Cerebral Hemodynamics and Metabolism During Cardiac Arrest and Cardiopulmonary Resuscitation Using Hyperspectral Near Infrared Spectroscopy

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Background: Maintaining cerebral oxygen delivery and metabolism during cardiac arrest (CA) through resuscitation is essential to improve the survival rate while avoiding brain injury. The effect of CA and cardiopulmonary resuscitation (CPR) on cerebral and muscle oxygen delivery and metabolism is not clearly quantified.

Methods and Results: A novel hyperspectral near-infrared spectroscopy (hNIRS) technique was developed and evaluated to measure cerebral oxygen delivery and aerobic metabolism during ventricular fibrillation (VF) CA and CPR in 14 pigs. The hNIRS parameters were measured simultaneously on the dura and skull to investigate the validity of non-invasive hNIRS measurements. In addition, we compared the hNIRS data collected simultaneously on the brain and muscle. Following VF induction, oxygenated hemoglobin (HbO2) declined with a 9.9 s delay and then cytochrome-c-oxidase (Cyt-ox) decreased on average 4.4 s later (P<0.05). CPR improved cerebral metabolism, which was reflected by an average 0.4 μmol/L increase in Cyt-ox, but had no significant effect on HbO2, deoxygenated hemoglobin (HHb) and tissue oxygen saturation (tSO2). Cyt-ox had greater correlation with HHb than HbO2.

Conclusions: Overall, hNIRS showed consistent measurements of hemodynamics and metabolism during CA and CPR.

Key Words: Cardiac arrest; Cardiopulmonary resuscitation; Cytochrome-c-oxidase; Metabolism; Near-infrared spectroscopy

Cardiac arrest (CA) is an abrupt and unexpected condition that results in the sudden drop in cardiac output and consequently cerebral perfusion. Approximately two-thirds of CA patients die from neurological injuries, which are due to prolonged cerebral ischemia and subsequent reperfusion despite cardiopulmonary resuscitation (CPR). Accurate measurement of cerebral oxygenation and metabolism during CA and CPR can provide important information on the cerebral response to CPR during CA, and test the effects of other resuscitation interventions. Near-infrared spectroscopy (NIRS) as a portable and non-invasive imaging technique is a good candidate for monitoring cerebral oxygenation under critical conditions such as CA. However, current NIRS technology has been evaluated in small observational CA studies with variable results.

Near-infrared spectroscopy has been progressively used for measurements of cerebral oxygenated hemoglobin (HbO2) and deoxygenated hemoglobin (HHb) since 1977, when Jöbsis proposed a new non-invasive optical imaging technique that used near-infrared light to measure concentrations of tissue chromophores according to their absorption coefficients. In the near-infrared window (650–1,050 nm), the main tissue chromophores are HbO2, HHb, water and oxidized cytochrome-c-oxidase (Cyt-ox; an intracellular oxygen metabolism index), among which water has the smallest and Cyt-ox has the greatest molar absorption.

Current commercial NIRS systems use only a few wavelengths in the near-infrared window, known as multispectral NIRS (mNIRS). Advancements in NIRS technology now utilize the whole near-infrared window, known as broadband or hyperspectral NIRS (hNIRS).

Both HbO2 and HHb are the intravascular chromophores reflecting oxygen delivery and have relatively high concentrations, which makes them easier to measure. Cyt-ox is the terminal enzyme in the mitochondrial electron transport chain that converts oxygen into water, leading to the production of adenosine triphosphate thus, measuring the redox changes of Cyt-ox represents the level of intracellular aerobic metabolism. Non-invasive measurement of...
Cyt-ox has previously been challenging, even in non-CA models due to the low concentration of this enzyme compared to hemoglobin; moreover, signal crosstalk between Cyt-ox and hemoglobin also compromises its measurement. The feasibility of hNIRS in the non-invasive measurement of cerebral microvascular oxygenation during CA has been previously investigated in a case series of 5 patients who experienced out-of-hospital CA. However, to the best of our knowledge, there were no studies on the measurement of Cyt-ox during CA and CPR, neither non-invasive nor using animal models allowing for invasive hNIRS measurements directly on the brain.

In this study, our objective was to develop and adapt hNIRS to detect cerebral hemodynamics and aerobic metabolism during CA and CPR using a novel algorithm based on the analytical solution to the diffusion equation (unlike most previous studies that used modified Beer-Lambert’s law) for the deconvolution of Cyt-ox. We evaluated the ability of this novel hNIRS method to measure cerebral oxygen delivery and metabolism during ventricular fibrillation (VF) and CPR, and measured hNIRS parameters directly on the brain dura. And in a limited number of cases, to investigate the validity of non-invasive hNIRS measurements, we also acquired hNIRS on intact skull simultaneously with invasive measurements on the dura. In addition, we carried out the analysis of the temporal correlations and delays between Cyt-ox and other chromophores.

Methods

Experimental Design
This was a pilot observational study of the hNIRS technique during CA and resuscitation. The required sample size was computed using the G-power 3.1 software for one-tail paired sample t-test, the effect size of 0.7 and the test power of 0.8, the minimum required sample size was 14. Fourteen healthy pigs (Yorkshire, female, 6–9 weeks, 33–39.3 kg) arrived in animal facility at least 5 days before the study. Pigs were housed in cages suitable for pigs in pairs when possible to provide an enriching and comfortable environment. When single housing was necessary, pigs were able to touch noses with pigs in neighboring pens. Double housing pens were 6’×5’×7’ (length × width × height), single housing pens were 3’×5’×7’ (length × width × height). Pigs were fed swine grower vegetarian diet (Harlan Teklad, Madison, WI, USA), 8–12 cups per day (depending on weight) divided into 2 meals during the day. Drinking water was purified by reverse osmosis and was provided ad libitum. Animals were fasted overnight and sedated with intramuscular ketamine (20 mg/kg), Xylazine (2 mg/kg) and atropine (1 ml/25 kg), and anesthesia was induced with 5% isoflurane. Once anesthetized, pigs were endotracheally intubated and maintained by continuous administration of isoflurane (1–3% mixed with 100% O2).

Pigs were connected to a ventilator (Ohio ventilator R.A.E. Technologies, Inc. Ontario, Canada) at a rate of 21 breaths/min, a tidal volume of 10–15 mL/kg, a minute ventilation of 5 L/min, and an inspiratory-to-expiratory ratio of 1:2. The depth of anesthesia was checked periodically (every 15 min) by monitoring heart rate and evaluating muscle tone, jaw reflex and movements. Ringers lactate infusion was administered as maintenance fluid at a rate of 2–4 mL/kg/h.

Three limb leads were placed for electrocardiography (ECG) recording. An electrode catheter (EP Technologies Inc., Sunnyvale, CA, USA) was positioned at the apex of the right ventricle via the right femoral vein to allow for intra-cardiac ECG recording and the induction of VF. The electrode was guided to the right ventricle through the inferior vena cava and right atrium, and VF was induced by applying a 2 s, 10 Volts, fully rectified, 60 Hz current. Mechanical ventilation was stopped after VF induction.

![Figure 1.](image-url)
CA was defined by identification of VF on ECG and absent pulsatile aortic systolic pressure. As shown in Figure 1A, pigs remained untreated in VF for 6 min followed by 8–14 min of CPR and, ultimately, a defibrillation shock of 200 J was applied. During CPR, chest compressions were performed by a LUCAS device (98–102 compressions/min; Jolife AB, Lund, Sweden); ventilation consisted of 6 breaths/min delivered with an Ambu bag (Ambu Inc., Glen Burnie, MD, USA).

Because after 3 min of CPR different animals were given a different dosage of epinephrine and defibrillation at different moments of time, for average changes measured by hNIRS in this study, we only included 2 min of CPR when the conditions were the same for all animals; the influence of epinephrine and defibrillation will be discussed in another paper.

All hNIRS data were collected by placing the optodes on the dura mater after removing the scalp and skull at the region of measurement. In 2 animals, hNIRS data were simultaneously collected over the skull (less-invasive). The skin was removed from the skull to make the thickness of the extra-cerebral layer close to that in adult humans. For 5 animals, another pair of hNIRS optodes were placed on the biceps femoris muscle.

The hNIRS data collection was started at least 5 min prior to VF induction, which was later used as the baseline, as shown in Figure 1A. The mean carotid flow (mCaF), mean femoral artery flow (mFeF) and mean aortic pressure (mAP) were also recorded during the experiments using solid-state high fidelity, micro manometer-tipped catheters (MiKro-TIPTM Transducer, Millar Instruments Inc., Houston, TX, USA).57 All protocols were approved by the Animal Care Committee of St. Michael’s Hospital (Toronto, ON, Canada) and conformed to the guide for the care and use of laboratory animals, US National Institutes of Health (NIH Publication N°. 85–23, revised 1996). The Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines88 were used as the guidelines for carrying out experiments and presenting the information.

**hNIRS System Setup**

A custom-made continuous-wave (CW) hNIRS system (shown in Figure 1B) was used to probe the brain and muscle. hNIRS data acquisition was performed by using broadband spectrometers, with a spectral resolution of 4 nm and a spectral range of 650–1,100 nm (QE65000 Ocean Optics, Dunedin, FL, USA) consisting of a holographic diffraction grating and a cooled charge-coupled device (CCD) detector (1,044×64 element) with 95% efficiency. A stabilized tungsten halogen light source (Thorlabs, OSL2BIR; Al-coated reflector bulb) with enhanced illumination in the near-infrared region was used as a broadband light source. One-meter-long optical fiber bundles with a 3-mm core diameter were used to transfer the light from the source to the tissue and also to collect the light transmitted through the tissue and to transfer it to the spectrometer.

Source detector distances on the muscle, skull and dura were 20, 30 and 10 mm, respectively. Considering the average thickness of the pig skull (9±1.2 mm), the source-detector separation on the brain was chosen such that the optimal hNIRS signal is collected and roughly interrogate similar volume69 of the brain in both invasive (dura) and less-invasive (skull) measurements.70 To minimize motion artefacts during VF and CPR vibrations, 7-mm thick rubber pads with punched holes were used to hold the optodes perpendicular to the tissue surface. For more support, the medical tape around the fibers was sutured to the surrounding skin layer. hNIRS data were sampled at 4 Hz.

**hNIRS Data Processing Algorithm**

The goal was to calculate the relative changes in the chromophore concentrations at each sampling time through the measured changes in the absorption coefficients ($\Delta\mu_a$), which was proportional to the changes in the concentrations of chromophores and their molar absorption (extinction) coefficients ($\varepsilon$):

$$\Delta\mu_a(\lambda) = \Delta[HbO_2]HbO_2 + \Delta[HHb]HHb + \Delta[Cyt-ox]Cyt-ox + \Delta[H_2O]H_2O$$

The extinction coefficient spectra were obtained from the “Tissue Spectra” database published by UCL.9,21,22 To calculate $\Delta\mu_a$ at every sampling time, the photon fluence as a function of source-detector distance ($\rho$), reduced scattering coefficient ($\mu_s'$) and absorption coefficient ($\mu_a$) was calculated using an analytical solution to the diffusion approximation (DA) for a semi-infinite medium with extrapolated boundary correction, as explained in detail by Fantini et al.31 The reduced scattering coefficients ($\mu_s'$) were assumed to be independent of chromophore concentrations and were calculated for each wavelength ($\lambda$) using the power law, as described by Yeganeh et al.10 Relative changes in the calculated fluence of each wavelength at each sampling point was then non-linearly fitted to the measured absorbance11 to estimate $\Delta\mu_a$:

$$\ln \frac{\Psi_{\lambda,s}(\mu_a + \Delta\mu_a, \mu'_s)}{\Psi_{\lambda,s}(\mu_a, \mu'_s)} = \text{non-linear-fit} \left[ \ln \frac{\text{Data}(\lambda, t)}{\text{Data}(\lambda, t_0)} \right]$$

The baseline concentrations of HHb, HbO2, and water concentration were estimated, as described by Yeganeh et al.10 As shown in Figure 1C, the data fitting was performed in 3 steps with different spectral ranges:

1. 700–900 nm for the calculation of $\Delta[HbO_2]$ and $\Delta[HHb]$;
2. 900–1,020 nm for the calculation of $\Delta[H_2O]$ and improvement of $\Delta[HbO_2]$;
3. 700–800 nm for the calculation of $\Delta[Cyt-ox]$.

In the third step of our algorithm, the first derivative of the absorbance vs. $\lambda$ was used to deconvolve Cyt-ox; therefore, for the deconvolution of Cyt-ox, redox changes the 700–800 nm spectral range at which maximum changes are observed in the first-order derivative, and which is used for data fitting. The usefulness of applying the derivatives of the extinction coefficient’s spectra has been reported.10 The schematics of the proposed data processing algorithm is shown in Figure 1C.

Tissue oxygen saturation ($tSO_2$) and total hemoglobin concentration (total Hb) were calculated as follows:

$$total\ Hb = HbO_2 + HHb \quad and \quad tSO_2 = \frac{HbO_2}{total\ Hb}$$

In addition, we have also used the algorithm27 based on the modified Beer-Lambert’s law (MBLL) to calculate the concentration of different chromophores and compare the results with the proposed new algorithm.
Data Analysis

Cerebral parameters including HbO₂, HHb, total Hb, tSO₂ and Cyt-ox calculated from hNIRS data were temporally aligned according to the instant of VF induction. Average traces were also calculated.

The correlation analysis was conducted over 20-s intervals starting from the onset of VF (defined by a 20% reduction of mAoP) in each experiment. In order to assess the correlation between different pairs of chromophores (including HbO₂ vs. Cyt-ox, HHb vs. Cyt-ox and tSO₂ vs. Cyt-ox), the Pearson linear correlation coefficient was calculated between all pairs.

To investigate how fast cerebral hemodynamics and oxygen metabolism were affected by VF and to quantify the delay (if any) between VF-induced changes, the instant at which the mAoP dropped by 20% of its baseline was considered as the reference (VF onset) point; the time interval after VF onset, during which the average baseline values of each chromophore (±0.01%) were maintained, was considered as the time lag.

To investigate the effects of VF and CPR on cerebral oxygenation and metabolism, 9-min intervals were isolated and divided into 3 epochs (including: 1 min baseline, 6 min during untreated VF and 2 min after start of CPR). All baseline values ("before VF") were calculated as the average over 1 min prior to VF induction; the last 1 min of untreated VF (before beginning of CPR) was averaged and was considered as "during VF"; the "during CPR" values were calculated as the average during the second minute of CPR. All calculated parameters in all experiments were averaged over the 9-min intervals. The individual and average changes in cerebral parameters were analyzed and compared against those of the muscle.

The statistical significance of the observed changes was assessed using a 1-tailed paired-sample t-test. The hNIRS measured values (averaged over 1-min intervals) before VF onset were compared with those during VF and CPR. Statistical significance was assumed at P<0.05. All statistical analyses were carried out in MATLAB (Mathworks, USA, version R2015a).

Results

Effects of VF and CPR on Cerebral Hemodynamics and Metabolism

The temporal traces of group-averaged HbO₂, HHb, total Hb, tSO₂ and Cyt-ox during untreated VF and 2 min after start of CPR are shown in Figure 2. All NIRS measurements were on the DURA. The arrows indicate the onset of ventricular fibrillation (VF) at 50 s and cardiopulmonary resuscitation (CPR) at 410 s. The error bars represent standard errors.

Figure 2. (A–F) Average changes in mean aortic pressure (mAoP in black) along with hyperspectral near-infrared spectroscopy (hNIRS)-derived cerebral parameters including (A) oxygenated hemoglobin (HbO₂), (B) deoxygenated hemoglobin (HHb), (C) cytochrome-c-oxidase (Cyt-ox), (D) tissue oxygen saturation (tSO₂), (E) total Hb and (F) mean carotid flow (mCaF). All NIRS measurements were on the DURA. The arrows indicate the onset of ventricular fibrillation (VF) (at 50 s) and cardiopulmonary resuscitation (CPR) (at 410 s). The error bars represent standard errors. (G, H) Two-sample comparison between the calculated redox changes of Cyt-ox using the proposed algorithm based on diffusion approximation (DA) and the algorithm based on the modified Beer-Lambert’s law (MBLL).
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Figure 2B concentration gradually increased during the entire VF epoch and slightly decreased during CPR. The increase in HHb during VF, while HbO2 remained almost constant, corresponded to a moderate increase of the total hemoglobin concentration (shown in Figure 2E), which began ~200 s after VF induction and added ~2 μmol/L (~3% of the baseline value) by the end of the untreated VF period.

Table 1 summarizes the mean changes of hNIRS parameters (HbO2, HHb, total Hb, tSO2, and Cyt-ox) along with Hb, tSO2, and Cyt-ox measured on the dura, and average changes in mAoP and mCaF during 9 min (baseline, untreated VF and CPR) are shown in Figure 2A-F. The time-course of VF-induced changes in HbO2, tSO2 and Cyt-ox were roughly similar to the changes in mAoP and mCaF, with all parameters falling during first 2–3 min after VF induction. HbO2 (Figure 2A) decreased and reached a nadir ~200 s after VF induction; however, HHb, tSO2, and Cyt-ox gradually declined and did not reach a plateau or nadir during 6 min of untreated VF. The average HHb
Comparing the Proposed and MBLL Algorithms

The time-course of the calculated changes in HbO₂ and HHb using the proposed algorithm were highly correlated (>95%) to those calculated using the MBLL algorithm. However, the Cyt-ox redox changes calculated using MBLL were inconsistent and sometimes quite unexpected.

Dura vs. Skull hNIRS Measurements

Changes in cerebral hNIRS parameters measured simulta-
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of Cyt-ox in the brain were significantly greater than that in the muscle. CPR resulted in opposite changes in Cyt-ox and HHb in the muscle compared to the brain (P<0.05); following CPR, Cyt-ox increased in the brain and decreased in the muscle; HHb decreased in the brain and increased in the muscle. From Table 2, one can see that CPR caused greater total Hb increase in the muscle than in the brain, which is in agreement with the femoral blood flow (mFeF) changes after CPR (see Figure 4F). The muscle tSO₂ increase after CPR was small and Cyt-ox continued to decline.

Comparison of the Brain vs. Muscle Hemodynamics and Metabolism
Figure 4 shows the time-courses of the average changes in the muscle hemodynamics and metabolism before and during VF and CPR (compare with Figure 2). The average changes induced by VF and CPR in the cerebral and the muscle tissues of 5 animals are presented in Table 2. VF resulted in similar trends in the muscle and the brain hemodynamics and metabolism; however, VF-induced changes of Cyt-ox in the brain were significantly greater than that in the muscle. CPR resulted in opposite changes in Cyt-ox and HHb in the muscle compared to the brain (P<0.05); following CPR, Cyt-ox increased in the brain and decreased in the muscle; HHb decreased in the brain and increased in the muscle. From Table 2, one can see that CPR caused greater total Hb increase in the muscle than in the brain, which is in agreement with the femoral blood flow (mFeF) changes after CPR (see Figure 4F). The muscle tSO₂ increase after CPR was small and Cyt-ox continued to decline.

Correlation Analysis
For all 14 animals, the temporal correlations between different pairs of chromophores were calculated and the correlation coefficients are listed in Table 3A. The absolute correlation between “Cyt-ox and HHb” and “Cyt-ox and tSO₂” was 0.98±0.001; the correlation between Cyt-ox and HbO₂ was +0.89±0.01.
Temporal Delay Analysis

Responses of different chromophores to the onset of VF showed different delay times, as shown in Figure 5A–C and in Table 3B. Following VF induction, HbO₂ dropped with a 9.5±3.1 s delay, HHb increased after 11.2±3.5 s, and tSO₂ fell after 10.1±3.10 s. Cyt-ox had the longest delay after VF induction, with a time lag of 14.2±5.5 s.

Discussion

This is the first animal study to show that hNIRS is able to measure HbO₂, HHb, tSO₂, total Hb, and Cyt-ox during CA and CPR. While the HbO₂ level represents the intravascular oxygenation, Cyt-ox reflects intracellular oxygen utilization, which is potentially a good index for cellular function. In addition, higher mitochondrial density in the brain gray matter compared to other tissues (such as skin and scalp) makes the cerebral Cyt-ox signal more brain-specific than the hemoglobin, which may be contaminated by hemodynamics in extra-cerebral layers.24,28

Bainbridge et al37 used hNIRS simultaneously with magnetic resonance spectroscopy to measure cerebral oxygen metabolism before and after hypoxia, and showed agreement between the 2 modalities. Subsequently, various hNIRS algorithms have been proposed to deconvolve Cyt-ox redox changes based either on MBLL28 or on DA.11,29 The results of the deconvolution of the Cyt-ox signal has been found to be strongly affected by the employed algorithm.30 Therefore, although hNIRS has shown to be a promising tool for the measurement of tissue oxygenation, the possibility of crosstalk between Cyt-ox and hemoglobin changes has questioned the reliability of the Cyt-ox measurements.30,31 Our novel hNIRS algorithm successfully avoided the crosstalk between the hemodynamics and metabolic changes. Compared to the MBLL algorithm, which was inconsistent in this study, the measured changes of Cyt-ox using our modified DA algorithm were consistent with the hemodynamic changes.

We also found that both invasive hNIRS measurements on the dura and less-invasive measurements on the skull showed similar changes of hNIRS parameters, including Cyt-ox, and were in agreement with the intravascular measurements of mAoP and mCaF. The observed minor differences in time delays of chromophores measured on the dura and through the skull could reflect differences between more local measurement on the dura and more integrative measurement through the skull (due to the scattering effect of the skull). This also agrees with the fact that skull traversing, shown in Figure 3, were similar to the group-averaged dura traversing shown in Figure 2A–F.

There have been few studies published on monitoring brain tissue and microvascular oxygenation during CA. Yu et al22 measured cerebral tissue oxygen tension during CA and CPR using an Oxysphor G4-based micro-sensor. Frisch et al32 used near infrared spectroscopy (NIRS) to measure the microvascular cerebral oxygen saturation (tSO₂) during and after CA and CPR in 4 patients. Nagdyman33 has monitored cerebral microvascular oxygenation and Cyt-ox using hNIRS during CA on 1 subject. Although these findings33 confirmed the potential usefulness of NIRS for brain monitoring during CA, due to the absence of baseline measurements, uncontrolled experiments and a limited number of samples, the results were inconsistent and statistically insignificant.

Our study provides new insights into brain hemodynamics and oxygen metabolism during CA. We found that upon VF induction, the level of cerebral HbO₂, HHb and tSO₂ underwent rapid changes before Cyt-ox began to fall. Cyt-ox may be partially uncoupled from hemodynamics and represent the ability of neurons to extract O₂, albeit short-lived, during hypoxic states. This is consistent with the findings by Vazquez et al,34 who showed a 4.8±3.1 s average temporal delay between oxygen diffusion into tissue and oxygen consumption in mitochondria. Interestingly, we also observed an increase in cerebral total Hb of ~200 s after VF induction (which was not observed in the muscle); this is consistent with a previous study by Shaik et al who showed increased vasodilator eicosanoids in the cerebral cortex during CA in rats.35 Increased cerebral total Hb only in the cerebral tissue during untreated VF may represent the cerebral autoregulation through cerebral vasodilation.

We also observed that there was a larger decrease in Cyt-ox in the brain compared to muscle during untreated VF, which may represent higher concentrations of Cyt-ox in the brain36 or the brain’s vulnerability to hypoxia.

During CPR, both HHb and HbO₂ in the muscle increased together, thus representing an increase in muscle blood volume; however, muscle Cyt-ox continued to decrease regardless, which may represent that CPR did not improve the muscle oxygen utilization. In contrast, in the brain, Cyt-ox increased significantly (by ~15%) correlating with a 16% increase in mCaF.

This study was limited by the number of experimental animals, which may affect our interpretation of the present study results. We were also not able to include less-invasive and muscle hNIRS monitoring on all study animals. Nevertheless, we met our objective to adapt hNIRS to detect cerebral and muscle microvascular oxygenation and aerobic metabolism in CA.

Conclusions

Our proposed hNIRS algorithm is able to consistently measure HbO₂, HHb and Cyt-ox with minimal crosstalk during CA and CPR. This hNIRS technology has provided new insights into the cerebral and muscle hemodynamics and metabolism during CA, and can be used to monitor cerebral oxygenation and aerobic metabolism in future CA studies.

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References


