expected to produce a greater yield and intact mitochondria. Bahnemann et al. found that the ultrasound method is superior in cell disruption efficiency, mitochondrial yield, and membrane integrity. The heat production from ultrasound cell disruption is a critical factor to consider. We found that keeping the tube on ice while performing sonication and applying the sonicator up and down in three cycles of 10 seconds was optimal. Frezza et al. found that, macroscopically, damaged mitochondria lead to cloudy sediment. We confirmed this finding and observed that vigorous cell disruption and homogenization create a cloudy suspension with little or no sedimentation. Another consideration is the utilization of trypsin in the protocol. Trypsin inactivates the rotenone-insensitive NADH-cytochrome c reductase system in the mitochondria’s outer membrane. High levels (1 mg/ml) of trypsin severely inhibit protein import but do not destroy the integrity of the outer membrane. Since trypsin may strip off the membrane, the isolated mitochondria’s integrity must be carefully checked.

Notably, we also learned that trimming the white fibrous tissue and lipids other than muscle tissue macroscopically is essential since contamination of tissues will lead to significantly lower yields. The macroscopic finding of the expected mitochondria pellet can be viewed in Figure 1. In this study, a small change in centrifugation speeds did not significantly affect mitochondria yield or quality. However, the cold chain is crucial when mitochondria isolation is in its early stages. Several studies have emphasized the importance of maintaining cold temperatures when working with fresh mitochondria. We also learned that the amount of organelle yield did not achieve the standard in the kit’s technical bulletin. First, the yield of mitochondria isolation...
Table 2. JC-1 Uptake Assay

<table>
<thead>
<tr>
<th>Condition</th>
<th>Fluorescence Units (FLU/mgP)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-ischemic (n=9)</td>
<td>11,529 ± 6,970</td>
</tr>
<tr>
<td>Ischemic (n=10)</td>
<td>9,945 ± 6,230</td>
</tr>
<tr>
<td>Post-reperfusion (n=10)</td>
<td>12,430 ± 9,936</td>
</tr>
</tbody>
</table>

Note: *: Mean ± SD

Table 3. SDH activity

<table>
<thead>
<tr>
<th>Condition</th>
<th>SDH Activity (nmol/min/L)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-ischemic (n=10)</td>
<td>18.12 (1.20–23.46)</td>
</tr>
<tr>
<td>Ischemic (n=10)</td>
<td>17.45 (1.08–65.92)</td>
</tr>
<tr>
<td>Post-reperfusion (n=10)</td>
<td>17.68 (0.74–33.57)</td>
</tr>
</tbody>
</table>

Note: In accordance with the DCIP value, *: Median (min-max)

Figure 4. JC-1 uptake (FLU/mgP).

Figure 5. TEM imaging shows (A) Slightly dilated mitochondria (red arrow), >1 µm. (B) Normal size mitochondria (blue arrow), 0.5-1 µm and ruptured oedematous mitochondria in between.

depends on the type of tissue. Brain and liver tissues are easier to isolate, while skeletal muscle tissue, which consists of hard and gelatinous components, is harder to isolate. Additionally, heart muscle tissue is chronically exposed to hypoxia in tetralogy of Fallot patients. Using electron transmission microscopy, Shinde et al. found that mitochondria from tetralogy of Fallot patients had a dilated, irregular shape and appeared to have ruptured. These slightly dilated mitochondria can be viewed in our TEM imaging, as shown in Figure 5. The normal size of mitochondria is 0.5-1 µm. This could be the reason for lower yield when tissues from tetralogy of Fallot patients are used for mitochondria isolation.

We chose JC-1 based on its ability to maintain membrane potential as an active mitochondrion. The mitochondria isolate in this protocol would be a sample source of clinical experimental study with a limited time frame. We would do several assays while maintaining fresh, functional mitochondria. Due to limited resources and infrastructure facilities in a developing country like Indonesia, JC-1 is a feasible measure of active and functional mitochondria.

SDH activity from isolated mitochondria showed a comparable value to results of previous work from Shinde et al. Isolation of mitochondria from fresh human heart tissue is rarely performed. This study shares our center’s first experience performing fresh heart muscle tissue from tetralogy of Fallot patients.

Limitations of the study

We know that measuring mitochondrial oxygen consumption using respirometry is the golden standard for assessing mitochondria viability. We are unable to perform a respirometry study and chose the JC-1 assay due to several reasons. The amount of heart tissue, i.e., infundibulum resected for mitochondria isolation, has become our main concern. Furthermore, isolated mitochondria are used for further study. Mitochondria oxygen consumption assay needs a significant number of isolated mitochondria using the conventional method. The current protocol of correction surgery for tetralogy of Fallot is performed at a younger age. Because of this, the infundibulum resected will be smaller than the previous experiences; therefore, the number of isolated mitochondria will not be adequate for conducting various assays, including, e.g., mitochondrial oxygen consumption and mitochondria swelling. We have followed the method described by the commercial kit and believe that the method has been validated for the isolation of mitochondria. Another reason is that certain assays like mitochondria swelling that need fresh samples, should be carried out immediately after mitochondria isolation. Since time limitation is another crucial factor, a faster method such as JC-1 could be more feasible.

CONCLUSION

Human mitochondria of heart tissues with intact membrane integrity have been successfully isolated using a commercial kit. Key factors must be considered: cold temperature maintenance, tissue contamination, and optimal sonicator application technique. The use of isolated mitochondria for further experiments should consider the effect of trypsin as applied in this method which may affect the life span of the isolated mitochondria.

ETHICAL CLEARANCE

The study was conducted after ethical clearance was granted by the Board of Bali Medical Journal 2023; 12(2): 2239-2244 | doi: 10.15562/bmj.v12i2.4599

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AUTHOR CONTRIBUTION
Contributors JKH, AW, NAZ, FDS, RFS, and MMD planned and concepted the study. JKH and AW, and NAZ conducted the study. JKH, AW, NAZ, FDS, and RFS processed and analyzed study data. FDS and RFS supervised the study. JKH, FDS, and RFS developed the theoretical framework. JKH, AW, NAZ, FDS, RFS, and MMD wrote the manuscript. All authors discussed the results and commented on the manuscript. All authors revised the manuscript.

CONFLICT OF INTERESTS
The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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DATA AVAILABILITY STATEMENT
The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

REFERENCES

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