The Role of Oxygen in Cardiopulmonary Resuscitation and Post Resuscitation Period – A Mitochondrial Perspective

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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Abstract

Sudden cardiac arrest is a leading cause of death in the United States and is responsible for more than 300,000 deaths every year. For the last fifty years, the guidelines of cardiopulmonary resuscitation (CPR) have been the standards to revive cardiac arrest patients. However, the major components of CPR have not changed since its birth and the success rate of CPR remains unsatisfied (5-18%). My work presented in the dissertation mainly focuses on the role of oxygen during the resuscitation and post-resuscitation periods as well as correspondent mitochondrial functions. There are four parts in the dissertation. In chapter 2, a rat CPR model was used to demonstrate that oxygen is critical during resuscitation in order to achieve return of spontaneous circulation (ROSC). Additionally, there was no significant difference between the treatment groups (21% oxygen vs. 100% oxygen resuscitation) in terms of survival rates or neurological outcome. In chapter 3, mitochondrial functions were examined in the hearts undergoing 15 minutes of cardiac arrest and 10 minutes of CPR as compared to the hearts undergoing 25 minutes of cardiac arrest. The results suggest that, despite the inherent low oxygen delivery, CPR after prolonged cardiac arrest preserves heart mitochondrial function and morphology. It demonstrates the beneficial effects of CPR from a subcellular perspective for the first time. Preservation of mitochondrial function is reflected by improved mitochondrial respiration, electron transport chain (ETC)
complex activities, and preservation of the ETC proteins. Meanwhile, complex I is the most sensitive ETC complex to ischemic injury and its activity is positively correlated with mitochondrial respiration.

In chapter 4, a rat cardiopulmonary bypass (CPB) model was done to study the effects of hyperoxygenation during the post-resuscitation period. After 25 minutes of normothermic cardiac arrest, hyperoxic reperfusion for one hour with CPB did show advantages compared to normoxic reperfusion in some aspects, including better systemic hemodynamics, less lactic acidosis and a small but significantly better cardiac relaxation. In addition, the benefits were also demonstrated by a better mitochondrial respiratory function. Finally, in chapter 5, a simplified method was developed to concentrate mitochondrial membrane complexes. It is a modified 2-D blue native/blue native PAGE (BN/BN-PAGE) which can be easily done with a mini-gel apparatus. Once completed, the concentrated protein complex in the gel strip is ready for SDS-PAGE or proteomic studies. Unwanted loss of protein complex is minimized, which is critical whenever the amount of mitochondrial sample is limited.
Dedicated to my wife Yun-Ying (澐潁), my adorable daughter Jenny and my late mom
Acknowledgments

I am really grateful to everyone who has ever helped me throughout my PhD study, especially my advisors, Dr. Mark Angelos and Dr. Yeong-Renn Chen, who have provided the wonderful research environment and valuable opinions. I also would like to thank the committee members, Dr. Alevriadou and Dr. Davis, for their intellectual comments on my work. Besides, I deeply appreciate Sverre’s numerous personal and scientific discussions, Chwen-Lih’s (from Chen’s lab) help on mitochondrial work and Alan’s laboratory management. Thanks to Dr. Tomas Drabek and Jason Stezoski, from Safar Center for Resuscitation Research, for technical assistance with the rat bypass model. This study was supported by grants from the American Heart Association (09PRE2170046, Yeh; 60015791, Angelos) and the National Institutes of Health (HL083237, Chen). Finally, without the endless love and support from my wife, this work would never have been accomplished.
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2004-2006…………………………………………….. Emergency physician, Puli Christian Hospital, Puli, Taiwan

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Publications


Fields of Study

Major Field: Biophysics
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Chapter 1: Introduction

1.1 Sudden Cardiac Arrest and Cardiopulmonary Resuscitation (CPR)

With a low survival rate ranging from 5 to 18 percent, sudden cardiac arrest is a leading cause of death in the United States and is responsible for more than 300,000 deaths every year [1]. Although all known heart diseases can result in sudden cardiac arrest, about 60%-80% of the victims have a preexisting coronary artery disease (CAD) and sudden cardiac arrest is the first manifestation of CAD in one-third of the patients. For children and young athletes, long QT syndrome and hypertrophic cardiomyopathy are the two major causes of sudden cardiac arrest [2]. The presenting rhythms of cardiac arrest can be classified as ventricular fibrillation (VF) or ventricular tachycardia (VT), pulseless electrical activity (PEA), and asystole. In general, victims with initial VF or VT have a higher chance of survival than the other two groups. Unfortunately, these shockable rhythms only present in less than one-third of the total cases (22.7% for inpatients, 33.2% for outpatients) and most patients, who present with PEA or asystole, are less likely to survive (Table 1.1) [3].
<table>
<thead>
<tr>
<th></th>
<th>Inpatient</th>
<th>Outpatient</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Location</strong></td>
<td>United States, Canada</td>
<td>United States, Canada, England, Norway, Sweden</td>
</tr>
<tr>
<td><strong>Dates included</strong></td>
<td>1/00–3/04</td>
<td>2/98–6/02 3/02–10/03 1/99–12/00</td>
</tr>
<tr>
<td><strong>Total No. (% survival)</strong></td>
<td>36902 (17.6)</td>
<td>5234 (6.4)</td>
</tr>
<tr>
<td><strong>% VF or VT (% survival)</strong></td>
<td><strong>22.7</strong> (36.0)</td>
<td><strong>33.2</strong> (16.1)</td>
</tr>
<tr>
<td>**% Pulseless electric activity</td>
<td>32.4 (11.2)</td>
<td>24.7 (2.7)</td>
</tr>
<tr>
<td>(% survival)**</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>% Asystole (% survival)</strong></td>
<td>35.3 (10.6)</td>
<td>39.0 (0.9)</td>
</tr>
<tr>
<td><strong>% Unknown rhythm</strong></td>
<td>9.6</td>
<td>3.0</td>
</tr>
</tbody>
</table>

**Table 1.1 Adult survival rates to hospital discharge after inpatient and outpatient arrest (from [3])**

Attempts to resuscitate the cardiac arrest patients can be traced back to the 18th century when people started using different maneuvers to clear the airway of drowning victims [3, 4]. Nevertheless, it was not until 1960 that closed-chest compression was introduced [5] and modern CPR, which includes airway, breathing, circulation and defibrillation, was born. For 50 years, the science of resuscitation has advanced a lot. With accumulation of current knowledge, the CPR guidelines are updated every few years to improve the survival rate of cardiac arrest patients [6]. However, the major components of CPR have not changed since its birth and the success rate of CPR remains unsatisfied. My work presented in the dissertation is a small step to elucidate the underlying mechanisms of CPR. Hopefully, these findings will contribute to the
resuscitation science and help substantially improve CPR techniques and survival in the long run.

1.2 The Role of Ventilation and Oxygen in CPR

Earlier studies in both cardiac arrest patients and animal models have shown two major detrimental effects of excessive ventilation. First, high ventilation rates, due to the positive pressure nature of ventilation of the CPR patient, result in increased intrathoracic pressure which impedes filling of the right heart and thereby limits CPR generated cardiac output [7]. Second, excessive ventilation rates are associated with longer and more frequent breaks in CPR, which interrupts flow and results in an overall decrease in coronary perfusion [8]. There has been a de-emphasis on ventilation as evidenced by the change in the 2005 American Heart Association guidelines [6] from a 15:2 compression:ventilation ratio to a 30:2 ratio and more recently the current recommendation of “compression only CPR” for bystanders [9]. In support of compression only CPR, a recent animal study noted that compression only CPR was associated with improved neurologic outcome when compared to CPR with a 30:2, compression:respiration ratio [10].

However, there may be certain advantages to maintaining at least some level of ventilation during CPR, particularly as applies to re-oxygenation. There are two potentially important benefits associated with positive pressure ventilation during CPR:
(1) amelioration of pulmonary atelectasis which can enhance CPR generated blood flow by decreasing pulmonary resistance and (2) improved oxygenation of the pulmonary blood which serves to increase tissue oxygen delivery during CPR [11]. Lately, an animal study showed improved brain tissue oxygen and improved neurologic outcome during CPR using 10 rather than 2 ventilations per minute [12]. The optimal strategy of ventilation during CPR is still unclear.

The role of oxygen has not been fully and independently resolved from ventilation during CPR and may be a critical component for successful return of spontaneous circulation (ROSC) and neurologic outcome. Despite the debate of using room air or 100% oxygen for neonatal resuscitation [13], there has been little effort to evaluate the optimal oxygen concentration independent of the rate of ventilation during adult CPR. Good quality chest compressions seem to improve the initial ROSC by improving blood flow and thereby increasing oxygen delivery to the globally ischemic heart. The primary determinant of myocardial oxygen delivery is the level of flow, which is generally very low under CPR conditions. The other major determinant of myocardial oxygen delivery is the oxygen content of the blood. Mouth to mouth ventilation during CPR has been shown to deliver a hypoxic fraction of inspired oxygen (FiO₂) of 0.16–0.17 [14]. Increasing blood oxygen content through the use of supplemental oxygen can increase oxygen delivery at every level of CPR generated flow and potentially improve the initial resuscitation of the heart.
1.3 Damage to Mitochondria during Cardiac Arrest

Ischemia, or lack of oxygen, during cardiac arrest causes both ultrastructural and functional abnormalities in mitochondria. The severity of ischemic damage is aggravated as ischemia time increases (Fig. 1.1) [15]. In an isolated working rat heart model, mitochondria start to swell with clearing of matrix density and separation of cristae after 15 minutes of global ischemia. After 40 minutes of arrest, the majority of mitochondria are severely swollen with loss of matrix density as well as widening space between cristae. In addition, subendocardial mitochondria are found to be more vulnerable to ischemic damage than epicardial mitochondria [16]. It is known that, during the initial stage of ischemia, the tricarboxylic acid (TCA) cycle is blocked and anaerobic glycolysis ensues, which results in the accumulation of lactate and breakdown of glycogen. As ischemia continues, glycolysis is finally inhibited due to lactic acidosis and accumulation of NADH [17]. In an isolated rabbit heart model, ischemia induces the decline of creatine phosphate and ATP levels. However, only creatine phosphate, not ATP, partially recovers during reperfusion. NADH/NAD ratio also increases during ischemia while NADPH/NADP ratio decreases. Additionally, glutathione disulfide (GSSG) increases significantly during reperfusion with a sustained low glutathione (GSH) level, which indicates a perturbed thiol redox state [18].
Fig. 1.1 The progression of ischemic damage to mitochondria in the isolated perfused heart (from [15])

The mitochondrial damage by cardiac arrest is also reflected by the deterioration of electron transport chain (ETC) complexes and impairment of calcium homeostasis. It is estimated that complex I activity starts to decline after 15 minutes of ischemia and complex III activity is affected after 25 minutes of ischemia. By 30 minutes of ischemia, cardiolipin content and complex IV activity start to fall, which is considered as the transition from reversible to irreversible myocardial damage [15].
1.4 The Role of Oxygen in Post-resuscitation (Reperfusion) Period

Just like the undetermined status of optimal oxygen during CPR, the optimal oxygen level for the post-resuscitation period remains unclear as well. It has been well recognized that when oxygen is re-introduced into the ischemic tissue, a burst of free radicals, including reactive oxygen species (ROS) or reactive nitrogen species (RNS), are generated within seconds to minutes. The release of free radicals at reperfusion is believed to account for the reperfusion injury [19, 20]. Nevertheless, these conclusions were derived from work in isolated hearts with initial full flow reperfusion or with an in vivo coronary artery ligation model to mimic myocardial infarction, which may not be the case in the setting of CPR. Extensive results from both animal experiments and clinical experience have shown that the blood flow generated by closed-chest compressions can only supply 10-20% of normal myocardial perfusion [21]. Therefore, hearts with cardiac arrest and successful CPR usually undergo a two-stage reperfusion: the “low flow” period during CPR and then full reperfusion after ROSC. This two-stage reperfusion phenomenon makes CPR distinct from the ischemia/reperfusion scenario of acute myocardial infarction, in which opening of an occluded coronary artery results in immediate full flow of the ischemic region. Whether the pattern of free radical generation has also changed during CPR and the post-resuscitation period is still to be answered.

Intuitively, people tend to implicate hyperoxygenation at reperfusion with increased production of ROS which may aggravate reperfusion injury. A series of
studies based on a ventricular fibrillation cardiac arrest and open-chest CPR model have demonstrated that hyperoxic reperfusion (FiO$_2$ = 1.0, pO$_2$ > 450 mmHg) impairs cerebral aerobic energy metabolism and causes more neuronal damage in the hippocampal CA1 region [22, 23]. In a Langendorff buffer-perfused heart model, hearts with hyperoxic reperfusion (pO$_2$ = 700 mmHg) after 30-min ischemia had impaired recovery compared to hearts with normoxic reperfusion (pO$_2$ = 500 mmHg) [24]. Other studies have reported similar adverse effects of hyperoxic reperfusion in kidneys [25] and lungs [26]. Recently in a clinical study, post cardiac arrest patients admitted to the ICU with initial hyperoxia (PaO$_2$ > 399 mmHg) have shown a significant higher hospital mortality than that of patients with initial normoxia or hypoxia [27].

Not all studies are in agreement with the deleterious effects of post resuscitation hyperoxygenation. A rabbit in vivo coronary ligation model showed no increase in infarct size with hyperoxic reperfusion [28]. Lately, clinical trials in acute myocardial infarction patients undergoing primary coronary intervention also demonstrated no harmful but possibly beneficial effects with regional hyperoxemic reperfusion [29, 30]. It is possible that hyperoxygenation after ischemia may cause different responses in different organs. Whether hyperoxygenation should be implemented during the post-resuscitation period is still unknown.
Chapter 2: Oxygen Requirement During CPR to Effect ROSC

2.1 Hypothesis

We hypothesized that limiting oxygen for the initial reperfusion period, i.e. the first minutes of CPR and the first 2 min following ROSC, a total time of roughly 3–4 min in our model, might improve cardiac resuscitation. Three different levels of supplemental oxygen (0%, 21%, 100%), independent of ventilation, were used to test the hypothesis.

2.1 Methods

2.1.1 Animal preparation (Fig. 2.1)

Sprague–Dawley rats weighing approximately 400–450 g (Harlan, Indianapolis, IN) were used in accordance with the guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No 85-23, revised 1996) and the approval of the University Laboratory Animal Resources Committee. Animals were anesthetized with ketamine (70 mg/kg) and xylaxine (10 mg/kg) IP and intubated with PE-200 (1.4 mm) tubing. Animals were ventilated with a FiO$_2$ of 0.21 at 50 breaths per minute and a tidal volume of 6 ml/kg using a rodent ventilator (Harvard
Model 683, South Natick, Mass) to maintain arterial blood gases in the normal range (pO₂ > 80 mmHg, pCO₂ 35–45 mmHg, and pH 7.35–7.45). The right jugular vein and right femoral or tail artery were cannulated with 24 gauge catheters for drug administration and continuous arterial pressure monitoring. Arterial pressure and heart rate were continuously recorded using an on-line data acquisition system (Digi-Med Heart Performance Analyzer). Rectal temperature was maintained between 36.5–37.5 °C throughout the duration of the experiment with a heating lamp.
Fig. 2.1 Animal preparation for CPR model

2.1.2 Experimental protocol

Using a standardized cardiac arrest model [31] adapted to the rat [32], cardiac arrest was induced by infusing a bolus of potassium chloride (0.04 mg/g KCl) into the jugular vein followed by a 0.2 ml bolus of saline to ensure that the full KCl dose was delivered to the heart. Ventilation was simultaneously discontinued. Cardiac arrest was
confirmed by asystole on the surface ECG and the loss of the arterial trace with a mean arterial pressure of <20 mm Hg. Uninterrupted cardiac arrest was maintained for 6 min at which time CPR was begun. Chest compressions were begun with a pneumatic chest compressor at a rate of 200–240/min and a compression depth of 17–20 mm, which represented approximately 40–50% of the AP chest diameter in these animals. All animals received epinephrine (0.01 mg/kg) through the jugular vein 1 min after starting CPR. As a drug of choice for resuscitation, epinephrine increases peripheral vasoconstriction and coronary blood flow. CPR was continued until the return of spontaneous circulation (ROSC), defined as the time of return of a spontaneous arterial blood pressure tracing with a mean arterial pressure of >60 mmHg without chest compressions.

Animals were block randomized (Fig. 2.2) at the onset of CPR to three groups; ventilation with (1) 0% oxygen (100% nitrogen), (2) 21% oxygen or (3) 100% oxygen. Minute ventilation, defined as the product of tidal volume and ventilation rate, was provided at 50% of baseline during CPR. At the time of ROSC, ventilation rate was increased to the pre-arrest rate. Fixed levels of oxygenation were maintained in groups until 2 min following ROSC, at which time all animals were changed to 100% oxygen. All ROSC survivors were ventilated for 60 min following ROSC with 100% oxygen and then were extubated. Arterial blood gases were determined at baseline (pre-arrest), 2 min ROSC and 60 min ROSC using a blood gas analyzer (GEM Premier 3000, Instrumentation Laboratory, IL). ROSC rates and time to ROSC were determined in each group.
Two minutes after ROSC, all animals received NaHCO$_3$ (1 meq/kg) to compensate for metabolic acidosis. Following ROSC, all animals received identical care consisting of mechanical ventilation at pre-arrest settings with 100% O$_2$ and continuous arterial blood pressure, heart rate, and temperature monitoring for 60 min. Fluid administration consisted of intermittent 1 ml boluses of normal saline if the animal developed hypotension (mean arterial pressure <60 mm Hg). At the end of the 60-min monitoring period, catheters were removed, cannulation sites were sutured, animals were allowed to awaken and were extubated. Neurologic testing using the neurologic deficit score (Table 2.1) [33] was performed at 6, 12, 24, 48 and 72 h following ROSC.

**Fig. 2.2 Cardiac arrest protocol.** Following 6 min of cardiac arrest, CPR was started with standardized chest compressions and ventilation with the prescribed fractional
inspiratory gas concentration. Two minutes after ROSC, all groups were ventilated with 100% oxygen for 60 min.

<table>
<thead>
<tr>
<th>Score</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>1. General behavioral deficit (worst, 40 points)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consciousness</td>
<td></td>
</tr>
<tr>
<td>Attempt to explore spontaneously</td>
<td>0</td>
</tr>
<tr>
<td>No attempt to explore spontaneously</td>
<td>20</td>
</tr>
<tr>
<td>Respirations</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Abnormal</td>
<td>20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2. Cranial nerve reflexes deficit (worst, 20 points)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olfactory (sniffing food)</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>0</td>
</tr>
<tr>
<td>Absent</td>
<td>4</td>
</tr>
<tr>
<td>Vision (follows hand)</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>0</td>
</tr>
<tr>
<td>Absent</td>
<td>4</td>
</tr>
<tr>
<td>Corneal</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>0</td>
</tr>
<tr>
<td>Absent</td>
<td>4</td>
</tr>
<tr>
<td>Whisker movement</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>0</td>
</tr>
<tr>
<td>Absent</td>
<td>4</td>
</tr>
<tr>
<td>Hearing (turns to clapped hands)</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>0</td>
</tr>
<tr>
<td>Absent</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3. Motor deficit (worst, 10 points)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Legs/tail movement</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Stiff</td>
<td>5</td>
</tr>
<tr>
<td>Paralyzed</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2.1 Rat neurologic deficit score (from [33])
Table 2.1 continued

<table>
<thead>
<tr>
<th>4. Sensory deficit (worst, 10 points)</th>
<th></th>
</tr>
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<tbody>
<tr>
<td>Legs/tail (on pinching)</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>0</td>
</tr>
<tr>
<td>Absent</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>5. Coordination deficit (worst, 20 points)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Travel ledge (place on beam)</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>0</td>
</tr>
<tr>
<td>Absent</td>
<td>5</td>
</tr>
<tr>
<td>Placing test (front paws reaching when lifted from the ground by tail)</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>0</td>
</tr>
<tr>
<td>Absent</td>
<td>5</td>
</tr>
<tr>
<td>Righting reflex (attempting to right self when placed on back)</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>0</td>
</tr>
<tr>
<td>Absent</td>
<td>5</td>
</tr>
<tr>
<td>Stop at edge of table</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>0</td>
</tr>
<tr>
<td>Absent</td>
<td>5</td>
</tr>
</tbody>
</table>

Total score: 0% normal 100% brain death

2.2.3 Data analysis

Data are presented as mean ± SD. ROSC rates were determined and compared between groups using Fisher’s exact test, which assumes random sampling and independent observations. Parametric data were analyzed within series using a one-way analysis of variance followed by a Tukey post-hoc test with $p < 0.05$ set as significant.
### 2.3 Results

<table>
<thead>
<tr>
<th>Groups</th>
<th>CPR AoDP (mm Hg)</th>
<th>Time to ROSC (secs)</th>
<th>ROSC rate</th>
<th>72-h survival rate</th>
<th>Fraction having normal neurologic function at 72 h (ND score = 0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% O₂ group</td>
<td>23 ± 6</td>
<td>80 (n = 1)</td>
<td>1/10*</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td>21% O₂ group</td>
<td>23 ± 10</td>
<td>115 ± 87</td>
<td>9/11</td>
<td>7/9</td>
<td>5/7</td>
</tr>
<tr>
<td>100% O₂ group</td>
<td>30 ± 14</td>
<td>95 ± 33</td>
<td>10/12</td>
<td>8/10</td>
<td>3/8</td>
</tr>
</tbody>
</table>

**Table 2.2 Group CPR and neurologic outcome.** Mean ± SD. AoDP = CPR generated aortic diastolic pressure. There were no significant differences between groups in mean AoDP during CPR. ROSC rate was significantly decreased in the 0% O₂ group. There were no differences between 21% and 100% oxygen groups in 72-h survival or neurologic deficit (ND) score at 72 h. *p < 0.001.

Thirty-three rats underwent cardiac arrest and were randomized to receive 0% O₂ (n = 10), 21% O₂ (n = 11) or 100% O₂ (n = 12) during CPR and the first 2 min following ROSC. All three groups were comparable at baseline in terms of amount of anesthesia received, baseline arterial blood pressure, heart rate and arterial blood gases. During CPR the mean aortic diastolic pressure was similar between all groups (Table

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16
2.2). ROSC was successful in only one rat in the 0% O₂ group in contrast to much higher ROSC rates in the other two groups (Fig. 2.3). Time to ROSC and survival to 72 h were similar in both oxygen groups (Table 2.2). Arterial PO₂ was significantly lower at ROSC 2 min in the 21% O₂ group compared to the 100% O₂ with no significant difference in pH or PaCO₂ (Fig. 2.4). Arterial blood gases at ROSC 2 min were only available in the one animal from the 0% O₂ group that obtained ROSC. The PaO₂ in this animal at ROSC 2 min was 5 mm Hg. During the post-resuscitation period each animal received approximate 3 ml normal saline. There were no differences in fluid administration between the two groups. Neurologic deficit (ND) scores were highest at 6 h, but had recovered to near normal by 72 h (ND = 2 ± 3%, 21% O₂ group vs. ND = 6 ± 8%, 100% O₂ group), (Table 2.2 and Fig. 2.5).
Fig. 2.3 **Group ROSC incidence.** Return of spontaneous circulation (ROSC) was high in the 21% oxygen (82%) and 100% oxygen (83%) groups, but significantly lower in the 0% O₂ group (10%, $p < 0.001$).
Fig. 2.4 Arterial blood gases during cardiac arrest and ROSC. Arterial blood gases were drawn pre-arrest (baseline), 2 min after ROSC and 60 min after ROSC. Arterial PO2 was significantly higher at 2 min ROSC in the 100% oxygen group (*p < 0.001). There were no differences between groups in either PaCO2 or pH. PaCO2 and pH values
were significantly higher and lower respectively at 2 min ROSC compared with baseline and 60-min ROSC values (**\(p < 0.001\)).

![Graph showing neurological deficit progression](image)

**Fig. 2.5 Progression of neurological deficit.** The neurologic deficits of both groups were worst at 6 h, but had recovered to near normal by 72 h.

### 2.4 Discussion

This study highlights the importance of oxygen during CPR in order to achieve initial resuscitation of the heart, i.e. return of spontaneous circulation (ROSC). We noted in contrast to our initial hypothesis, that ventilation without oxygen for even a
short period of time during CPR precluded successful ROSC. This finding was observed in a group of animals ventilated with 0% O\textsubscript{2} for approximately 3–4 min during CPR compared to animals ventilated with either 21% O\textsubscript{2} or 100% O\textsubscript{2}. In this study, oxygenation as a variable was independent of ventilation. By design, all animals were ventilated according to a standardized protocol during CPR and had similar PaCO\textsubscript{2} values on arterial blood gases. Similarly, CPR was delivered in a standardized method and CPR generated aortic diastolic pressure was similar in all groups. Despite standardization of CPR together with a relatively short cardiac arrest duration of 6 min, ventilation without oxygen during CPR was a major determinant of ROSC success. Previous large animal cardiac arrest studies suggest that during a relatively short CPR time, particularly following a short cardiac arrest duration, the need for supplemental oxygenation (or ventilation) is not critical [8, 10]. The discrepancy may result from the passive ventilation of room air caused by chest compressions in these experiments as compared to ventilation of 0% O\textsubscript{2} in our study. In fact a strategy of no ventilation, continuous chest compression, is now advocated by the American Heart Association as the initial approach to the pulseless, apneic patient by the lay rescuer [9]. In considering this initial approach to the cardiac arrest patient, chest compressions alone may allow for some gas exchange, however the effects of this limited gas exchange on adequacy of oxygenation are not known. This study did not investigate no-ventilation chest compressions but points out the importance of oxygen, independent of ventilation, in the early moments of resuscitation, a time period when no ventilation chest compressions might be employed.
The rationale for limiting oxygen at the onset of CPR is that reduced oxygen levels at reperfusion will reduce oxidant stress and reactive oxygen species generation in ischemic tissues with the reintroduction of oxygen. It is well established in the laboratory that reperfusion of the ischemic heart results in a burst of reactive oxygen species formation which can result in tissue injury [34]. This response to re-oxygenation can theoretically be ameliorated by controlling oxygenation. Previous animal studies have noted increased lipid peroxidation when ischemic hearts were reperfused with hyperoxia while on cardiopulmonary bypass [35, 36]. A similar effect is seen in the neonatal swine model, where hyperoxic re-oxygenation with 100% O2 for 1-h results in increased myocardial oxidative stress as measured by increased levels of oxidized glutathione [37]. Limiting initial oxygen at reperfusion is a potential strategy to ameliorate the oxidative stress associated with reperfusion. A gradual increase in partial pressure of oxygen during reperfusion has been shown to decrease oxidative stress [38]. This principle seems to also have application to cardiac arrest. In a canine cardiac arrest model, oximetry guided re-oxygenation during the first hour following ROSC showed improved neurologic function and a reduction in injured CA-1 neurons in the brain at 24 h [23].

Previous cardiac arrest studies, which have looked at PO2 levels during CPR, noted a reduction in the ROSC rate following a 6-min ventricular fibrillation (VF) swine cardiac arrest model with 10 min of controlled hypo-oxygenation (10% O2/90% N2) compared with 85% O2/15% N2 and 95% O2/5% CO2 [39]. However in another study, Zwemer et al., noted poorer neurologic recovery in a canine VF cardiac arrest
model which received CPR with 100% O\textsubscript{2} compared with 21% O\textsubscript{2} or 100% O\textsubscript{2} with antioxidant pre-treatment using tirilazad mesylate \cite{40}. To explore the strategy of limited re-oxygenation during cardiac arrest, Zwemer et al. in another study, used hypoxic ventilation of FiO\textsubscript{2} = 0.085 and FiO\textsubscript{2} = 0.12 during CPR in their dog model and failed to show an improvement in neurologic outcome compared to FiO\textsubscript{2} = 0.21 \cite{41}. Idris et al. in a swine VF cardiac arrest model with and without ventilation during 9 min of CPR noted a depressed arterial PaO\textsubscript{2} equal to 38 ± 7 mm Hg and a significant reduction in ROSC success in the non-ventilated group compared with the ventilated group \cite{42}. In our study, neurologic deficit scores did not differ between 21% and 100% oxygen groups at 72 h. Failure to show differences in neurologic outcome is likely limited due to the very brief period (few minutes) of controlled oxygen during CPR. Following 2 min of ROSC, both groups were ventilated with 100% oxygen until extubation, at which time all animals remained on room air until 72 h.

The issue of oxygenation during CPR, particularly after a short cardiac arrest, has likely been less studied due to the residual amount of oxygen available in the stagnant blood. Arterial blood gases, drawn during cardiac arrest and CPR often show surprisingly high oxygen content in the arterial blood \cite{43}. It must however be appreciated that these gases are generally samples from the large arteries, i.e. femoral arteries, where oxygen utilization even in stasis is small relative to the tissue capillary beds, where the relatively large capillary surface area allows for much more oxygen diffusion due to the oxygen gradient. As CPR is effective, this static volume of blood found in the aorta and larger arteries with its relatively high oxygen content is moved

\[23\]
into the capillary beds. It seems, at least initially, oxygen offloading to the tissues can continue and frequently can allow for ROSC. This is in contrast to our study, where ventilation with no O₂ (100% N₂) for a very short period of time did not allow for ROSC. The main difference in our study compared with previous studies is that not only was there no ventilation with oxygen, but there was active ventilation with 100% N₂ which allowed for active removal of residual oxygen from the lungs and a rapid increase in the N₂ gradient between alveoli and the pulmonary circulation. In summary, the results of this study highlight the need for some threshold level of oxygen tension in order to achieve ROSC, even after a short period of cardiac arrest and CPR.

Limitations in this study include the absence of direct measurements of reactive oxygen species (ROS) and tissue oxygen tension which would allow a direct determination of the influence of tissue oxygen tensions on localized ROS formation in our model. Based on arterial blood gases, we note the effect of FiO₂ in the arterial blood, but do not have an exact measure of interstitial or cellular oxygen tension which would better approximate the oxygen tension available for mitochondrial use. Further studies are needed to understand the extent of tissue and cellular hypoxia and the associated oxygen requirement during resuscitation. Based on our past work, we note a compartment specific increase of ROS generation at reperfusion with higher PaO₂ [44]. However, in addition to the initial ROS burst with hyperoxic reperfusion of the ischemic heart, past work has also shown that hypoxic reperfusion may also induce a large burst of ROS formation at reperfusion [45]. This balance between hyperoxia and hypoxia induced effects may be an important determinant for successful ROSC.
2.5 Future direction

To improve this project, biochemical analyses, including glutathione (GSH)/glutathione sulfide (GSSG) ratio, thiobarbituric acid reactive substances (TBARS) assay, or anti-nitrotyrosine staining in tissue slices, can be conducted to examine the difference of oxidative stress between the treatment groups. Aggravation of the ischemic insult by a longer cardiac arrest (10-15 min) may enable us to tell the difference of using room air and 100% O₂ during resuscitation. However, cardiopulmonary bypass (CPB) resuscitation might be needed to achieve an acceptable ROSC and survival rate. Finally, ROS detection in the blood can be performed by the chemiluminescence (Lucigenin) or the fluorescence (Amplex Red) assay.
Chapter 3: Preservation of Mitochondrial Function with CPR

3.1 Hypothesis

The concept that hearts with cardiac arrest and CPR (or low flow reperfusion) behave differently from hearts with conventional ischemia/reperfusion was supported in our previous study using isolated buffer-perfused hearts. Hearts with 15 min of global ischemia, followed by 5 min of 10% baseline flow and then full flow reperfusion, showed improved postischemic contractile function and bioenergetic capacity compared to hearts with 20 min of global ischemia and full reperfusion [46]. These findings suggest that CPR-type low flow reperfusion, although insufficient to meet the oxygen demand and sustain full contractility of the ischemic heart, may be able to alleviate further injury caused by ischemia and improve post-resuscitation cardiac function at full reperfusion.

Mitochondria are thought to be progressively damaged during ischemia and injured further when full flow reperfusion resumes (reviewed by Lesnefsky et al [15]). However, very little is known about the effects of CPR on mitochondrial function. Recently, Rea et al [47] proposed that the low flow generated by CPR may not only slow the ischemic injury but may also attenuate reperfusion injury by “priming” the heart with oxidative substrates which activate protective mechanisms. The proposed
beneficial effects of CPR might be directly or indirectly linked to mitochondria, since they play a pivotal role in determining cardiac function. In the present study, we hypothesize that CPR preserves mitochondrial function during cardiac arrest. Mitochondrial function following cardiac arrest with and without CPR in a whole animal rat cardiac arrest model was determined by measuring respiration and the activities of electron transport chain (ETC) complexes. Western blotting of mitochondrial ETC proteins was performed to determine if changes in complex activity were related to alterations in protein expression. Finally, morphological changes of mitochondria were examined by transmission electron microscopy (TEM).

3.2 Methods

3.2.1 The animal CPR model (Fig. 2.1, p11)

Male Sprague-Dawley rats (400-450 g), purchased from Harlan Laboratories (Indianapolis, IN), were cared for in accordance with the National Institutes of Health (NIH) guidelines and with approval of the Institutional Animal Care and Use Committee. Animals were orally intubated after an i.p. injection of ketamine/xylazine (70/10 mg/kg), followed by mechanical ventilation (model 683, Harvard Apparatus, Holliston, MA) with room air at a rate of 35 strokes/min, a tidal volume of 8 ml/kg, and a positive end expiratory pressure (PEEP) of 3 cm H$_2$O. The right femoral artery and right jugular vein were cannulated with 24-gauge catheters for arterial pressure
monitoring (HPA-210a, Digi-Med, Louisville, KY) and drug administration, respectively. The core body temperature was maintained at 37 °C by a thermistor-controlled heat lamp and peripheral oxygenation was evaluated by pulse oximetry (NPB-40, Nellcor, Boulder, CO). Cardiac rhythm was acquired using a three lead ECG with electrocardiograph leads inserted subcutaneously into the chest wall (Lifepak 9, Physio-Control, Redmond, WA).

Fig. 3.1 The experimental protocol.
Immediately following 20 min of instrumentation, animals were randomly divided into four groups (Fig. 3.1): 1) BL (instrumentation only, no cardiac arrest), 2) CA\textsubscript{15} (15 min cardiac arrest without CPR), 3) CA\textsubscript{25} (25 min cardiac arrest without CPR) and 4) CPR (15 min cardiac arrest, followed by 10 min CPR). Heparin (1000 U/kg, i.v.) was administered to prevent blood coagulation in all groups. Cardiac arrest was induced by a bolus injection of KCl (0.07 mg/g) via the jugular vein and ventilation was stopped. In the CPR group, chest compressions were initiated at 15 min cardiac arrest by a pneumatic rodent thumper (Michigan Instruments, Grand Rapids, MI) at a rate of 200-240 /min and a compression depth of 17-20 mm. This depth represented 40-50% of the anterior-posterior diameter of the animal’s chest. During CPR, the ventilation rate was at 60% of baseline with 100% O\textsubscript{2} and no PEEP. At the end of experiment, the heart was excised and mitochondria were immediately isolated for further analysis.

3.2.2 Isolation of heart mitochondria

Mitochondrial isolation was based on Costa et al [48] with some modifications. All isolation procedures were performed at 0-4 °C. The excised heart was trimmed and washed in isolation buffer (250 mM sucrose, 10 mM HEPES and 1 mM EGTA, pH 7.2). The left ventricle with the interventricular septum was selected and minced thoroughly in 1 ml isolation buffer containing 1 mg protease (type XXIV, Sigma-Aldrich, St. Louis, MO) to increase mitochondrial yield. The addition of protease in the isolation buffer helped the disruption of myocardium and extraction of mitochondria. The suspension
was then added to 7 ml isolation buffer containing 2 mg/ml of bovine serum albumin (BSA) and homogenized with a motorized Teflon pestle. The homogenate was centrifuged at 1500 g for 3 min twice and both supernatants were added together and centrifuged at 9000 g for 10 min. This crude mitochondrial pellet was washed and re-suspended in 10 ml isolation buffer without BSA. The suspension underwent another centrifugation of 2300 g for 3 min and the supernatant was decanted and centrifuged again at 9000 g for 10 min. The final pellet was re-suspended in isolation buffer and the protein concentration was determined by the Lowry method [49]. Mitochondrial yield, expressed as mg mitochondrial protein/g wet tissue, was determined by dividing the product of protein concentration and total volume by tissue wet weight. Fresh mitochondrial samples were used for respiration and cytochrome a assay. Part of the mitochondrial preparation was stored immediately at –80 ºC for immunoblotting analysis and spectrophotometric assays.

### 3.2.3 Measurement of cytochrome a

The content of cytochrome a was assayed to determine relative mitochondrial purity in the samples as cytochrome a only exists in mitochondria. One hundred microliters of mitochondrial protein were incubated with 400 µl assay buffer (50 mM Tris, 0.3% deoxycholate, 0.3% KCl, pH 8.0) in a quartz cuvette. After being fully oxidized by addition of 1 µl 100 mM K₃Fe(CN)₆, the baseline absorbance spectrum (500-650 nm) was measured on a Shimadzu 2401 UV/VIS spectrophotometer.
(Columbia, MD). A few grains of solid sodium dithionite were then added to fully reduce the cytochromes of mitochondria and the absorbance spectrum was measured again. The concentration of cytochrome \( a \) was determined by the difference in absorbance at 605 nm with an extinction coefficient of 12 mM\(^{-1}\)cm\(^{-1}\) \[^{[50]}\].

3.2.4 Estimation of mitochondrial extraction and breakage ratios

Citrate synthase (CS), a mitochondrial matrix marker enzyme, was used to estimate the percentage of mitochondria extracted from the myocardium and the percentage of broken mitochondria during the isolation process. The CS activity by volume was assayed \[^{[51]}\] in the following samples: 1) the 1\(^{st}\) tissue homogenate after Teflon pestle homogenization, 2) the supernatant after the first centrifugation at 1500 g, 3) the suspension before the first centrifugation at 9000 g, and 4) the supernatant after the first centrifugation at 9000 g. Mitochondrial extraction ratio was defined as the CS activity of sample 2 divided by the CS activity of sample 1, while mitochondrial breakage ratio was defined as the CS activity of sample 4 divided by the CS activity of sample 3.
3.2.5 Mitochondrial respiration

The oxygen consumption of fresh isolated mitochondria was measured by a Clark-type oxygen electrode (Apollo 4000, World Precision Instruments, Sarasota, FL) in a closed chamber with magnetic stirring. Five hundred micrograms of mitochondrial protein were incubated in 1 ml respiration buffer (225 mM sucrose, 5 mM MgCl$_2$, 10 mM KH$_2$PO$_4$, 20 mM KCl, 10 mM Tris and 5 mM HEPES, pH 7.4) at 30 ºC. After one minute of equilibration, respiration substrates for complex I (glutamate/malate: 10 mM/5 mM) or complex II (succinate/rotenone: 6 mM/1.2 µM) were added to the respiration buffer to initiate state 2 respiration. The rates of state 3 and state 4 respiration were measured after the addition of 300 nmol ADP (state 3) and following the depletion of ADP during which the respiration slowed down (state 4). The respiratory control ratio (RCR) was then calculated from the ratio of state 3 to state 4 respiration. For measuring the mitochondrial respiration under the condition of uncoupling, 0.5 µM carbonylcyanide-<i>p</i>-trifluoromethoxy-phenylhydrazone (FCCP) was used in the study.

3.2.6 Spectrophotometric assays of electron transport chain (ETC) complex activity

The activities of ETC complexes were assayed spectrophotometrically using sonicated, thawed mitochondria samples at room temperature as detailed by Han et al [52] and Chen et al [53].
3.2.6.1 Complex I (NADH-ubiquinone reductase) activity assay

The electron transfer activity of complex I was assayed by ubiquinone-stimulated NADH oxidation. Twenty-five micrograms of mitochondrial protein were mixed with 500 µl assay buffer (20 mM potassium phosphate buffer, 2 mM NaN₃, 0.8% sodium cholate, 0.15 mM NADH, pH 8.0) in a quartz cuvette. The reaction was initiated by the addition of 0.1 mM ubiquinone-1 (Q₁) and the change of absorbance for NADH was measured at 340 nm (ε = 6.22 mM⁻¹cm⁻¹). The specificity of the assay was validated by rotenone-sensitive inhibition. Complex I activity was expressed as the oxidation rate of NADH (nmol NADH oxidized/min/mg protein).

3.2.6.2 NADH-ferricyanide reductase (NFR) activity assay

NFR activity was measured to estimate the function of the peripheral arm of complex I (Fig. 3.2). In the assay mixture, 0.5 mM K₃Fe(CN)₆ was used to replace ubiquinone-1 as the electron acceptor in the assay mixture and the decrease of ferricyanide absorbance was recorded at 420 nm (ε = 1.0 mM⁻¹cm⁻¹) [54]. The enzyme-mediated reaction was not sensitive to rotenone and NFR activity was expressed as the reduction rate of ferricyanide (nmol ferricyanide reduced/min/mg protein).
3.2.6.3 Complex II (succinate-ubiquinone reductase) activity assay

Complex II activity was analyzed by ubiquinone-2 (Q\textsubscript{2}) stimulated dichlorophenolindophenol (DCPIP) reduction. Ten micrograms of mitochondrial protein were incubated at 30 °C for 10 min with 500 µl assay buffer (50 mM phosphate buffer, 20 mM sodium succinate, 0.1 mM EDTA, 75 µM DCPIP, pH 7.4) to fully activate complex II [51]. The reaction was initiated by adding 50µM Q\textsubscript{2} and the decrease of absorbance at 600 nm (\(\epsilon = 21 \text{mM}^{-1} \text{cm}^{-1}\)) was measured. Inhibition of the reaction by the thenoyltrifluoroacetone (TTFA) was used to confirm the specificity of assay. Complex II activity was expressed as the reduction rate of DCPIP (nmol DCPIP reduced/min/mg protein).
3.2.6.4 Complex III (ubiquinol-cytochrome c reductase) activity assay

Complex III activity was evaluated by ubiquinol-mediated ferricytochrome c reduction at 550 nm ($\varepsilon = 18.5 \text{ mM}^{-1}\text{cm}^{-1}$) upon addition of 2 $\mu$g protein to 500 $\mu$l assay buffer (50 mM phosphate buffer, 1mM EDTA, 50 $\mu$M cytochrome c, 0.1% sodium cholate, 0.1 mM KCN and 25 $\mu$M ubiquinol-2, pH 7.0). Inhibition of the assay with Antimycin A was used to verify the specificity of the assay. Complex III activity was expressed as the reduction rate of ferricytochrome c (nmol ferricytochrome c reduced/min/mg protein).

3.2.6.5 Complex IV (cytochrome c oxidase) activity assay

Ten micrograms of mitochondrial protein were added to 600 $\mu$l assay buffer. The assay buffer contained 50 mM phosphate buffer and 60 $\mu$M ferrocytochrome c (pH 7.4). Complex IV activity was determined by the decrease in the rate of absorbance of ferro-cytochrome c at 550 nm ($\varepsilon = 18.5 \text{ mM}^{-1}\text{cm}^{-1}$). Potassium cyanide was used as the inhibitor to validate the assay. Complex IV activity was expressed as the oxidation rate of ferrocytochrome c (nmol ferrocytochrome c oxidized/min/mg protein).
3.2.7 Western blotting of mitochondrial ETC complex proteins

To prevent unexpected degradation of mitochondrial proteins by residual proteases, 1 mM phenylmethysulphonyl fluoride (PMSF, dissolved in isopropanol) was added in the homogenization step during mitochondrial isolation to block protease activity. The samples (100 µg) were pretreated with dithiothreitol (DTT) for disruption of protein disulfide bonds and then loaded on a pre-cast NuPAGE 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA) for SDS-PAGE. After electrophoresis, protein bands were transferred to a nitrocellulose membrane. The membrane was first stained with Ponceau S to verify the success of protein transfer and then blocked with 5% nonfat dry milk in Tween-Tris Buffered Saline (TTBS) for 1 hr. Following blocking, the blots were incubated overnight at 4 °C with the primary antibody of interest. The antibodies included in-house generated anti-complex I 51 kDa, anti-complex II 70 kDa [56], anti-OxPhos Complex III FeS subunit, anti-OxPhos Complex IV subunit I (Invitrogen, Carlsbad, CA) and anti-superoxide dismutase 2 (FL-222, Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was then washed with TTBS three times for 10 min each, followed by incubation with HRP-conjugated secondary antibody for 1 hr at room temperature. Secondary antibodies were removed by three 10 min washes of TTBS and the labeled proteins were identified using Amersham ECL Western blotting reagents (GE Healthcare, Buckinghamshire, UK) for chemiluminescence detection.
3.2.8 Transmission electron microscopic studies

To investigate the mitochondrial damage in situ between groups, we examined the ultrastructure of myocardium by transmission electron microscopy (TEM). A small piece (< 1 cubic millimeter) of subendocardial tissue was excised from the left ventricle at the end of the experiment and fixed in 2% glutaraldehyde (0.1 M phosphate buffer, pH 7.4) for three hours. Each specimen was post-fixed in 1% OsO$_4$ for one hour and dehydrated through a graded ethanol series (50-100%). After treatment of propylene oxide, the specimen was embedded in Eponate 12 resin, sectioned at a thickness of 80 nm, and stained by 2% aqueous uranyl acetate followed by lead citrate. The grids were then observed in a Technai G2 Spirit transmission electron microscope (FEI Company, Hillsboro, OR). Mitochondria were imaged at 6800X, 18500X and 68000X. For the morphometric analysis, total mitochondria in five micrographs (18500X) per group were counted and the mitochondrial disruption rate was calculated. Relative matrix density (normalized to the density of myofibrils) and mitochondrial size \[
\frac{\text{pixels of a single mitochondrion}}{\text{pixels of the whole micrograph}} \times \text{real size of the micrograph}
\] were analyzed from 100 mitochondria randomly delineated in 5-8 micrographs (6800X and 18500X) per group by NIH ImageJ software.
3.2.9 Data analysis

All data were expressed as mean ± SEM. Statistical analyses were carried out with one-way ANOVA and followed by Tukey’s post hoc test where appropriate. Significance was assigned at P < 0.05.

3.3 Results

3.3.1 Quality of CPR

In the CPR group, there was a slow, steady decline of aortic end-diastolic pressure in the 10 min CPR period. The aortic end-diastolic pressure during CPR was 28.7 ± 3.6, 18.7 ± 0.9 and 15.7 ± 2.0 mmHg at 1, 5, 9 min, respectively. All animals had some organized electrical activity during CPR but none achieved return of spontaneous circulation (ROSC).

3.3.2 Mitochondrial yield and cytochrome a content of isolated mitochondria

The results suggested that as the ischemia time increased, mitochondrial yield reciprocally decreased (Table 3.1). The baseline group had the highest yield while the CA25 group had the lowest yield which was less than half of the baseline. Hearts from the CPR groups had about the same mitochondrial yield as those from the CA15 group,
suggesting that intervention with CPR preserved mitochondrial integrity. In a different set of experiments (n = 3/group), the citrate synthase activity, as an indicator of mitochondrial mass, was assayed to estimate the extraction and breakage ratios of mitochondria among groups. The BL group had the highest extraction ratio and the least breakage ratio while the CA\textsubscript{25} group showed the opposite findings (Fig. 3.3). There was no difference between the CA\textsubscript{15} and CPR groups in regard to both mitochondrial extraction and breakage. The findings imply that mitochondria in the CA\textsubscript{25} group sustained more disruption and that less mitochondria could be recovered during the isolation process. However, the cytochrome \textit{a} content was similar between groups, indicating that the relative purity of mitochondrial preparation was unaffected by isolation process or treatment (Table 3.1).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mitochondrial yield (mg/gm wet tissue)</th>
<th>Cytochrome \textit{a} content (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL</td>
<td>20.26 ± 0.82</td>
<td>768 ± 17</td>
</tr>
<tr>
<td>CA\textsubscript{15}</td>
<td>13.43 ± 0.38 *</td>
<td>749 ± 14</td>
</tr>
<tr>
<td>CA\textsubscript{25}</td>
<td>8.54 ± 0.54 *\dagger</td>
<td>712 ± 12</td>
</tr>
<tr>
<td>CPR</td>
<td>13.40 ± 0.77 *\dagger</td>
<td>734 ± 18</td>
</tr>
</tbody>
</table>

Table 3.1 Mitochondrial yield and cytochrome \textit{a} content in different groups.

Mitochondrial yields were statistically different among groups (\*BL vs. CA\textsubscript{15}, BL vs. CA\textsubscript{25}, BL vs. CPR: p < 0.001, \daggerCA\textsubscript{15} vs. CA\textsubscript{25}: p < 0.001, \ddaggerCA\textsubscript{25} vs. CPR, p < 0.001).

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However, there was no significant group difference (p > 0.05) in terms of cytochrome $a$ content, indicating that the purity of mitochondrial samples was consistent among groups. (Data expressed as mean ± SEM, n = 8/group)

Fig. 3.3 Extraction and breakage ratios of mitochondria during isolation process.

The BL group had the highest extraction ratio and the least breakage ratio while the CA$_{25}$ group showed the opposite findings. There was no difference between the CA$_{15}$
and CPR groups in regard to both mitochondrial extraction and breakage. (Data expressed as mean ± SEM, n=3/group, *p < 0.001, †p < 0.01, ‡p < 0.05)

3.3.3 Respiration parameters in isolated mitochondria

A representative trace of the mitochondrial respiration experiment is showed in Fig. 3.4A. When glutamate/malate (10 mM/5 mM) was used as complex I substrates for NAD-linked respiration in isolated heart mitochondria, the rates of state 3 and FCCP-stimulated respiration (uncoupling conditions) in the CA25 group were significantly lower when compared with the other three groups (Fig. 3.4B). The CA25 group also had the highest state 4 respiration of all groups (Fig. 3.4B, middle), which contributed to a further decline of the respiratory control ratio (RCR: state 3/4 respiration) in the CA25 group (36% of baseline, Fig. 3.4C). In contrast, mitochondrial function in the CPR group was not significantly different from those of the BL and CA15 groups in regard to state 3, state 4 and FCCP-stimulated respiration (Fig. 3.4B). When succinate/rotenone (6 mM/1.2 µM) was used to measure the FAD-linked respiration, the results showed a very similar pattern (Table 2). The RCR, state 3 and FCCP-stimulated respiration were all markedly depressed in the CA25 group (p < 0.001), but preserved in the CPR group. No significant difference in state 4 respiration was found between groups.
Fig. 3.4 Mitochondrial respiration: representative trace and results.
Glutamate (10mM) and malate (5mM) were used as respiration substrates. (A) A representative trace of the mitochondrial respiration experiment. (B) State 3, state 4 and FCCP-stimulated respiration (nmol O₂ consumed/min/mg protein) in isolated mitochondria. The CA₂₅ group had significantly lower state 3 and FCCP-stimulated respiration but higher state 4 respiration than the other three groups. (C) Respiratory control ratio (RCR: state 3/ state 4 respiration). The CA₂₅ group had the lowest RCR, while the CPR group, not different from the CA₁₅ group, had a higher RCR than the CA₂₅ group. (Data expressed as mean ± SEM, n=8/group, *p < 0.001, †p < 0.01, ‡p < 0.05)
<table>
<thead>
<tr>
<th>Groups</th>
<th>State 3</th>
<th>State 4</th>
<th>FCCP-stimulated</th>
<th>RCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL</td>
<td>289.8 ± 7.6</td>
<td>115.7 ± 2.6</td>
<td>293.1 ± 12.8</td>
<td>2.51 ± 0.09</td>
</tr>
<tr>
<td>CA₁₅</td>
<td>263.9 ± 15.2</td>
<td>128.9 ± 10.0</td>
<td>279.3 ± 15.2</td>
<td>2.08 ± 0.09</td>
</tr>
<tr>
<td>CA₂₅</td>
<td>115.5 ± 16.1*†</td>
<td>113.3 ± 7.1</td>
<td>147.4 ± 17.2*†</td>
<td>1.00 ± 0.11*†</td>
</tr>
<tr>
<td>CPR</td>
<td>270.7 ± 12.5‡</td>
<td>126.1 ± 3.8</td>
<td>289.5 ± 14.0‡</td>
<td>2.16 ± 0.11‡</td>
</tr>
</tbody>
</table>

**Table 3.2 Parameters of mitochondrial respiration using succinate/rotenone (6 mM/ 1.2 µM) as respiration substrates.** The pattern is the same as seen in Fig. 2.

The CA₂₅ group had significantly lower state 3, FCCP-stimulated respiration and RCR than the other three groups (*BL vs. CA₂₅: p < 0.001, †CA₁₅ vs. CA₂₅: p < 0.001, ‡CA₂₅ vs. CPR: p < 0.001). No significant difference of state 4 respiration was found among groups. Data are expressed as mean ± SEM (nmol O₂/min/mg protein), n = 8/group.

### 3.3.4 Mitochondrial ETC complex activities

Complex I activity was depressed approximately 55% from baseline in the CA₂₅ group compared with 21% depression in the CA₁₅ and CPR groups (Fig. 3.5A).

Similarly, the CA₂₅ group had the most severe impairment in the other three enzymatic activities, including a 23% reduction from baseline in NADH-ferricyanide reductase (NFR) activity (Fig. 3.5B), a 19% reduction from baseline in complex II activity (Fig. 3.5C) and a 28% reduction from baseline in complex III activity (Fig. 3.5D). However, the CPR group showed less injury in the ETC activities than the CA₂₅ group and was
not different from the CA₁₅ group (Fig. 3.5B-3.5D). There were no significant differences in the enzymatic activity of complex IV among all four groups in our study (Fig. 3.5E).

continued

**Fig. 3.5 ETC complex activities in isolated mitochondria.** The CA₂₅ group had the lowest activity for complex I-III in all groups. The CA₁₅ and CPR groups were not significantly different from each other. (A) Complex I activity (nmol NADH oxidized/min/mg protein) in the CA₂₅ group had a 55% reduction from baseline and the CA₁₅ and CPR groups had only about a 21% reduction from baseline.
(B) The NADH-ferricyanide reductase (NFR) activity (nmol ferricyanide reduced/min/mg protein) in the CA_{25} group showed a 23% reduction from baseline.  (C) Complex II activity (nmol DCPIP reduced/min/mg protein) in the CA_{25} group had a 19% reduction from baseline.  (D) Complex III activity (nmol cytochrome c reduced/min/mg protein) in the CA_{25} group had a 28% reduction from baseline.  (E) Complex IV activity (nmol ferrocytochrome c oxidized/min/mg protein) was not statistically different between groups.  (Data expressed as mean ± SEM, n=8 in all groups, *p < 0.001, †p < 0.01, ‡p < 0.05)

3.3.5 Western blotting of mitochondrial complexes

Mitochondrial samples were probed with antibodies against the ETC complexes to determine the level of protein expression.  Superoxide dismutase-2 (SOD-2, MnSOD)
was used as the loading control for Western blotting (Fig. 3.6A) due to its stability in the tissue homogenates from normal, ischemic, and post-ischemic myocardium (data not shown). The blots were scanned and analyzed by NIH ImageJ software and all densitometric measurements were normalized to baseline. The CA$_{25}$ group showed a weaker signal of immunoblotting for all complexes except complex IV (Fig. 3.6A). After normalization to SOD-2, the CA$_{25}$ group had a 22% loss of complex I 51kDa subunit (p < 0.05, Fig. 3.6B), a 15% loss of complex II 70 kDa subunit (p < 0.05, Fig. 3.6B) and a 28% loss of complex III FeS subunit (p < 0.05, Fig. 3.6C) when compared to the BL group. In contrast, the signal intensity of the CA$_{15}$ or CPR group was not significantly different from that of the BL group in regard to all probed proteins, thus supporting the hypothesis of preservation of ETC complexes by CPR (Fig. 3.6B & 3.6C).

(A)

![Western blot and densitometry of isolated mitochondria in different groups.](image)

Fig. 3.6 Western blot and densitometry of isolated mitochondria in different groups.
Fig. 3.6 continued

(A) Western blot of mitochondrial proteins probed for ETC complexes. There was less complex I 51 kDa subunit, complex II 70 kDa subunit and complex III FeS subunit in the CA25 group than the other groups. (B&C) Densitometry of western blots.
(normalized to SOD-2) showed approximately 22% loss of complex I 51 kDa subunit, 15% loss of complex II 70 kDa subunit and 28% loss of complex III FeS subunit in the CA25 group compared to baseline. There was no difference in complex IV protein expression between groups. (Data expressed as mean ± SEM, n = 4/group, *p < 0.05)

3.3.6 TEM analysis of in situ mitochondria

TEM images of subendocardial mitochondria with 18500X and 68000X magnification are shown in Fig. 3.7A & 3.7B, respectively. Mitochondria of the BL group were compact, with a higher matrix density and multiple visible matrix dense granules. After 15 min of cardiac arrest, we observed the swelling of mitochondria, which was accompanied with the decrease of matrix density and separation of cristae. The morphological changes of mitochondria were more significant in the CA25 group. Swollen mitochondria with substantial clear areas inside were noted and the cristae were disintegrated. Some mitochondria were disrupted, with irregular shapes and loss of outer and inner membranes. Mitochondria in the CPR group exhibited a modest damage pattern. The severity of mitochondrial disruption, cristae separation and matrix clearing of the CPR group was between that of the CA15 and CA25 groups. Except in the BL group, the matrix dense granules were rarely seen in mitochondria from the other three groups.
Fig. 3.7 Representative electron micrographs of subendocardial mitochondria.
Fig. 3.7 continued
(B)

(A) Ultrastructural changes of mitochondria, including swelling, decreased matrix
density and cristae disintegration, were more discernible in the CA\textsubscript{25} group
(magnification at 18500X, scale bar = 500 nm). (B) A close look at single
mitochondrion of each group (magnification at 68000X, scale bar = 100 nm).
Morphometric analysis of the electron micrographs further supported our visual impression. Four quantitative parameters were used to assess mitochondrial injury; mitochondrial population density (Fig. 3.8A), disruption rate (Fig. 3.8B), relative matrix density (Fig. 3.8C) and size (Fig. 3.8D). In the CA15 group, the population density declined (72% of baseline, \( p < 0.05 \)), the disruption rate increased (1.3%, \( p > 0.05 \)), the relative matrix density decreased (85% of baseline, \( p < 0.001 \)) and the average size increased (124% of baseline, \( p < 0.01 \)). The CA25 group had the greatest injury profile, including the lowest population density (57% of baseline, \( p < 0.001 \)) and relative matrix density (71% of baseline, \( p < 0.001 \)), the highest disruption rate (31.8%, \( p < 0.001 \)) and the largest average size (146% of baseline, \( p < 0.001 \)). In the CPR group the values of mitochondrial population density (66% of baseline, \( p < 0.01 \)), disruption rate (10.0%, \( p < 0.001 \)), relative matrix density (77% of baseline, \( p < 0.001 \)) and average size (128% of baseline, \( p < 0.01 \)) were all intermediary between the CA15 and CA25 groups.
Fig. 3.8 Morphometric analysis of TEM images. (A) Mitochondrial population density, calculated from total mitochondria in 5 different micrographs (6800X) per group. (B) Mitochondrial disruption rate, calculated from total mitochondria in 5
different micrographs (6800X) per group. (C) Relative mitochondrial matrix density (normalized to the electron density of myofibrils), analyzed from 100 random mitochondria in 5-8 micrographs (6800X and 18500X) per group. (D) Mitochondrial size, analyzed from 100 random mitochondria in 5-8 micrographs (6800X and 18500X) per group. (Data expressed as mean ± SEM, *p < 0.001, †p < 0.01, ‡p < 0.05)

3.4 Discussion

In the current study using a rodent CPR model, we have demonstrated that 10 min of CPR following an untreated 15 min cardiac arrest (25 min total) maintains mitochondrial function at the level seen with 15 min cardiac arrest alone. Preservation of mitochondrial function during the CPR period is further supported by the preservation of mitochondrial morphology seen with TEM. Despite the low blood flow and oxygen supply with CPR, the mitochondrial injury induced by 15 min of ischemia did not progress further during the 10 min CPR period. This was in sharp contrast to the significant decrease in mitochondrial function and altered morphology seen after 25 min of cardiac arrest without CPR. These findings suggest that CPR is sufficient to interrupt or slow the ongoing ischemic insult imposed on mitochondria, and possibly extend the viability of the myocardium during cardiac arrest.

Mitochondrial function has been shown to decline progressively during ischemia. This dysfunction following ischemia is mainly associated with impaired complex I activity [57-61]. In isolated perfused hearts, complex I activity begins to deteriorate
after 15 min of ischemia, and the onset of irreversible myocardial damage occurs at around 30 min of ischemia (Fig. 1.1) [15]. Consistent with these previous studies, we found that complex I activity is more susceptible to ischemia than the other ETC complexes. Twenty-five minutes of ischemia caused about 55% reduction of complex I activity and 23% loss of NFR activity, which could be mitigated with the intervention of CPR. In addition, the immunoblotting analysis showed that a 22% reduction of the hydrophilic 51 kDa flavoprotein subunit, which is the first component of complex I to accept the electrons from NADH through flavin mononucleotide (FMN) [62]. This reduction was similar to the degree of impairment seen in NFR activity. Since the decrease of NFR activity (23%) and protein expression of 51 kDa subunit (22%) only accounts for 40% of the decline of complex I activity (55%), there must be other factors contributing to the reduction of complex I activity during ischemia. These factors may include the loss of FMN [59], tissue acidosis and ATP depletion [63], reactive oxygen species and cardiolipin damage [60], and conformational change from active form to a “de-active” form [64]. Furthermore, it has been reported that the dissociation of flavoprotein from complex I can be induced in vitro under an acidic environment [65, 66]. Ischemic acidosis which develops during cardiac arrest might also facilitate the process of flavoprotein dissociation, which, in turn, hinders electron transfer and impairs complex I activity. Damage to the iron-sulfur protein fraction and/or hydrophobic protein fraction of complex I could be another factor associated with the decline of complex I activity during cardiac arrest. Whether these potential contributing factors contribute to the beneficial effects of CPR remains to be explored.
In support of our hypothesis that mitochondrial function is positively related to complex I activity, we observed an excellent linear relationship (adjusted $R^2 = 0.985$, Fig. 3.9) between mitochondrial RCR (Fig. 3.4B) and complex I activity (Fig. 3.5A). Intervention at complex I, such as blockade of electron transfer either by rotenone, amobarbital [61], or nitrite [67] has been investigated as a strategy to preserve mitochondrial function during ischemia. Considering the possible factors associated with the loss of complex I activity, development of a combinatory approach may increase the chance of ROSC and survival. A strategy to preserve complex I activity during cardiac arrest and CPR, might include the use of reversible complex I blockers and antioxidants to synergistically prevent more mitochondrial damage.

Compared with complex I, the injuries of complex II and III in the current study were found to be less severe after 25 min of ischemia. In addition, the decreases of enzymatic activity (complex II: 19%; complex III: 28%) are accompanied with comparable levels of depression in protein expression (complex II: 15%; complex III: 28%). Rouslin [57] has reported no change of complex II activity and a 26% decrease of complex III activity in hearts subjected to 60 min of autolysis. Others have reported a 20% reduction of complex III activity in isolated buffer-perfused hearts subjected to 25-30 min of ischemia [68, 69]. The discrepancy in our complex II activity may be related to the full activation by pre-incubation with succinate, which was not performed in Rouslin’s protocol.
It has been shown \textit{in vitro} that the oxidative injury of complex II impairs the protein-protein interaction with complex III \cite{56}, which interaction is also expected to be affected by damaged complex III. That may help explain why, in our study, the depression of FAD-linked respiration was about the same as the depression of NAD-
linked respiration in the CA\textsubscript{25} group although the level of complex II injury was less severe than that of complex I.

Observations from the TEM images of in situ mitochondria were in agreement with the biochemical results. The ultrastructural changes of subendocardial mitochondria, including swelling, loss of matrix density and cristae disintegration, correlated well with the time of cardiac arrest, which was compatible with previous work [16]. With the intervention of CPR, the ischemic damage to mitochondria was ameliorated in every aspect of morphological features. These visual findings were confirmed by the morphometric analysis, which exhibited a similar pattern. Twenty-five minutes of cardiac arrest resulted in significant mitochondrial loss (represented by population density and disruption rate) and swelling (represented by matrix density and average size). In contrast, all parameters were improved in the CPR group, indicating the preservation of mitochondrial quantity and morphology. It has been suggested that the functional recovery during reperfusion correlates with mitochondrial morphological grading [16]. Thus, it is reasonable to assume that the CPR group would have a better functional recovery than the CA\textsubscript{25} group.

Our study was limited by the absence of outcome data to show if preserved mitochondrial function could be translated into improved myocardial function following successful resuscitation. Additional work is needed to elucidate the relationship between mitochondrial preservation, ROSC rate and post-resuscitation cardiac function. Another potential limitation in extrapolating our results to clinical cardiac arrest is the use of the KCl-induced cardiac arrest model, which likely has different mitochondrial
oxygenation implications than the ventricular fibrillation or asphyxia models of cardiac arrest. However, the KCl-arrested heart does simulate clinical asystole and pulseless electrical activity (PEA) cardiac arrest, which now constitutes greater than 50% of out-of-hospital cardiac arrests [70]. The strength of this model is the ability to study mitochondrial changes under actual in vivo cardiac arrest conditions.

In conclusion, our results show that, despite the inherent low oxygen delivery, CPR after prolonged cardiac arrest preserves heart mitochondrial function and morphology. It demonstrates the beneficial effects of CPR from a subcellular perspective for the first time. Preservation of mitochondrial function is reflected by improved mitochondrial respiration, ETC complex activities, preservation of the ETC proteins. Additionally, complex I is the most sensitive ETC complex to ischemic injury and its activity is positively correlated with mitochondrial respiration. Future work is needed to address how preservation of complex I activity during cardiac arrest might further preserve mitochondrial function and possibly improve the current low success rate of clinical resuscitation.

3.5 Future direction

By using the method developed in chapter 5, it would be possible to isolate and concentrate enough complex I from each group and analyze the post-translational protein modifications. Such analysis would help us understand the molecular mechanisms how CPR could preserve mitochondrial complex I activity. Co-
administration of drugs during CPR to further improve or protect mitochondrial function would be another possible direction of this project.
Chapter 4: Hyperoxygenation at Reperfusion in a Cardiopulmonary Bypass (CPB) Model

4.1 Hypothesis

In the clinical setting, patients who have been successfully resuscitated from cardiac arrest are often kept on 100 % oxygen until they are transferred to an intensive care unit, which may result in several consecutive hours of hyperoxemia [71]. As described in chapter 1.4, various studies have suggested that the heart may respond differently to post-resuscitation hyperoxemia as compared to the brain. Whether hyperoxygenation during the post-resuscitation period is harmful or not remains undetermined. In the latest 2010 AHA guidelines, this knowledge gap has been addressed; “There is insufficient clinical evidence to support or refute the use of inspired oxygen concentration titrated to arterial blood oxygen saturation in the early care of cardiac arrest patients following sustained ROSC.” and “Studies evaluating combined myocardial infarction and cardiac arrest are needed to evaluate the impact of post-cardiac arrest arterial hyperoxemia on cardiovascular outcomes.” [72].

Mitochondria are considered one of the major sources of reactive oxygen species (ROS) production in the post-ischemic myocardium. The ROS generated during reperfusion can result in additional mitochondrial and cardiac damage beyond
that caused by ischemia [15]. In this study, we examine the hypothesis that hyperoxygenation during post-resuscitation period may further impair functional recovery and mitochondrial function via increased ROS production due to higher oxygen tension (Fig. 4.1).

**Fig. 4.1** The proposed relationship between hyperoxic reperfusion after CPR, ROS formation and mitochondrial dysfunction
4.2 Methods:

4.2.1 The animal cardiopulmonary bypass (CPB) model (Fig. 4.2)

Male Sprague-Dawley rats (400–450 g), purchased from Harlan Laboratories (Indianapolis, IN), were cared for in accordance with the National Institutes of Health (NIH) guidelines and with approval of the Institutional Animal Care and Use Committee. Animals were inducted by an i.p. injection of ketamine/xylazine (35/5 mg/kg) and tracheostomy was done for mechanical ventilation with room air at a rate of 35 strokes/min, a tidal volume of 8 ml/kg, and a positive end expiratory pressure (PEEP) of 3 cm H₂O (model 683, Harvard Apparatus, Holliston, MA). The left femoral artery and left femoral vein were cannulated first with 22-gauge catheters for arterial pressure monitoring (HPA-410a, Digi-Med, Louisville, KY) and continuous anesthetic infusion (ketamine 18-36 mg/kg/hr + xylazine 0.4-0.8 mg/kg/hr, ketamine:xylazine ratio = 45:1) to keep adequate depth of anesthesia during instrumentation. Volatile anesthetics were not chosen because of the concerns about mitochondrial depression [73, 74]. The left jugular vein was cannulated by PE-60 tubing for central venous pressure monitoring (LPA, Digi-Med, Louisville, KY) and drug administration. Then the right carotid artery was cannulated with PE-25 (OD 0.91 mm, ID 0.46 mm) tubing and advanced into the left ventricle for hemodynamic monitoring (HPA-410a, Digi-Med, Louisville, KY). Finally, a 14-gauge catheter with 5 spiral holes was inserted into the right jugular vein and a 22-gauge catheter was inserted into the right femoral artery for the cardiopulmonary bypass circuit. The circuit included a digital peristaltic pump.
(Masterflex Model 7550-90, Cole-Parmer, Vernon Hills, IL) and a membrane oxygenator (made by Martin Humbs, Ingenieurburo für Feinwerktechnik, Germany). The core body temperature was maintained at 37 °C by a thermistor-controlled heat lamp and peripheral oxygenation was evaluated by pulse oximetry (NPB-40, Nellcor, Boulder, CO). Cardiac rhythm was acquired using a three lead ECG with electrocardiograph leads inserted subcutaneously into the chest wall (Lifepak 9, Physio-Control, Redmond, WA). The total instrumentation time was about 1-1.5 hours.

Fig. 4.2 The animal preparation for the CPB model. Blue arrows indicate the blood flow direction of the cardiopulmonary bypass circuit.
After the instrumentation was done, there was a 10-minute baseline in which heparin (1000 U/kg) was administered (10-min before arrest) and an arterial blood gas was drawn (5 min before arrest). There were 5 groups in the study: 1) normoxic sham (21% oxygen for the whole experiment); 2) hyperoxic sham (100% oxygen for the last hour of the experiment); 3) ROSC 3 (25 min cardiac arrest, resuscitated by CPB with 100% oxygen until 3 minutes after ROSC); 4) normoxic reperfusion (NR, 25 min cardiac arrest, CPB with 100% oxygen until ROSC 3 and then switched to 40%-50% oxygen until 60 minutes after ROSC); and 5) hyperoxic reperfusion (HR, 25 min cardiac arrest, CPB with 100% oxygen until 60 minutes after ROSC) (Fig. 4.3). In the treatment groups, a bolus injection of KCl (0.07 mg/g) was given via the jugular vein to induce cardiac arrest and ventilation was stopped as well. After 25 minutes of normothermic cardiac arrest, the animals were resuscitated with CPB. The CPB circuit was primed with 20 ml of Plasma-lyte A pH 7.4 (Baxter, Deerfield, IL) and oxygenated with 100% oxygen in the beginning. The flow rate for CPB was set at 120 ml/kg/min. We defined ROSC as the appearance of regular, sustained left ventricular contractions with a developed pressure > 40 mmHg as measured by the LV catheter. Once ROSC is obtained, the ventilation was resumed at a reduced rate (25 strokes/min). Continuous intravenous phenylephrine infusion was also started and titrated to maintain the mean arterial pressure > 65 mmHg or the systemic diastolic pressure > 60 mmHg. The maximal infusion dosage of phenylephrine was 1.5 µg/kg/min to avoid unnecessary inotropenic effects even if the desired range of blood pressure could not be maintained. At ROSC 3, sodium bicarbonate (1 meq/kg) was given to ameliorate severe acidosis.
Arterial blood gases, analyzed by GEM Premier 3000 (Instrumentation Laboratory, IL), were drawn at 3, 15, 30 and 60 minutes after ROSC, respectively. Intermittent boluses of Plasma-lyte A fluid was added to the reservoir to compensate for the vascular leakage into the interstitial tissue space and keep the bypass flow constant throughout the experiment. There was no more anesthetic use after KCl-induced cardiac arrest in the three treatment groups.

**Fig. 4.3 The experimental design.** There were 5 groups in the study: 1) normoxic sham (21% oxygen for the whole experiment); 2) hyperoxic sham (100% oxygen for the last hour of the experiment); 3) ROSC 3 (25 min cardiac arrest, resuscitated by CPB with 100% oxygen until 3 minutes after ROSC); 4) normoxic reperfusion (25 min
cardiac arrest, CPB with 100% oxygen until ROSC 3 and then switched to 40%-50% oxygen until 60 minutes after ROSC); and 5) hyperoxic reperfusion (25 min cardiac arrest, CPB with 100% oxygen until 60 minutes after ROSC).

4.2.2 Isolation of heart mitochondria

The procedures of mitochondrial isolation are described in chapter 3.2.2 except that type XXIV protease was replaced by 250 U (1mg) collagenase [75] (type II, Invitrogen, Carlsbad, CA) in 1ml buffer without EGTA (sucrose 250 mM, HEPES 10 mM, pH 7.2). The protein concentration of isolated mitochondria was determined by an UV absorption method [76] on a UV-VIS spectrophotometer (HP 8452a, Agilent Technologies, Santa Clara CA) when mitochondrial samples were dissolved in 0.02% n-dodecyl β-D-maltoside PBS buffer. Unlike SDS, n-dodecyl β-D-maltoside has a low UV absorbance [77] and makes it suitable for the protein quantification by UV absorbance assay.

4.2.3 Mitochondrial respiration and swelling assay

The protocol for mitochondrial respiration is detailed in chapter 3.2.5. As for the mitochondrial swelling assay, 0.25 mg of mitochondrial protein was incubated in 1 ml swelling assay buffer (250 mM sucrose, 10 mM HEPES, 6 mM succinate, 1.2 μM
rotenone, and 250 µM CaCl$_2$, pH 7.4 at room temperature). The change of light absorbance at 520 nm, indicating calcium-induced swelling in energized mitochondria, was measured with a UV-VIS spectrophotometer at room temperature (HP 8452a, Agilent Technologies, Santa Clara CA) every 10 min over a one-hour period.

4.3 Results:

4.3.1 Resuscitation and arterial blood gases

The CPB resuscitation time, defined as the start of CPB circuit to ROSC, is comparable in both treatment groups (NR group: 138 ± 32 sec, HR group: 132 ± 32 sec, n = 6/group), so is the total volume of Plasma-lyte A added during the experimental period (NR group: 122 ± 17 ml, HR group: 119 ± 10 ml, n = 6/group). As for the arterial blood gases, both groups showed the same change of blood pH during the experimental period without significant difference (Fig. 4.4A). The HR group maintained a blood oxygen level above 400 mmHg while the NR group had a blood oxygen level at around 100 mmHg after ROSC 3 (Fig. 4.4B). Both groups had an improved base excess level during the one-hour reperfusion. However, the HR group had a significantly higher base excess level than the NR group after ROSC 30 (Fig. 4.4C), suggesting a decreased level of lactate in the HR group. Finally, both groups were able to maintain the hemoglobin level above 6 g/dL until the end of the experiment (NR group: 6.4 ± 0.2 g/dL, HR group: 6.5 ± 0.4 g/dL at ROSC 60).
Fig. 4.4 Values of arterial blood gases.
Fig. 4.4 continued

(C) Base Excess

(D) Hemoglobin Level
A) pH; B) Arterial oxygen tension; C) Base excess; D) Hemoglobin level. (Data expressed as mean ± SEM, n = 6/group). The hyperoxic reperfusion group was significantly different from the normoxic reperfusion group regarding oxygen tension (at ROSC 15, 30, 60) and base excess levels (at ROSC 15, 60). Blood pH and hemoglobin levels were not statistically different between the groups.

### 4.3.2 Hemodynamics

Both normoxic and hyperoxic reperfusion groups showed similar functional recovery in terms of left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), and dP/dt (Fig. 4.5A & Fig. 4.5B). However, the hyperoxic reperfusion group had a significantly higher negative dP/dt_max compared to the normoxic reperfusion group at ROSC 60 (54.2 ± 2.3% vs. 48.9 ± 2.1%, n = 6/group, p < 0.05), indicating a better diastolic function (relaxation) in the hyperoxic reperfusion group (Fig. 4.5C). In addition, the hyperoxic reperfusion group had a significantly better mean arterial pressure than the normoxic reperfusion group despite the same degree of vasopressor support (Fig. 4.5D). For both groups, five out of six animals received the maximal dose of phyenylephrine during the whole reperfusion period.
Fig. 4.5 Parameters of hemodynamics.
Fig. 4.5 continued

(C) Negative $dP/dt_{\text{max}}$

(D) Mean Arterial Pressure
A) Left ventricular systolic pressures (LVSP) and end-diastolic pressures (LVEDP). B) dP/dt_{max}; C) Negative dP/dt_{max}. At ROSC 60, the HR group had a significantly better negative dP/dt_{max} than the NR group (54.2 ± 2.3\% vs. 48.9 ± 2.1\%); D) Mean arterial pressure. The HR group had a significantly higher MAP than the NR group at ROSC 30, 40 and 50. (Data expressed as mean ± SEM, n = 6/group, *p < 0.05)

4.3.3 Mitochondrial swelling assay

Among all groups, mitochondria from the ROSC 3 group showed the least resistance to calcium-induced swelling over the 60 min incubation period, implying that the ROSC 3 group has the most damaged mitochondria (Fig. 4.6). After one hour of normoxic or hyperoxic reperfusion, the severity of calcium-induced swelling was significantly improved as compared with the ROSC 3 group (p < 0.01). Nevertheless, there was no statistical difference of mitochondrial swelling between the HR and NR groups, or between the sham groups.
Fig. 4.6 Mitochondrial swelling assay. *After 60 min of incubation, the ROSC 3 group had the most significant decline of Abs$_{520}$ compared to other groups ($p < 0.01$). Both HR and NR groups had significantly worse mitochondrial swelling than the sham groups ($p < 0.01$). However, there was no statistical difference between the HR and NR groups, or between the sham groups. (Data expressed as mean ± SEM, n = 6/group)
4.3.4 Mitochondrial respiration

The NAD-linked respiration, using glutamate/malate (10mM / 5mM) as substrates, in isolated mitochondria demonstrated that both NR and HR groups had comparable state 3 respiration (Fig. 4.7A) but the NR group had a significantly higher state 4 respiration (Fig. 4.7B), which resulted in the worse respiration control ratio (RCR) of the NR group (Fig. 4.7C). The findings suggested that the mitochondria in the NR group were less coupled than those in the HR group. The ROSC 3 group had the lowest state 3 respiration (Fig. 4.7A) but not the worst state 4 respiration (Fig. 4.7B). The FCCP-stimulated respiration of the ROSC 3 group was also not significantly different from the other groups (Fig. 4.7D). The data implied that complex I was compromised in the ROSC 3 group and recovered at reperfusion in both treatment groups. In addition, mitochondria in the HR group were more coupled than those in the NR group.
Fig 4.7 Mitochondrial respiration using glutamate/malate (10mM/5mM) substrates
Fig. 4.7 continued

(C)

Respiration Control Ratio (state 3/4 respiration)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Value (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxic Sham</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>Hyperoxic Sham</td>
<td>4.5 ± 0.4</td>
</tr>
<tr>
<td>ROSC 3</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>Normoxic Reparfusion</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>Hyperoxic Reparfusion</td>
<td>3.5 ± 0.2</td>
</tr>
</tbody>
</table>

(D)

Glu/Mal FCCP respiration

<table>
<thead>
<tr>
<th>Condition</th>
<th>Value (nmol O₂/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxic Sham</td>
<td>180 ± 5</td>
</tr>
<tr>
<td>Hyperoxic Sham</td>
<td>170 ± 6</td>
</tr>
<tr>
<td>ROSC 3</td>
<td>160 ± 7</td>
</tr>
<tr>
<td>Normoxic Reparfusion</td>
<td>150 ± 8</td>
</tr>
<tr>
<td>Hyperoxic Reparfusion</td>
<td>140 ± 9</td>
</tr>
</tbody>
</table>
(A) State 3 respiration. The ROSC 3 group had the lowest state 3 respiration (*p < 0.001, †p < 0.01, ‡p < 0.05); (B) State 4 respiration. (*p < 0.01); (C) Respiration control ratio. The HR group had a significant better RCR than the NR group. (*p < 0.05); (D) FCCP-stimulated respiration. There was no statistical difference among the groups. (Data expressed as mean ± SEM, n=6/group)

4.4 Discussion

In the present study, we used a CPB resuscitation model to study the effects of hyperoxegenation in the post cardiac-arrest period. After 25 minutes of normothermic cardiac arrest, hyperoxic reperfusion for one hour with CPB did show advantages compared to normoxic reperfusion in some aspects, including better systemic hemodynamics, less lactic acidosis and a small but significant improvement in cardiac relaxation. In addition, hyperoxic reperfusion demonstrated better mitochondrial respiratory function. For most in vivo cardiac arrest and resuscitation animal studies, the cardiac arrest time is quite limited because of the difficulties of getting ROSC by conventional CPR. The use of CPB in our cardiac arrest model has enabled us to extend the time limit to 25 minutes and still achieve ROSC, which is important given that more cardiac arrest patients are now rescued by CPB with a longer arrest time [78]. It is crucial to determine whether hyperoxegenation at reperfusion would benefit the short-term or long-term outcome in these patients.
There are several limitations in our study. First, it is difficult to extrapolate the short-term benefits into long-term outcome because of the non-survival experimental design. An extraordinary amount of crystalloid fluid was administered to replenish the loss of intravascular volume due to third spacing, in order to keep adequate bypass flow. Except phenylephrine, no other inotropic agent was used in order to minimize the drug influence on the heart performance. In addition, we did not correct the anemia due to hemodilution in the experiment. It has been shown in Sprague-Dawley rats that the oxygen delivery is relatively constant when the hemoglobin level is greater than 6 g/dL and the critical hemoglobin level is estimated to be 2.4 g/dL [79]. In our model, the hemoglobin levels of both groups at ROSC 60 were still above 6 g/dL, presumably enough for adequate oxygen delivery. Whether the benefits of hyperoxygenation will remain or disappear when the anemia is corrected is unknown.

As mentioned in chapter 1.4, whether hyperoxygenation at reperfusion period is beneficial is still debatable. While hyperoxic reperfusion was shown to cause more injuries in the brain after cardiac arrest [22, 23], there are more studies which have demonstrated the benefits of hyperbaric oxygen in different cerebral ischemic models, including cardiac arrest [80, 81]. If the hyperbaric hyperoxic reperfusion after ischemia does show benefits but not the normobaric hyperoxic reperfusion, then there must be other factors, in addition to oxygen, involved in such discrepancies. Furthermore, is there an organ-specific response to post-ischemic hyperoxygenation? These are the questions which could not be answered in this project.
4.5 Future direction

Several experiments could be done in the future to study the inter-relationships between hyperoxic reperfusion and mitochondrial function. First, the activities of ETC complexes are assayed to identify the key complex(es) responsible for the improvement of mitochondrial respiration after hyperoxic reperfusion. Second, electrophoresis by blue-native PAGE is performed to separate the ETC complexes for immunoblotting of protein modifications. Three types of protein modifications are tested, including carbonylation, glutathionylation and nitrosylation. Finally, immunostaining for nitrotyrosine residues in the tissue slices are conducted to demonstrate the oxidative stress status after hyperoxic reperfusion.
Chapter 5: Electrophoretic Concentration of Mitochondrial Membrane Protein Complexes

5.1 Introduction

Blue native PAGE (BN-PAGE), first developed by Schägger and von Jagow [82], has been successfully applied not only to the separation of mitochondrial respiratory protein complexes but also to the study of mitochondrial proteomics and disorders [83-85]. After BN-PAGE, the protein complex of interest is excised from the gel and further analyzed by SDS-PAGE for immunoblotting [86]. Nevertheless, due to the inherent hydrophobicity of mitochondrial membrane proteins, no more than 400 µg of sample can be loaded into a 1 cm wide, 1.6 mm thick well in the gel for BN-PAGE [86]. Otherwise, the proteins aggregate and cannot enter the stacking gel. The limited protein load in BN-PAGE has made the subsequent 2-D SDS-PAGE on a mini-gel system difficult. In order to obtain a sufficient amount of protein for SDS-PAGE, a wide blue native gel strip is required and must be rotated 90 degrees to be embedded into the SDS stacking gel. However, this long stacking gel occupies too much space and hampers the resolution of the resolving gel. Alternatively, electroelution or passive diffusion of protein from the gel strips with subsequent centrifugal concentration can be done but it is time-consuming and not applicable when the amount of sample is limited. In our experience, only about 150 µg of complex I could be obtained from 4 mg of rat
heart mitochondrial protein. Furthermore, a significant dissociation of complex I after the long isolation process was noted on the SDS-PAGE gel. Here we present an electrophoretic concentration method for mitochondrial membrane proteins using a mini-gel system. The protein complex of interest can be concentrated in a gel strip less than 1 cm wide, which can be easily sealed for SDS-PAGE. As a result, more protein is transferred to the 2-D SDS-PAGE and the sensitivity and accuracy of western blotting is improved.

5.2 Methods and Results:

Rat heart mitochondria were prepared as described previously [87] and frozen at -80 °C until use. The electrode, gel and solubilization buffers for BN-PAGE were made according to Wittig et al. [86]. All gels were hand-cast and run using a mini-gel system (Bio-Rad Mini-Protean, Hercules, CA). For BN-PAGE and electrophoretic concentration, all procedures were performed at 0-4 °C. One milligram of mitochondria was first resuspended in 300 μl buffer (250 mM sucrose, 10 mM HEPES, 1 mM EGTA and 1 mM PMSF, pH 7.2), followed by three cycles of freezing and thawing, and then pelleted at 16100 g for 20 min. The mitochondrial pellet was solubilized in 77.5 μl solubilization buffer (50 mM imidazole, 500 mM 6-aminohexanoic acid and 1 mM EDTA, pH 7.0 at 4°C), added with 22.5 μl 10% n-dodecyl β-D-maltoside (DDM, detergent/protein ratio (w/w): 2.25:1) and incubated on ice for 30 minutes. The solution was centrifuged at 20000 g for another 30 min to
remove insolubles and the supernatant was added with 10 μl loading buffer (solubilization buffer with 50% glycerol and 5% Coomassie blue G-250).

On a 1.5 mm-thick blue native gel composed of 4-6% acrylamide resolving gel and 3% acrylamide stacking gel, half of the sample (0.5 mg mitochondrial protein) was loaded onto each well (Fig. 5.1A). A lower concentration of gradient gel was selected because complex I was the desired protein complex in our study. For other mitochondrial membrane complexes of interest, a different acrylamide concentration may be chosen for better resolution. The BN-PAGE was run at 70 V for 80 min until the complex I band was distinctively separated. To verify the position of the complex I band, a duplicate gel was stained for the in-gel complex I activity [88]. Both short and long complex I gel strips were cut by a razor blade and only the long gel strip underwent the electrophoretic concentration. Finally, the short gel strip from the initial BN-PAGE and the concentrated gel strip were analyzed by SDS-PAGE to estimate the efficiency of electrophoretic concentration (Fig. 5.1).

The Y-shaped blue native gel (Fig. 5.1B) for electrophoretic concentration was cast by inserting two 1.5 mm-thick trapezoidal glass spacers between the plates. The neck was filled with 8% acrylamide resolving gel (2.0 cm x 0.8 cm) topped with a layer of 3% acrylamide stacking gel (4.5 cm in height). The excised gel strip was immobilized by 1% agarose gel. Then the gel underwent a 3-hour BN-PAGE in which the voltage was set at 100 V and the cathode buffer was added with 0.01% DDM and 0.001% SDS. After electrophoresis, all complex I in the gel strip was concentrated at the top of the resolving gel because of the small pore size (Fig. 5.1C). A duplicate
concentration gel was destained overnight with 5% methanol and 7.5% acetic acid to demonstrate excellent migration of complex I and indiscernible dissociation (Fig. 5.1D). We noticed that the addition of minimal SDS was needed to facilitate the complete migration of complex I from the gel strip while a concentration greater than 0.001% caused protein dissociation.

To evaluate the recovery rate of complex I after electrophoretic concentration, the short gel strip from the initial BN-PAGE and that of the concentration gel were analyzed by standard Tris-glycine SDS-PAGE [89]. The gel strips were soaked in 1% SDS for 15 min and embedded in a 1.5 mm-thick mini-gel with 8-16% gradient gel (4.5cm) and 4% stacking gel (1cm). After SDS-PAGE, the gel was stained for one hour using a modified colloidal coomassie blue G-250 staining method [90]. Destained with water overnight, the gel was scanned and densitometrically analyzed by ImageJ software (National Institutes of Health) for comparison of total protein content in both gel strips [91]. As seen in Fig. 5.1E, the protein bands in both lanes were comparable. There were no protein bands missing in the lane of the concentrated gel strip. The recovery rate of complex I, determined as the ratio of the total protein content of the concentrated gel strip to the total protein content of the short gel strip from BN-PAGE, was 87.7 ± 0.8 % (mean ± SD, n = 3).
Fig. 5.1 The procedures of electrophoretic concentration and evaluation of concentration efficiency. A) Mitochondrial protein complexes separated by BN-PAGE. Five hundred micrograms of mitochondrial protein were loaded onto each well. The first band of BN-PAGE was identified as complex I (marked in white dashed rectangle). B) The concentration gel. The excised complex I gel strip was sealed in a Y-shaped gel composed of 3% stacking gel and 8% resolving gel. C) The concentration gel after second BN-PAGE. A visible dark band of concentrated complex I was located at the top of the resolving gel. D) Destaining of the concentration gel. There was no
residual protein identified in the gel strip and stacking gel and only one band (complex I) was noted in the resolving gel.  E) Second SDS-PAGE. The concentrated gel strip has the identical protein band pattern as the non-concentrated gel strip. Gel photos A-D were taken by a Canon A570 IS digital camera and gel photo E was scanned by an Epson Perfection V30 scanner. Original color images were transformed into grayscale images and resized by FastStone Image Viewer.

5.3 Discussion

There have been a few reports applying gel electrophoresis to protein concentration [92-95]. However, none of them can be used for the concentration of high-molecular-weight protein complexes because of the SDS-PAGE system. Although conceptually similar to funnel-well SDS-PAGE [92], our system is superior for its low-neck design, which reduces both the total electrical resistance of the gel and heat production. Additionally, there is no overlay of sample gel strips which could hinder the migration of hydrophobic, large-sized protein molecules. This concentration method does not require special equipment, i.e., a funnel tube, or involve cumbersome electroelution and centrifugal concentration procedures. It is a modified 2-D BN/BN-PAGE which can be easily done with a mini-gel apparatus. Once completed, the concentrated protein complex in the gel strip is ready for SDS-PAGE or proteomic studies. Unwanted loss of protein complex is minimized, which is critical whenever the amount of mitochondrial sample is limited. In conclusion, the proposed method is a
simple and effective way to concentrate mitochondrial membrane protein complexes in preparation for the subsequent experimentation.

5.4 Future direction

Concentration of other mitochondrial membrane complexes could be conducted using a different pore size in the gel. This method can be readily applied to concentrate other cellular membrane proteins as well.
Chapter 6: Conclusion

These CPR-related projects presented in the dissertation have shed light on the importance of oxygen and the interesting role of mitochondria during cardiac arrest and post-reperfusion period. It would be imperative to further elucidate the mechanisms of oxygen sensing pathway in mitochondria and develop a mitochondria-oriented protection strategy in cardiac arrest and CPR. An accurate measurement of tissue oxygen tension should be exploited to reflect the status of cellular oxygenation. In addition, the difference of normobaric hyperoxygenation and hyperbaric hyperoxygenation at reperfusion should be thoroughly examined. Oxygen is undoubtedly important in reversing the dying process during the ischemic insult. What people don’t know and pursue constantly is the best strategy to minimize the reperfusion injury and increase the success of resuscitation. Hopefully the findings in the dissertation can serve as the basis for future CPR studies to substantially improve the survival rate of cardiac arrest victims.
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