Methodological Issue of Isolating Mitochondria in Acute Injury: A Rat Cardiac Arrest Model

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Abstract

Background Mitochondrial studies are key to understanding the pathophysiology of cardiac arrest (CA), however there is a potential risk of sampling bias during the mitochondrial isolation process. This study aimed to evaluate the dysregulation of mitochondrial respiratory function after CA while testing the sampling bias induced by the mitochondrial isolation method.

Methods and Results Adult rats were subjected to 10-minunte asphyxia-induced CA. Thirty minutes after resuscitation, brain and kidney mitochondria from sham and CA group animals were isolated (n=8, each). The mitochondria quantity, expressed as protein concentrations (isolation yields), was determined and then oxygen consumption rates were measured. ADP-dependent (state-3) and ADP-limited (state-4) respiration activity were compared between the groups. The mitochondrial quantity was evaluated by citrate synthase (CS) activity and cytochrome c concentration measured independently from isolation yields. The state-3 respiration activity and isolation yield in the CA group declined significantly as compared to those in the sham group (brain, $p < 0.01$; kidney, $p < 0.001$). The CS activity in the CA group declined significantly as compared to that of the sham group (brain, $p < 0.01$; kidney, $p < 0.01$). Likewise, cytochrome c levels in the CA group had decreasing trends (brain, $p = 0.08$; kidney, $p = 0.25$).

Conclusions CA decreased mitochondrial respiration activity and the quantity of mitochondria isolated from the tissues. Due to the nature of fragmented or damaged mitochondria membranes caused by this acute injury model, it is plausible that the mitochondrial function measured in the acute injury animal model might be underestimated.

Introduction

Cardiac arrest (CA) is a major public health issue affecting approximately 600,000 patients each year in the United States\(^1\). Various pathophysiological changes occur during and after CA. These include mitochondrial dysfunction followed by multiple organ failure and prolonged neurological dysfunction, which increases the mortality of CA patients\(^2\). Some research suggests therapies effective against mitochondrial dysfunction may improve the mortality and neurocognitive impairment after CA in a rodent animal model\(^3,4\). These results imply that mitochondria might be an effector or a target to improve survival of CA patients. Thus, studies focusing on mitochondrial pathophysiology are imperative to understand the mechanism underlying the complex biological responses preceded by ischemia/reperfusion injury.

Mitochondrial research using experimental models places an important role on its way of sampling, and mitochondrial isolation is the foremost method of understanding the pathophysiology of mitochondrial dysfunction\(^5,6\). Therefore, the assessment of mitochondrial purity is in high demand nowadays and analyses of different mitochondrial isolation methods have been reported by several researchers\(^7,8\).
Picard et al. investigated the potential complications arising from isolation methods by structurally and functionally disrupting the mitochondria. Their data indicate that the functional alterations induced by mitochondrial isolation are attributed to a disruption of mitochondrial morphology induced by mechanical homogenization and a loss of soluble proteins and of other molecules from the mitochondrial matrix. Given animal models with acute injuries likely have mitochondria disruption in nature, Picard's concern may become more significant in these experimental settings. However, although Picard discussed a potential risk of sampling bias because of the loss of disrupted mitochondria during the isolation process, no studies have shown such a bias in acute injury animal models.

We previously reported responses to resuscitation of tissues including brain and kidney following prolonged CA by examining mitochondrial respiration using isolated mitochondria. Herein, we evaluated mitochondrial dysfunction of brain and kidney tissues after CA while testing the sampling bias induced by our mitochondrial isolation method by measuring multiple indicators of the purity of mitochondrial samples.

**Materials And Methods**

The Institutional Animal Care and Use Committees of Feinstein Institutes for Medical Research approved this study protocol. All methods were performed in accordance with the Guide for the Care and Use of Laboratory Animals, American Veterinary Medical Association Guidelines on Euthanasia, and all other related regulations. We performed all instrumentation and surgical preparation according to our previously described protocol. This report is in compliance with the Animal Research, Reporting of *In Vivo* Experiments guidelines.

**Animal preparation and interventions**

This is a non-randomized, prospective, experimental controlled study. We performed all instrumentation according to the previously described protocol. In brief, the total number of 16 adult male Sprague-Dawley rats (450–550 g, Charles River Laboratories) were anesthetized with 4% isoflurane (Isosthesia, Butler-Schein AHS) and intubated with a 14-gauge plastic catheter (Surflo, Terumo Medical Corporation). We used male rats to avoid potential differences among animal subjects that may be caused by hormonal or genetic differences rather than differences from the experimental intervention (i.e., to minimize potential sources of variability). Rats were mechanically ventilated (Ventilator Model 683, Harvard Apparatus) and anesthesia was maintained with 2% isoflurane and a fraction of inspired O\textsubscript{2} (FIO\textsubscript{2}) equivalent to 0.3. Core temperature was maintained at 36.5 ± 1.0 °C during the surgical procedure. Animals were assigned into two groups: CA group and sham group (n=8, for each group). The CA group included rats successfully resuscitated with cardiopulmonary resuscitation (CPR) from a 10-minute asphyxia. The sham group included rats treated without asphyxia or CPR. After instrumentation, neuromuscular blockade was achieved by slow intravenous administration of 2 mg/kg of vecuronium bromide (Hospira) for the CA group rats and asphyxia was induced by turning off the ventilator. After 10
minutes, mechanical ventilation was restarted at an FIO₂ of 1.0 and manual chest compression CPR was delivered. Chest compressions were performed with 2 fingers over the sternum at a rate of 260 to 300 per minute. Immediately after beginning CPR, a 20 µg/kg bolus of epinephrine was given to rats through a venous catheter. Following return of spontaneous circulation, defined as systolic blood pressure > 60 mmHg, CPR was discontinued. For rats in the sham group, the same surgical procedures were performed, including vecuronium and epinephrine injections. Mechanical ventilation was discontinued 30 minutes after CPR, after which rats were euthanized, and tissues were collected for mitochondrial experiments.

All surgical procedures including resuscitation were performed by one investigator and therefore blinding procedures were not applied. Allocation concealment is not possible in nature of using acute injury model as opposed to healthy control animals. Therefore, we used sham-surgery animals as our control group in order to reduce the risk of exaggerated effects. The other investigator independently and unbiasedly performed the following biochemical assays.

Isolation of Brain and Kidney Mitochondria and Evaluation of Mitochondrial Respiratory Function

Mitochondria samples were collected from the sham group and the CA group. All procedures were performed at 4°C. The brain and the kidney mitochondria were isolated according to the modified procedure of Scholte et al. Briefly, tissues were immediately placed in mitochondrial isolation buffer composed of 210 mM mannitol, 70 mM sucrose, 10 mM HEPES (pH 7.3), and 0.2 mM EGTA with 0.2% w/v fatty acid free-BSA (MESH-BSA). Spinal cord, extra ventricular tissue of the brain, and fats of both tissues were removed in MESH-BSA buffer. Next, tissues were blot-dried on filter papers, weighed, and placed in MESH-BSA buffer. After mincing and washing, the tissues were diluted in MESH-BSA buffer, and subsequently homogenized using a teflon/glass motor-driven homogenizer (Model BDC2010, Caframo Lab Solutions) at 8 strokes for the brain, and 3 strokes for the kidney. Homogenates were centrifuged at 5,600×g for 1 minute and supernatants were poured into a polycarbonate tube and centrifuged at 12,000×g for 6 minutes. For brain samples, homogenization was performed twice, and pooled supernatants were centrifuged. The supernatants of the brain homogenization were poured out gently until the synaptosomes layer reached the top, and the remaining loose pellets were suspended with 20 ml of 12.5% percoll (GE Healthcare) in MESH buffer without BSA and centrifuged at 12,000×g for 6 minutes. The kidney homogenization was not required this process due to the lack of myelin synaptosome structures. For both brain and kidney samples, supernatants were gently decanted with pipettes without disturbing mitochondrial pellets (usually ~200 µL buffer remains). Finally, pellets were resuspended in 20 mL of MESH buffer and centrifuged at 12,000×g for 6 minutes. After mitochondria pellets were collected and their volumes measured, mitochondria concentrations were determined via BCA assay using BSA as protein standard, and then isolation yields (mg protein/g tissue) were calculated.

Subsequently, oxygen consumption was measured using a Strathkelvin oxygen electrode (30°C). Isolated mitochondria samples were diluted into an oxygen electrode mix buffer containing 80 mM KCl, 50 mM
MOPS, 1 mM EGTA, 5 mM KH2PO4, and 1 mg defatted BSA/mL at pH 7.4\textsuperscript{11}. ADP-dependent (state-3), ADP-limited (state-4), and DNP-dependent (uncoupled) respirations were measured in 150 µL of the mitochondrial suspension (0.5 mg/mL) using glutamate and malate as substrates. Rates of substrate oxidation were expressed as nmol/min/mg protein. Respiratory control ratio (RCR) was calculated as the ratio of state-3 to state-4 respiration.

**Citrate Synthase Activity Assay**

Citrate synthase (CS) activity of each isolated mitochondria sample was measured using CS activity assay kit (MAK193, Sigma-Aldrich), as per manufacturer’s technical bulletin. Briefly, after preparing reagents, isolated mitochondria samples were diluted with a CS assay buffer. Diluted samples, GSH standard solutions, and positive controls were applied to 96-well plates, and then reaction mixes containing CS developer and CS substrate mix were added. Specific absorbance at 412 nm was measured every 5 minutes continuously for 30 minutes. Finally, the amount of GSH was calculated from a standard curve, and CS activity was calculated according to the GSH amount and the reaction time.

**Cytochrome C ELISA**

Cytochrome c concentration of each isolated mitochondria sample was measured using cytochrome c profiling ELISA kit (ab110172, abcam), as per manufacturer’s instructions. Briefly, after preparation of reagents, isolated mitochondria samples were diluted with a solution containing 0.1% sodium dodecyl sulfate (SDS) and centrifuged at 15,000\(\times\)g for 20 minutes. Supernatants of samples and standards were applied to the supplied 96-well microplates and incubated for 3 hours. After antigen-antibody reaction using a detector antibody against cytochrome c and HRP label, HRP development solution was added. Absorbance data was detected at 600 nm using a plate reader (Spark\textsuperscript{®}, TECAN). Finally, cytochrome c concentration of each sample was calculated according to the standard curve.

**Statistical Analysis**

Data are shown as a mean and standard deviation (SD) for continuous variables. An unpaired two-tailed Student t-test was used to compare two independent groups. Two-tailed P values were calculated, and \(p < 0.05\) was considered statistically significant. SPSS 25.0 (IBM, Armonk) was applied for statistical analyses.

**Results**

**Cardiac Arrest Decreases Mitochondrial Respiratory Function**
Table 1 and Table 2 show basal characteristics and oxygen consumption rates of the isolated mitochondria from the brain and kidney, respectively. Brain tissue weight was heavier in the CA group compared to the sham group (2.03 ± 0.05 and 1.91 ± 0.06 g, \( p < 0.01 \)), but kidney tissue weight was the same for both groups (1.63 ± 0.09 and 1.62 ± 0.09 g, \( p = 0.78 \)). Isolated mitochondria volume of CA group was significantly lower than that of the sham group in brain and kidney tissues (brain, 146 ± 42 and 196 ± 44 µl, \( p < 0.05 \); kidney, 554 ± 68 and 692 ± 113 µl, \( p < 0.05 \), respectively). Likewise, isolation yield of the CA group was significantly lower than that of the sham group in both tissues (brain, 2.71 ± 0.49 and 4.29 ± 0.82 mg protein/g tissue, \( p < 0.001 \); kidney, 18.4 ± 1.7 and 22.6 ± 1.3 mg protein/g tissue, \( p < 0.001 \), respectively).

### Table 1

| Measurement                        | Sham (n = 8) | CA (n = 8) | \( p \) value |
|------------------------------------|--------------|------------|--------------|
| Rat weight (g ± SD)                | 509 ± 26     | 513 ± 18   | 0.7247       |
| Tissue weight (g ± SD)             | 1.91 ± 0.06  | 2.03 ± 0.05| 0.0010**     |
| Mitochondria volume (µl ± SD)      | 196 ± 44     | 146 ± 42   | 0.0369*      |
| Isolation yield (mg protein/g tissue ± SD) | 4.29 ± 0.82 | 2.71 ± 0.49| 0.0004***    |
| State-3 activity (nmol/min/mg protein ± SD) | 286 ± 50     | 209 ± 26   | 0.0016**     |
| State-4 activity (nmol/min/mg protein ± SD) | 45.4 ± 12.6  | 43.9 ± 14.7| 0.8296       |
| RCR ± SD                           | 6.57 ± 1.42  | 5.17 ± 1.63| 0.0881       |

*CA, cardiac arrest; SD, standard deviation; RCR, respiratory control ratio

*\( p < 0.05 \) in all variables

**\( p < 0.01 \) in all variables

***\( p < 0.001 \) in all variables
Table 2
Basal Characteristics and Oxygen Consumption Rates of the Isolated Mitochondria from the Kidney

| Kidney       | Sham (n = 8) | CA (n = 8) | p value |
|--------------|--------------|------------|---------|
| Measurement  |              |            |         |
| Rat weight   | 509 ± 26     | 513 ± 18   | 0.7247  |
| Tissue weight| 1.62 ± 0.09  | 1.63 ± 0.09| 0.7780  |
| Mitochondria volume | 692 ± 113  | 554 ± 68   | 0.0105* |
| Isolation yield | 22.6 ± 1.3  | 18.4 ± 1.7 | < 0.0001*** |
| State-3 activity | 269 ± 55  | 148 ± 37   | 0.0001*** |
| State-4 activity | 42.8 ± 19.9 | 27.6 ± 6.5 | 0.0602 |
| RCR ± SD     | 6.94 ± 1.96  | 5.75 ± 2.58| 0.3166  |

CA, cardiac arrest; SD, standard deviation; RCR, respiratory control ratio

*< 0.05 in all variables

***< 0.0001 in all variables

The state-3 respiration activity of the brain and kidney mitochondria in the CA group declined significantly as compared to those in the sham group (brain, 209 ± 26 and 286 ± 50 nmol/min/mg protein, p < 0.01; kidney, 148 ± 37 and 269 ± 55 nmol/min/mg protein, p < 0.001, respectively). In contrast, we did not observe significant differences in state-4 respiration activity in either tissue. As a result, the RCR showed decreasing trends after CA in both tissues.

Cardiac Arrest Decreases Citrate Synthase Activity in the Tissue Samples

Figure A shows the results of CS activity assay of isolated mitochondria from the brain and the kidney. The CS activity of brain and kidney mitochondria in the CA group declined significantly compared to the activity in the sham group (brain, 2.40 ± 1.01 and 4.19 ± 0.89 µmol/min/g tissue, p < 0.01; kidney, 5.07 ± 1.92 and 7.73 ± 1.07 µmol/min/g tissue, p < 0.01, respectively).

The Results of Cytochrome C in the Tissue Samples
Figure B shows the results of cytochrome c profiling ELISA for isolated mitochondria from the brain and the kidney. Although there was no statistical difference, cytochrome c levels of the brain and kidney mitochondria in the CA group decreased compared to levels in the sham group (brain, 10.8 ± 4.2 and 17.0 ± 8.4 μg/g tissue, \( p = 0.08 \); kidney, 146 ± 26 and 162 ± 27 μg/g tissue, \( p = 0.25 \)).

**Discussion**

Our data indicates that 10-minute asphyxia CA followed by 30-minute resuscitation decreases mitochondrial respiratory function of the brain and the kidney. These results are in line with our previous reports and other investigators who studied mitochondrial dysfunction in ischemia/reperfusion injury models\(^{12}\). Although the underlying mechanism of decreasing isolation yield in tissues remains unclear, alteration of cell viability and mitochondrial physiological activity after injury might have affected the mitochondrial volume after isolation\(^5\). The current study is highlighted by our findings indicating a decrease in mitochondrial quantity, which was measured by three independent assays, in our acute injury rodent model. Our finding is of importance and should be valued in the broad area of mitochondrial research using acute injury model due to the lack of knowledge of potential bias generated by mitochondrial isolation methods.

Besides yield (protein amounts) as an indicator of mitochondrial quantity in the isolated mitochondrial sample, it is possible that protein contents of isolated mitochondria – from pure mitochondria, cytosol, and any other organelle – could affect the differences in results obtained from sham (non-injured) and CA (injured) rats. In other words, the purification method of mitochondria could affect sample volume after injury. In order to verify a decrease in mitochondria quantity in injured tissues, we performed CS activity assay and cytochrome c ELISA independently from the number of mitochondrial yields of our mitochondria samples.

CS plays a central role in the mitochondrial oxidative capacity during the first step of the citric acid cycle, and is commonly utilized as a quantitative enzyme marker for the presence of intact mitochondria. Cytochrome c is a key protein for cellular respiration that contains an iron porphyrin cofactor. Cytochrome c is involved primarily in the electron transport chain of mitochondrial inner membrane, and is widely believed to be localized solely in the mitochondrial inner membrane space under normal physiological conditions. These assays are independent and mitochondria specific, which is why we used them as independent indicators of mitochondrial quantity in isolated mitochondria samples.

Collectively, our findings suggest that CA might cause decreased mitochondrial respiration activity and reduced mitochondria purification amounts in the brain and the kidney. Even though the quantity of mitochondria in injured tissues decreased, mitochondrial respiration activity was standardized and adjusted by the number of yield according to a widely accepted method.

Cytochrome c is also known to be extruded into the soluble cytoplasm through pores in the outer membrane during the early phase of apoptosis. In clinical settings, Donnino et al. reported non-survivors
of post cardiac arrest patients had higher cytochrome c levels in plasma samples as compared to survivors\textsuperscript{13}. Accordingly, if we consider that isolated mitochondria in CA rats could release cytochrome c and mitochondria fragments to the cytosol and bloodstream, then the result of isolated mitochondria from CA rats containing lower amounts of cytochrome c might represent a potential selection bias of the samples, in which we lose the information from damaged mitochondria by the isolation procedure. It may be inferred that if we could obtain the information of damaged/fragmented mitochondria in our mitochondria functional analysis, the results could be even worse than what we have observed given the fact that mitochondria require intact inner and outer membrane to complete oxidative phosphorylation.

This study is subject to several limitations. First, our isolation procedure can enhance or attenuate the trend in decrease of mitochondria quantity in injured tissues. Multiple isolation procedures are still being reported until today particularly in regard to centrifugation speed. The initial centrifugation is key to separating mitochondria from other cytosolic organelles, for which the centrifugation speed ranges from 400x$g$ to 30,700x$g$\textsuperscript{14,15}. We did not test other isolation procedures that might worsen or improve selection bias of mitochondria samples. Second, we did not perform mitochondrial DNA measurement, which might strengthen the result of evaluating the quantity of isolated mitochondria.

**Conclusions**

In our isolated mitochondria samples from the brain and the kidney, CA decreased mitochondrial respiration activity and the quantity of isolated mitochondria. It is important to evaluate the quantity of mitochondria and to use its value for adjusting the result of mitochondrial function in order to standardize and compare the results between experimental conditions.

**Declarations**

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None

**Source of Funding**

None

**Authors’ Contributions**

K. Shinozaki has full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. T. Aoki and Y. Okuma contributed equally to this work. K. Shinozaki and LB. Becker designed the conception of the study; K. Shinozaki, T. Aoki, and Y. Okuma performed acquisition of data; T. Aoki and Y. Okuma analyzed data; all authors made interpretations of
data; T. Aoki drafted and K. Shinozaki critically edited the manuscript; K. Shinozaki supervised the project. All authors added intellectual content of revisions to the paper and gave full approval of the version to be published.

Conflict of Interest and Sources of Funding

KS and LBB have a patent right of metabolic measurements in critically ill patients. KS has a grant/research support from Nihon Kohden Corp.. LBB has a grant/research support from Philips Healthcare, the NIH, Nihon Kohden Corp., Zoll Medical Corp, PCORI, BrainCool, and United Therapeutics and owes patents including 7 issued patents and several pending patents involving the use of medical slurries as human coolant devices to create slurries, reperfusion cocktails, and measurement of respiratory quotient. Other authors have no known conflicts of interest associated with this study and there has been no significant financial support for this work that could have influenced its outcome.

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