

Cite this article as: Mavroudis CD, Karlsson M, Ko T, Hefti M, Gentile JI, Morgan RW *et al.* Cerebral mitochondrial dysfunction associated with deep hypothermic circulatory arrest in neonatal swine. *Eur J Cardiothorac Surg* 2018; doi:10.1093/ejcts/ezx467.

Cerebral mitochondrial dysfunction associated with deep hypothermic circulatory arrest in neonatal swine[†]

Constantine D. Mavroudis^{a,*}, Michael Karlsson^b, Tiffany Ko^c, Marco Hefti^d, Javier I. Gentile^a, Ryan W. Morgan^b, Ross Plyler^b, Kobina G. Mensah-Brown^c, Timothy W. Boorady^c, Richard W. Melchior^e, Tami M. Rosenthal^e, Brandon C. Shade^e, Kellie L. Schiavo^e, Susan C. Nicolson^b, Thomas L. Spray^a, Robert M. Sutton^b, Robert A. Berg^b, Daniel J. Licht^c, J. William Gaynor^a and Todd J. Kilbaugh^b

^a Department of Cardiothoracic Surgery, The Children's Hospital of Philadelphia, Philadelphia, PA, USA

^b Department of Anesthesia and Critical Care Medicine, The Children's Hospital of Philadelphia, Philadelphia, PA, USA

^c Department of Neurology, The Children's Hospital of Philadelphia, Philadelphia, PA, USA

^d Department of Pathology, University of Iowa Carver College of Medicine, Iowa City, IA, USA

^e Department of Perfusion Services, The Children's Hospital of Philadelphia, Philadelphia, PA, USA

* Corresponding author. Department of Cardiothoracic Surgery, The Children's Hospital of Philadelphia, Philadelphia, PA, USA. Tel: +1-215-7769285; fax: +1-215-5902715; e-mail: constantine.mavroudis@uphs.upenn.edu (C.D. Mavroudis).

Received 9 September 2017; received in revised form 15 November 2017; accepted 2 December 2017

Abstract

OBJECTIVES: Controversy remains regarding the use of deep hypothermic circulatory arrest (DHCA) in neonatal cardiac surgery. Alterations in cerebral mitochondrial bioenergetics are thought to contribute to ischaemia-reperfusion injury in DHCA. The purpose of this study was to compare cerebral mitochondrial bioenergetics for DHCA with deep hypothermic continuous perfusion using a neonatal swine model.

METHODS: Twenty-four piglets (mean weight 3.8 kg) were placed on cardiopulmonary bypass (CPB): 10 underwent 40-min DHCA, following cooling to 18°C, 10 underwent 40 min DHCA and 10 remained at deep hypothermia for 40 min; animals were subsequently rewarmed to normothermia. 4 remained on normothermic CPB throughout. Fresh brain tissue was harvested while on CPB and assessed for mitochondrial respiration and reactive oxygen species generation. Cerebral microdialysis samples were collected throughout the analysis.

RESULTS: DHCA animals had significantly decreased mitochondrial complex I respiration, maximal oxidative phosphorylation, respiratory control ratio and significantly increased mitochondrial reactive oxygen species ($P < 0.05$ for all). DHCA animals also had significantly increased cerebral microdialysis indicators of cerebral ischaemia (lactate/pyruvate ratio) and neuronal death (glycerol) during and after rewarming.

CONCLUSIONS: DHCA is associated with disruption of mitochondrial bioenergetics compared with deep hypothermic continuous perfusion. Preserving mitochondrial health may mitigate brain injury in cardiac surgical patients. Further studies are needed to better understand the mechanisms of neurological injury in neonatal cardiac surgery and correlate mitochondrial dysfunction with neurological outcomes.

Keywords: Deep hypothermic circulatory arrest • Congenital heart surgery • Neuroprotection • Mitochondria • Basic science

INTRODUCTION

Despite advancements in surgical techniques, anaesthetic protocols, cardiopulmonary bypass (CPB) methods and postoperative care, neurological complications remain a major source of morbidity in neonates undergoing complex cardiac surgery, and their incidence has not decreased significantly in the last 30 years [1, 2].

There is an ongoing debate regarding the relative safety of using deep hypothermic circulatory arrest (DHCA) during complex neonatal cardiac surgeries. The theoretical basis behind the safe conduct of DHCA lies in the hypothermia-mediated decrease in the cerebral metabolic rate, allowing brief interruptions of circulation for performing certain surgical procedures. Several studies on animals have shown deleterious effects of DHCA, but its use continues in clinical practice because human data have yet to demonstrate any significant short- or long-term neurological morbidity associated with its use compared with other perfusion strategies [3, 4].

[†]Presented at the 31st Annual Meeting of the European Association for Cardio-Thoracic Surgery, Vienna, Austria, 7–10 October 2017.

Previous studies in DHCA have used downstream markers of intrinsic and extrinsic apoptotic pathways such as caspases and cytochrome *c* translocation as indicators of neurological injury [5, 6]. These studies indirectly implicate mitochondrial function as being an important contributor to neurological injury, but there have been no direct assessments of cerebral mitochondrial function in cardiac surgical models. Further evidence of metabolic and mitochondrial dysfunction leading to brain injury comes from cerebral microdialysis (cMD) assessments in neurocritical care patients. cMD is used in these patients to assess the real-time changes in cerebral metabolism and global cerebral health and has been used in the animal models of circulatory arrest and cardiac surgery [4, 7].

Cerebral mitochondrial dysfunction has been shown to be a critical component driving secondary brain injury after ischaemia-reperfusion (IR) [8, 9]. In swine models of cardiac arrest and traumatic brain injury, alterations in the mitochondrial function correlated with neurological injury [10]. We believe that mitochondria are at the fulcrum of brain IR injury and represent a prime mechanistic target to identify strategies for innovative neuro-monitoring and therapeutic development. However, the role that mitochondrial bioenergetics play in IR brain injury during paediatric cardiac surgery remains a critical knowledge gap. The overarching goal of this research is to determine how differing CPB strategies affect cerebral mitochondrial bioenergetics and IR injury in a swine model of neonatal cardiac surgery. We hypothesized that animals exposed to DHCA would exhibit significant damage to the mitochondrial electron transport chain (ETC), increased production of mitochondrial reactive oxygen species (ROS) and cerebral metabolic crisis and neuronal injury measured by cMD compared to animals treated with deep hypothermic continuous perfusion (DHCP).

MATERIALS AND METHODS

Animals and overall study design

All procedures were approved by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. One-week-old female piglets ($n=24$; mean weight 3.6 kg) were premedicated with 20 mg/kg ketamine, followed by inhaled isoflurane in 100% oxygen via a snout mask. They were then intubated and oxygen was weaned to room air. The animals were randomized to 3 different treatment groups: DHCA ($n=10$), DHCP ($n=10$) and CPB at normothermia (control) ($n=4$).

Perioperative monitoring

Ventilator settings were as follows: tidal volume 10–11 ml/kg, positive end-expiratory pressure 6 cm H₂O and respiratory rate titrated to achieve end-tidal carbon dioxide 35–45 mmHg to minimize potential confounding changes in cerebral blood flow and acid-base status. The right femoral artery and vein were cannulated for arterial pressure monitoring and central venous access. Intravenous fentanyl (25–200 µg/kg/min) and dexmedetomidate (0.5–2 µg/kg/min) were started and isoflurane was weaned to approximately 0.5–1% to simulate human anaesthetic protocols and minimize confounding toxicity and cerebral blood

flow changes associated with higher doses of isoflurane [11, 12]. Rectal and nasopharyngeal temperature probes were placed.

Operative methods and experimental protocol

Median sternotomy and central, aortoatrial cannulation for CPB was performed using a 10-Fr arterial cannula and a 20-Fr venous cannula, respectively. Heparin bolus of 1000 U/kg was given, and CPB was initiated once the activated coagulation time reached >480 s. The CPB circuit comprised a heart-lung machine (Jostra HL20, Maquet Cardiovascular, Wayne, NJ, USA), a membrane oxygenator and hard-shell venous reservoir (Capiiox FX05, Terumo Cardiovascular, Ann Arbor, MI, USA), a sterile 0.25-inch tubing and a haemoconcentrator (Capiiox, Terumo Cardiovascular, Ann Arbor, MI, USA). The circuit was primed as follows: 400 ml Plasma-Lyte A, 300 ml fresh whole blood from donor swine, 500 IU heparin, 2 mEq/kg sodium bicarbonate, 1 mg/kg furosemide and 450 mg calcium gluconate. Target CPB flow rates were 150 ml/kg/min while maintaining carbon dioxide levels within 35–45 mmHg, mean arterial pressure >35 mmHg, activated coagulation time >480 s, partial pressure of arterial oxygen >250 mmHg and haematocrit >28%. Animals were randomized to the DHCA, DHCP or control groups. Subjects were cooled no quicker than 1°C per minute with a temperature gradient no greater than 10°C. pH stat was used during cooling and while cold. At a nasopharyngeal temperature of 18°C, DHCA animals underwent 40 min of DHCA, while DHCP animals were maintained at a flow of 125–150 ml/kg/min to a mean arterial blood pressure between 30 and 40 mmHg for 40 min. Animals were rewarmed to normothermia, using alpha stat once the temperature reached 27°C. Control animals were maintained at normothermia on CPB at 150 ml/kg/min.

Measurement of cerebral microdialysis

cMD was measured in the left frontal cortex (CMA 71 Elite, mDialysis, Sweden). Probes were placed 0.5–1 cm deep in the brain parenchyma. Sterile saline was perfused at 1 µl/min, and after a 30-min calibration period, samples were collected in 20–30-min intervals throughout CPB to correspond with the initiation of CPB, cooling, deep hypothermia, rewarming and end of study periods. Samples were immediately frozen at -80°C. Pyruvate, lactate, glycerol and glucose levels were analysed in a blinded fashion using the automated ISCUS Flex™ Microdialysis Analyzer (mDialysis) and data were processed using the LABpilot software (mDialysis).

Tissue extraction and preparation

Following rewarming and 30 min of normothermic CPB, craniectomy was performed to expose the brain while the animals remained on CPB. Cortical tissue sections were rapidly extracted and placed in ice-cold buffer (320 mM sucrose, 10 mM Trizma base and 2 mM ethylene glycol tetraacetic acid). Brain tissue preparation for high-resolution mitochondrial respirometry was immediately performed as previously described [13].

Measurement of mitochondrial respiration

Mitochondrial respiratory function was analysed *ex vivo* in brain cortex homogenates using high-resolution respirometry (Oxygraph-2k,

Oroboros Instruments) with a substrate–uncoupler–inhibitor titration (SUIT) protocol as previously described [13]. The SUIT protocol measures oxidative phosphorylation capacity with electron flow through both complex I (CI) and complex II (CII), as well as the convergent electron input (CI + CII) using the nicotinamide adenine dinucleotide-linked substrates malate (5 mM) and pyruvate (5 mM), as well as succinate (10 mM), both in the presence of adenosine diphosphate (1 mM), corresponding to State 3 respiration in classical mitochondrial terminology. Oxidative phosphorylation produces adenosine triphosphate (ATP), which is the primary fuel for performing basic function. During times of stress and injury, increased maximal oxidative phosphorylation is necessary for neuronal salvage and repair because of high-energy requirements. Oligomycin, an inhibitor of the ATP synthase, induces uncoupled respiration without ATP-synthase activity (LEAK) respiration ($LEAK_{CI+CII}$) or State 4_o. If LEAK respiration is increased, the electrochemical gradient across the mitochondrial membrane is uncoupled, resulting in the production of adequate ATP. Maximal convergent non-phosphorylating respiration of ETC_{CI+CII} is evaluated by titrating the protonophore, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone. ETC_{CI+CII} is a sort of stress test for mitochondria, as a marker of mitochondrial respiratory reserve. Non-phosphorylating respiration through CII (ETC_{CII}) is achieved through the addition of rotenone (2 mM). The CIII inhibitor antimycin-A (1 µg/ml) is added to measure the residual oxygen consumption, and this value is subtracted from each of the previously measured respiratory states. CIV activity is measured by the addition of *N,N,N',N'*-tetramethyl-phenylenediamine (0.5 mM) together with ascorbate (0.8 mM). The CIV inhibitor sodium azide (10 mM) is added to reveal the chemical background that is subtracted from the *N,N,N',N'*-tetramethyl-phenylenediamine-induced oxygen consumption rate. A respiratory control ratio (RCR) was calculated as a ratio of oxidative phosphorylation (OXPHOS) and LEAK respiration, reflecting the ATP-generating capacity of the mitochondria that takes into account the mitochondrial inner membrane stability. RCR is a marker of overall mitochondrial health.

Measurement of reactive oxygen species in brain tissue

Measurements of ROS generation in brain tissue was measured using the O2k-Fluorescence LED2-Module (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria), permitting simultaneous measurements of hydrogen peroxide (H₂O₂) production and mitochondrial respiration, utilizing an Amplex UltraRed assay as previously described [14]. In short, Amplex UltraRed (N-acetyl-3, 7 dihydroxyphenoxazine) (5 mM), in the presence of horseradish peroxidase (1 U/ml), reacts with H₂O₂ to produce the fluorescent compound resorufin. The addition of superoxide dismutase (SOD) (10 U/ml) ensures that all superoxide is converted into H₂O₂. Calibration of the fluorometric signal was done prior to each measurement by the addition of 100 nM H₂O₂. Mitochondrial ROS generation is the predominate source of ROS and leads to alterations in redox signalling, oxidative damage to proteins and lipids, additional mitochondrial dysfunction and ultimately a major cause of ongoing secondary brain injury.

Citrate synthase activity

Citrate synthase (CS) was measured as a marker of mitochondrial content and used in addition to tissue weight for normalization of brain tissue as previously described [15]. Chamber contents

from the brain high-resolution respirometry measurements were frozen for subsequent CS activity measurements. A commercially available kit (Citrate Synthase Assay Kit, CS0720, Sigma-Aldrich, St Louis, MO, USA) was used according to the manufacturer's instructions to determine the CS activity (µmol/ml/min).

Statistics and data analysis

Laboratory technicians analysing the mitochondrial function and cMD were blinded to the treatment groups. Statistical analysis was carried out using GraphPad Prism software (GraphPad, San Diego, CA, USA). The Student's *t*-test was used for normally distributed data. RCRs were not normally distributed, and the Mann-Whitney non-parametric test was used. Differences were considered significant where $P < 0.05$. Normally distributed data are presented as mean ± standard error of the mean unless otherwise noted.

RESULTS

Baseline characteristics, blood gas chemistry and haemodynamic variables

Perioperative and operative variables are listed in Table 1. Animals in the DHCA group were slightly smaller than the animals in the DHCP group and had higher baseline haematocrit, but there were no differences in baseline characteristics. During rewarming and at the end of the experiment, the animals in the DHCA group had significantly higher serum lactate levels than the animals in the DHCP group ($P = 0.002$ and 0.001 , respectively). There were no significant differences in blood gas variable levels between DHCP and DHCA animals. Mean arterial blood pressure did not vary significantly between the animal groups (data not shown).

Brain cerebral mitochondrial respiration

CI respiration (OXPHOS_{CI}) in animals treated with DHCP was significantly higher when compared with DHCA ($P < 0.03$) and controls (Fig. 1). Mitochondrial maximal coupled OXPHOS_{CI+CII}, stimulated by both CI and CII substrates, was significantly higher in the cortex of DHCP animals when compared with DHCA animals ($P < 0.02$) and controls ($P < 0.02$) (Fig. 2). RCRs were measured and reported as measures of overall mitochondrial performance (Table 2). There were no differences in the inner membrane stability (LEAK respiration) or CII respiration among groups. Respiratory ratios evaluating phosphorylation coupling efficiency, overall mitochondrial health and the ability to produce ATP were calculated for DHCA, DHCP and control animals. The RCR for OXPHOS_{CI+CII} (OXPHOS_{CI+CII}/LEAK) was calculated to determine the efficiency of coupled phosphorylating respiration, a global measure of overall mitochondrial function and was significantly decreased in the cortex ($P < 0.02$) in DHCA animals compared with DHCP and control animals (Fig. 2).

Reactive oxygen species generation

Mitochondrial ROS production, measured during maximal oxidative phosphorylation and normalized to mitochondrial content (H₂O₂/CS), was significantly increased in the DHCA treatment

Table 1: Baseline group characteristics and intraoperative blood gas variables

Variables	DHCA (n=10)	DHCP (n=10)	Control (n=4)	P-value DHCA vs DHCP
Weight (kg)	3.6 (0.6)	4.1 (0.5)	3.6 (0.2)	0.048
Cooling duration (min)	27.6 (5.6)	25.1 (4.9)	n/a	0.261
Rewarming duration (min)	28.2 (5.4)	27.4 (5.9)	n/a	0.705
Baseline Hct (%)	33.9 (4.1)	29.9 (2.8)	35.3 (4.0)	0.017
Hct cooling (%)	31.3 (3.4)	30.6 (4.6)	34.7 (4.7)	0.701
Hct hypothermia (%)	29.0 (3.4)	29.3 (6.4)	33.7 (5.5)	0.902
Hct rewarming (%)	35.3 (7.1)	32.0 (6.7)	32.7 (2.5)	0.306
Hct end (%)	35.0 (4.6)	37.6 (3.5)	32.3 (3.8)	0.201
Baseline pH	7.5 (0.1)	7.4 (0.1)	7.5 (0.0)	0.255
pH cooling	7.2 (0.1)	7.2 (0.1)	7.5 (0.0)	0.36
pH hypothermia	7.2 (0.2)	7.1 (0.1)	7.5 (0.0)	0.266
pH rewarming	7.5 (0.1)	7.5 (0.1)	7.4 (0.0)	0.856
pH end	7.5 (0.0)	7.4 (0.1)	7.4 (0.0)	0.472
Baseline PaO ₂ (mmHg)	227.2 (55.0)	284.2 (89.3)	304.3 (66.0)	0.096
PaO ₂ cooling (mmHg)	362.1 (94.8)	360.8 (77.1)	250.3 (64.7)	0.974
PaO ₂ hypothermia (mmHg)	299.9 (33.2)	327.3 (58.5)	310.7 (42.4)	0.233
PaO ₂ rewarming (mmHg)	191.3 (47.0)	214.2 (51.0)	305.0 (6.1)	0.309
PaO ₂ end (mmHg)	199.6 (71.8)	183.0 (75.9)	301.7 (34.6)	0.75
Baseline PaCO ₂ (mmHg)	41.2 (7.2)	45.8 (17.2)	43.3 (2.1)	0.423
PaCO ₂ cooling (mmHg)	74.0 (14.2)	76.7 (19.8)	40.3 (1.2)	0.73
PaCO ₂ hypothermia (mmHg)	80.8 (29.1)	86.5 (16.9)	39.3 (2.1)	0.602
PaCO ₂ rewarming (mmHg)	35.8 (4.7)	33.3 (9.4)	41.3 (0.6)	0.452
PaCO ₂ end (mmHg)	38.6 (6.2)	38.1 (7.2)	41.3 (0.6)	0.882
Baseline lactate (mmol/l)	3.7 (1.1)	3.5 (1.2)	4.4 (1.0)	0.655
Lactate cooling (mmol/l)	3.7 (1.2)	3.6 (1.1)	4.6 (1.1)	0.875
Lactate hypothermia (mmol/l)	3.9 (1.4)	3.8 (1.2)	4.7 (1.2)	0.794
Lactate rewarming (mmol/l)	6.6 (1.4)	4.2 (1.6)	6.0 (1.3)	0.002
Lactate end (mmol/l)	7.1 (1.7)	4.4 (0.9)	6.0 (1.2)	0.001

All values are represented as mean (SD).

DHCA: deep hypothermic circulatory arrest; DHCP: deep hypothermic continuous perfusion; PaCO₂: partial pressure of arterial carbon dioxide; PaO₂: partial pressure of arterial oxygen.

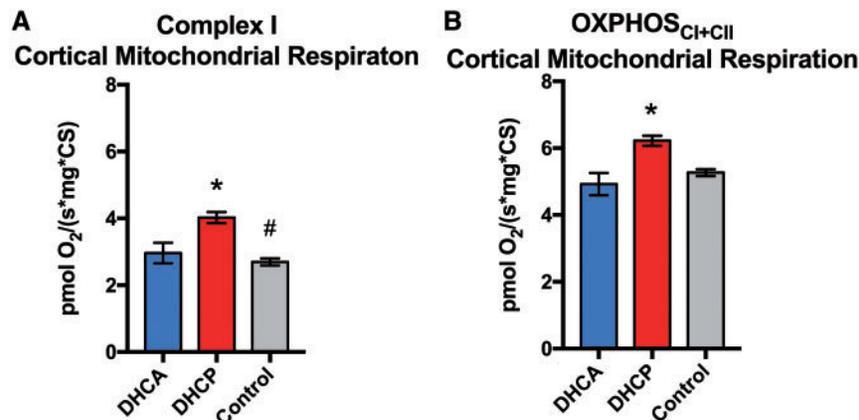


Figure 1: Cortical brain tissue mitochondrial respiration. (A) CI respiration: oxidative phosphorylation with CI substrates malate and pyruvate. CI respiration is significantly higher in DHCP when compared with DHCA ($P < 0.03$) and control ($P < 0.002$). (B) Maximum OXPHOS with the addition of succinate (CI and CII substrates). OXPHOS respiration is significantly higher in DHCP when compared with DHCA ($P < 0.02$) and control ($P < 0.002$). Values are expressed as mean \pm SEM. CI: complex I; CII: complex II; CS: citrate synthase; DHCA: deep hypothermic circulatory arrest; DHCP: deep hypothermic continuous perfusion; OXPHOS: oxidative phosphorylation; SEM: standard error of the mean.

group when compared with the DHCP [5.65 ± 0.52 pmol/(s · ml) vs 1.83 ± 0.18 pmol/(s · ml), $P < 0.007$], and controls [0.98 ± 0.07 pmol/(s · ml), $P = 0.0003$]. Additionally, DHCP ROS production was significantly increased compared with the controls ($P < 0.02$) (Fig. 3).

Microdialysis

cMD samples were taken during different phases of CPB (Table 3). There was a significant increase in the extracellular lactate/pyruvate

Cortical Respiratory Control Ratio (OXPHOS/LEAK)

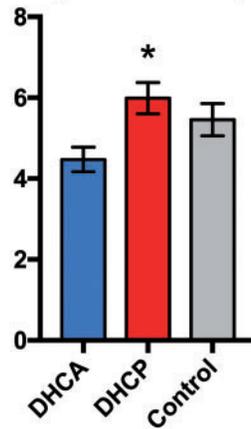


Figure 2: Cortical RCR, a metric of global mitochondrial function (OXPHOS/LEAK respiration). RCR is significantly higher in DHCP compared with DHCA. * $P < 0.01$. Values are expressed as mean \pm SEM. ATP: adenosine triphosphate; DHCA: deep hypothermic circulatory arrest; DHCP: deep hypothermic continuous perfusion; LEAK: uncoupled respiration without ATP-synthase activity; OXPHOS: oxidative phosphorylation; RCR: respiratory control ratio; SEM: standard error of the mean.

Cortical Mitochondrial Reactive Oxygen Species

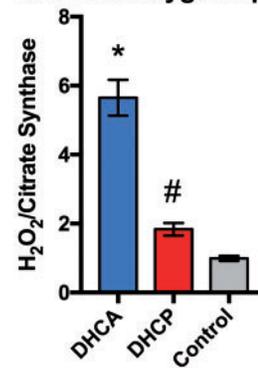


Figure 3: Mitochondrial reactive oxygen species (mtROS) production, a measure of mitochondrial dysfunction, at maximal oxidative phosphorylation (OXPHOS_{CI+CIII}). mtROS is significantly increased in DHCA compared with DHCP (* $P < 0.007$) and control ($P = 0.0003$). mtROS is significantly increased in DHCP compared with control ($^{\#}P < 0.02$). Values are expressed as mean \pm SEM. DHCA: deep hypothermic circulatory arrest; DHCP: deep hypothermic continuous perfusion; H₂O₂: hydrogen peroxide; OXPHOS: oxidative phosphorylation; SEM: standard error of the mean.

Table 2: Mitochondrial respiration variables

Respiratory parameters (normalized to CS)	DHCA (n = 10)	DHCP (n = 10)	Control (n = 4)
OXPHOS _{CI}	2.96 \pm 0.31	4.1 \pm 0.16*	2.69 \pm 0.11 [#]
OXPHOS _{CI+CIII}	4.92 \pm 0.33	6.22 \pm 0.15*	5.27 \pm 0.10
LEAK _{CI+CIII}	0.89 \pm 0.06	0.87 \pm 0.07	0.85 \pm 0.03
ETC _{CI+CIII}	4.45 \pm 0.32	5.03 \pm 0.34*	4.89 \pm 0.38
ETC _{CIII}	2.93 \pm 0.13	3.08 \pm 0.07	3.30 \pm 0.12
CIV	10.43 \pm 0.67	10.9 \pm 0.72	11.05 \pm 0.81
RCR _{OXPHOS CI+CIII}	4.83 (3.65–5.17)	6.58 (5.28–6.83)*	5.53 (4.65–6.18)

Summary of mitochondrial respiratory states (pmol O₂/s · mg) normalized to CS activity, a marker of mitochondrial content expressed as mean \pm SEM. RCR is presented as median (IQR).

* $P < 0.05$: statistical comparisons between DHCA and DHCP.

[#] $P < 0.05$: statistical comparisons between DHCP and control.

ATP: adenosine triphosphate; CI: complex I; CIII: complex III; CIV: complex IV; CS: citrate synthase; DHCA: deep hypothermic circulatory arrest; DHCP: deep hypothermic continuous perfusion; ETC: electron transport chain; IQR: interquartile range; LEAK: uncoupled respiration without ATP-synthase activity; OXPHOS: oxidative phosphorylation; RCR: respiratory control ratio; SEM: standard error of the mean.

ratio, a marker of metabolic stress, starting during deep hypothermia and continuing through the end of CPB in animals undergoing DHCA. Additionally, extracellular glycerol, a measure of cellular injury, was significantly increased in DHCA animals when compared with DHCP, starting during the rewarming phase and persisting through the end of the study (Fig. 4A and C). Glycerol levels were significantly increased in DHCP animals compared with the controls at the end of the study. Glucose levels did not differ significantly between the DHCA and the DHCP groups but were significantly decreased during rewarming in DHCA and DHCP animals compared with the control animals during the rewarming period (Fig. 4B).

DISCUSSION

The objective of this study was to evaluate the alterations in mitochondrial bioenergetics, cerebral metabolism and markers of neuronal injury following DHCA in a swine model. DHCA animals had a significant decrease in mitochondrial oxidative phosphorylation mediated by CI dysfunction and a significant increase in ROS production compared to those with continuous perfusion. Increasing evidence points to mitochondrial health as the critical convergence point of cellular survival in other forms of neurological injury. This study provides novel data on cerebral mitochondrial bioenergetics in a large animal model of CPB. Longitudinal cMD measurements of *in vivo* cerebral metabolism confirmed significant ongoing cerebral metabolic dysfunction and neurological injury in DHCA subjects compared with baseline, DHCP and controls. Our multimodal *in vivo* and *ex vivo* data provide evidence of primary cellular and mitochondrial failure following DHCA compared to subjects with continuous perfusion.

Our findings of both mitochondrial dysfunction and its immediate downstream effects on metabolic dysfunction and neuronal injury in animals treated with DHCA are consistent with prior animal studies demonstrating the deleterious effects of DHCA. Tang *et al.* [16] demonstrated increased cerebral interleukin 6, apoptotic index and up-regulation of the toll-like receptor 4/nuclear factor κ B (TLR4/NF- κ B) pathways in DHCA animals, suggesting increased apoptosis. Ultimately the intrinsic apoptotic pathway of neuronal injury is mediated by mitochondrial health, and other groups have demonstrated increases in caspase activation and other stigmata of the intrinsic apoptotic pathway in neurons of animals treated with DHCA [5, 6]. Our study is the first to provide the primary evidence of mitochondrial dysfunction with DHCA, which helps in understanding the mechanisms of neuronal death following DHCA and also provides clear mechanistic targets for future neuromonitoring techniques and neurotherapeutics to mitigate this pathophysiological cascade that leads to neuronal death. An example may be the addition of alternative mitochondrial substrates such as cell-permeable succinate that bypasses CI dysfunction to promote oxidative phosphorylation. We have shown the proof of

Table 3: Cerebral microdialysis data

Cerebral microdialysis	DHCA (n = 10)	DHCP (n = 10)	Control (n = 4)	P-value DHCA vs DHCP
Initiation LPR, mean ± SEM	14.4 ± 2.96	13.8 ± 5.43	12.6 ± 1.34	0.99
Cooling LPR, mean ± SEM	34.51 ± 14.4	12.4 ± 5.87	11.57 ± 0.57	0.67
Deep hypothermia LPR, mean ± SEM	71.81 ± 4.50	23.89 ± 6.53	16.39 ± 3.39	0.029
Rewarming LPR, mean ± SEM	132.60 ± 13.1	35.56 ± 3.87	17.83 ± 14.4	0.0001
End CPB LPR, mean ± SEM	60.83 ± 3.70	30.04 ± 6.40	16.41 ± 1.55	0.001
Initiation glucose (mg/dl), mean ± SEM	24.55 ± 7.73	23.81 ± 3.65	22.1 ± 4.02	0.99
Cooling glucose (mg/dl), mean ± SEM	15.15 ± 6.74	13.0 ± 3.93	16.41 ± 6.91	0.99
Deep hypothermia glucose (mg/dl), mean ± SEM	10.3 ± 3.71	13.62 ± 3.91	21.37 ± 6.04	0.99
Rewarming glucose (mg/dl), mean ± SEM	5.23 ± 2.15	4.37 ± 1.01	17.44 ± 6.40	0.99
End CPB glucose (mg/dl), mean ± SEM	13.33 ± 6.08	13.52 ± 1.65	14.76 ± 2.87	0.99
Initiation glycerol (µM), mean ± SEM	34.27 ± 5.19	26.4 ± 3.56	22.51 ± 3.32	0.99
Cooling glycerol (µM), mean ± SEM	39.34 ± 6.06	22.3 ± 1.90	23.39 ± 2.72	0.18
Deep hypothermia glycerol (µM), mean ± SEM	34.65 ± 7.38	19.6 ± 4.07	29.59 ± 2.98	0.32
Rewarming glycerol (µM)	49.6 ± 6.44	19.49 ± 6.11	31.75 ± 7.12	0.0016
End CPB glycerol (µM), mean ± SEM	75.3 ± 7.46	38.98 ± 6.12	22.66 ± 1.53	0.0001

Summary of cerebral microdialysis values during each phase of CPB.

CPB: cardiopulmonary bypass; DHCA: deep hypothermic circulatory arrest; DHCP: deep hypothermic continuous perfusion; LPR: lactate/pyruvate ratio; SEM: standard error of the mean.

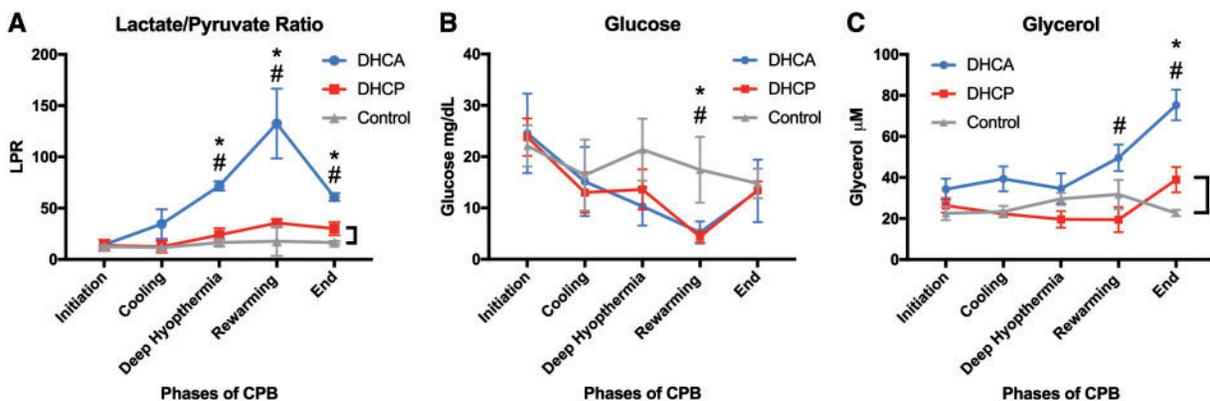


Figure 4: Cerebral microdialysis (cMD) measurements during CPB. **(A)** LPRs, a measure of metabolic stress, were significantly increased from baseline measurements at the initiation of CPB in animals undergoing DHCA during deep hypothermia ($*P < 0.005$), rewarming ($*P < 0.0001$) and at the end of CPB ($*P < 0.04$). LPRs in DHCA animals were significantly increased compared with DHCP and controls during the same time intervals ($*P < 0.0001$; $P < 0.001$; $P < 0.01$). DHCP LPR was significantly increased compared with the controls at the end of CPB (bracket, $P < 0.05$). **(B)** Brain glucose was significantly decreased from baseline measurements at the initiation of CPB during rewarming in both DHCA ($*P < 0.03$) and DHCP ($*P < 0.02$). DHCA and DHCP brain glucose was significantly lower than the controls during rewarming ($\#P < 0.05$ and $P < 0.05$, respectively). **(C)** Glycerol levels, a marker of cell membrane damage and ongoing cellular injury, were significantly increased from baseline measurements at the initiation of CPB in animals undergoing DHCA compared with the end of CPB ($*P < 0.0001$). Brain glycerol in DHCA animals was significantly increased compared with DHCP during rewarming ($\#P < 0.004$) and at the end of CPB ($*P < 0.002$). Glycerol was significantly increased in DHCP animals compared with the controls at the end of CPB (bracket, $P < 0.05$). Values are expressed as mean ± SEM. CPB: cardiopulmonary bypass; DHCA: deep hypothermic circulatory arrest; DHCP: deep hypothermic continuous perfusion; LPR: lactate/pyruvate ratios; SEM: standard error of the mean.

concept using cell-permeable succinate prodrugs in treating human cells with inhibited CI function [17] and we plan further investigations into the treatment of IR injury.

In our assessment of mitochondrial function, we evaluated the ETC, oxidative phosphorylation and inner membrane integrity using high-resolution respirometry. These novel techniques allow for functional measurements of different respiratory states of the entire ETC to determine OXPHOS and uncoupled ETC capacities, and CI, CII and CIV functions, as well as LEAK respiration. Damage to the ETC and oxidative phosphorylation results in increased ROS production, which leads to cellular damage and dysfunction, especially in the brain with its high oxidative metabolic requirements [18–20]. Our findings are consistent with our own data from a

model of paediatric cardiac arrest, as well as that of others, which demonstrate that ETC function, particularly mitochondrial CI, is reduced after IR injury [8, 18]. Mitochondria play a central role in cellular bioenergetics by coupling substrate oxidation and phosphorylation to produce ATP. Mitochondria also generate ROS, which at low levels participate in normal physiological signalling. However, when a pathological state ensues, ROS generation may overwhelm the antioxidant defence. It has not been fully elucidated how hypothermia and, especially, deep hypothermia affect mitochondrial function, but in the context of cardiac arrest, it has been suggested that hypothermia limits injury by preventing mitochondrial permeability [21], a pathophysiological mechanism that may lead to mitochondrial swelling and cell death. Hypothermia

also decreases the metabolic requirements and the cellular requirements for ATP. Despite these putative protective effects, our data suggest that the protective effects of hypothermia alone may not be sufficient to mitigate mitochondrial dysfunction and neuronal injury in DHCA.

Our cMD data are consistent with previous studies that demonstrated an increase in lactate/pyruvate ratio and glycerol levels in DHCA-treated swine [4, 7]. Our study differs from previous studies because we used smaller piglets [~ 4 kg instead of 8–10 kg and a shorter duration of DHCA (40 min instead of 60 min)]. As such, our model is more akin to neonatal cardiac surgical procedures, where patients are in the 1st week of life and DHCA times are closer to 40 min. Despite the decreased DHCA duration, we demonstrated significant evidence of cerebral injury, metabolic dysfunction and mitochondrial bioenergetic failure. Our *in vivo* microdialysis data supplement and contextualize the *ex vivo* changes in mitochondrial function.

Our findings suggest a possible mechanism behind neurological injury after DHCA that may be mitigated by maintaining perfusion and provide additional preclinical data on the deleterious effects of DHCA. Our findings demonstrate obvious cellular and mitochondrial dysfunction, but further studies are required to assess the irreversibility and clinical correlation of such neuronal injury, making clinical extrapolation difficult. Despite these and other groups' data, the use of DHCA remains a subject of debate because of lack of human data to support the measurable differences in neurological outcomes associated with its use in comparison with other perfusion strategies.

Limitations

Our study has several limitations. It is important to consider the methods demonstrated in this study to contextualize our findings. We chose the duration of 40 min for DHCA to best approximate DHCA time for a complicated neonatal cardiac surgical procedure such as the Norwood procedure. We chose pH stat during hypothermia and while cooling to optimize cerebral blood flow during these time periods according to both our institutional protocol and its demonstrated advantages [22, 23]. By maintaining corporeal circulation in our DHCP animals and not selectively perfusing the brain, our study did not address other questions such as proper flow rate or perfusion strategies such as selective antegrade cerebral perfusion.

CONCLUSIONS

In this preliminary study of the mitochondrial effects of DHCA versus maintaining continuous perfusion, we found that significant mitochondrial dysfunction occurred following DHCA and have corroborated these findings with cMD evidence of neuro-metabolic failure in a high-fidelity swine model. These findings are consistent with other preclinical studies characterizing the deleterious effects of DHCA and offer insight into the poorly understood mechanism of IR injury that may lead to neuronal death and neurological disability. Future work will compare the mitochondrial response to DHCA and selective antegrade cerebral perfusion in an effort to further understand the optimal perfusion strategy for neuroprotection in neonatal cardiac surgery.

Conflict of interest: none declared.

REFERENCES

- [1] International Cardiac Collaborative on Neurodevelopment (ICCON) Investigators. Impact of operative and postoperative factors on neurodevelopmental outcomes after cardiac operations. *Ann Thorac Surg* 2016;102:843–9.
- [2] Gaynor JW, Stopp C, Wypij D, Andropoulos DB, Atallah J, Atz AM *et al.* Neurodevelopmental outcomes after cardiac surgery in infancy. *Pediatrics* 2015;135:816–25.
- [3] Algra SO, Jansen NJ, van der Tweel I, Schouten AN, Groenendaal F, Toet M *et al.* Neurological injury after neonatal cardiac surgery: a randomized, controlled trial of 2 perfusion techniques. *Circulation* 2014;129:224–33.
- [4] Liang MY, Tang ZX, Chen GX, Rong J, Yao JP, Chen Z *et al.* Is selective antegrade cerebral perfusion superior to retrograde cerebral perfusion for brain protection during deep hypothermic circulatory arrest? Metabolic evidence from microdialysis. *Crit Care Med* 2014;42:e319–28.
- [5] Pastuszko P, Pirzadeh A, Reade E, Kubin J, Mendoza A, Schears GJ *et al.* The effect of hypothermia on neuronal viability following cardiopulmonary bypass and circulatory arrest in newborn piglets. *Eur J Cardiothorac Surg* 2009;35:577–81; discussion 581.
- [6] Arnaoutakis GJ, George TJ, Wang KK, Wilson MA, Allen JG, Robinson CW *et al.* Serum levels of neuron-specific ubiquitin carboxyl-terminal esterase-L1 predict brain injury in a canine model of hypothermic circulatory arrest. *J Thorac Cardiovasc Surg* 2011;142:902–10.e1.
- [7] Salazar JD, Coleman RD, Griffith S, McNeil JD, Steigelman M, Young H *et al.* Selective cerebral perfusion: real-time evidence of brain oxygen and energy metabolism preservation. *Ann Thorac Surg* 2009;88:162–9.
- [8] Han F, Da T, Riobo NA, Becker LB. Early mitochondrial dysfunction in electron transfer activity and reactive oxygen species generation after cardiac arrest. *Crit Care Med* 2008;36:S447–53.
- [9] Ikeda K, Marutani E, Hirai S, Wood ME, Whiteman M, Ichinose F. Mitochondria-targeted hydrogen sulfide donor AP39 improves neurological outcomes after cardiac arrest in mice. *Nitric Oxide* 2015;49:90–6.
- [10] Kilbaugh TJ, Bhandare S, Lorom DH, Saraswati M, Robertson CL, Margulies SS. Cyclosporin A preserves mitochondrial function after traumatic brain injury in the immature rat and piglet. *J Neurotrauma* 2011;28:763–74.
- [11] Schlunzen L, Cold GE, Rasmussen M, Vafaei MS. Effects of dose-dependent levels of isoflurane on cerebral blood flow in healthy subjects studied using positron emission tomography. *Acta Anaesthesiol Scand* 2006;50:306–12.
- [12] Li CX, Patel S, Auerbach EJ, Zhang X. Dose-dependent effect of isoflurane on regional cerebral blood flow in anesthetized macaque monkeys. *Neurosci Lett* 2013;541:58–62.
- [13] Kilbaugh TJ, Karlsson M, Duhaime AC, Hansson MJ, Elmer E, Margulies SS. Mitochondrial response in a toddler-aged swine model following diffuse non-impact traumatic brain injury. *Mitochondrion* 2016;26:19–25.
- [14] Karlsson M, Hara N, Morata S, Sjoval F, Kilbaugh T, Hansson MJ *et al.* Diverse and tissue specific mitochondrial respiratory response in a mouse model of sepsis-induced multiple organ failure. *Shock* 2016;45:404–10.
- [15] Cung TT, Morel O, Cayla G, Rioufol G, Garcia-Dorado D, Angoulvant D *et al.* Cyclosporine before PCI in patients with acute myocardial infarction. *N Engl J Med* 2015;373:1021–31.
- [16] Tang ZX, Chen GX, Liang MY, Rong J, Yao JP, Yang X *et al.* Selective antegrade cerebral perfusion attenuating the TLR4/NF- κ B pathway during deep hypothermia circulatory arrest in a pig model. *Cardiology* 2014;128:243–50.
- [17] Ehinger JK, Piel S, Ford R, Karlsson M, Sjoval F, Frostner EA *et al.* Cell-permeable succinate prodrugs bypass mitochondrial complex I deficiency. *Nat Comms* 2016;7:12317.
- [18] Kilbaugh TJ, Sutton RM, Karlsson M, Hansson MJ, Naim MY, Morgan RW *et al.* Persistently altered brain mitochondrial bioenergetics after apparently successful resuscitation from cardiac arrest. *J Am Heart Assoc* 2015;4:e002232.
- [19] Ayoub IM, Radhakrishnan J, Gazmuri RJ. Targeting mitochondria for resuscitation from cardiac arrest. *Crit Care Med* 2008;36:S440–6.
- [20] Gazmuri RJ, Radhakrishnan J. Protecting mitochondrial bioenergetic function during resuscitation from cardiac arrest. *Crit Care Clin* 2012;28:245–70.
- [21] Jahandiez V, Cour M, Bochaton T, Abrial M, Loufouat J, Gharib A *et al.* Fast therapeutic hypothermia prevents post-cardiac arrest syndrome through cyclophilin D-mediated mitochondrial permeability transition inhibition. *Basic Res Cardiol* 2017;112:35.
- [22] Dahlbacka S, Alaoja H, Makela J, Niemela E, Laurila P, Kiviluoma K *et al.* Effects of pH management during selective antegrade cerebral perfusion on cerebral microcirculation and metabolism: alpha-stat versus pH-stat. *Ann Thorac Surg* 2007;84:847–55.
- [23] Duebener LF, Hagino I, Sakamoto T, Mime LB, Stamm C, Zurakowski D *et al.* Effects of pH management during deep hypothermic bypass on cerebral microcirculation: alpha-stat versus pH-stat. *Circulation* 2002;106:1103–8.