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Experimental paper

Elevated serum neurologic biomarker profiles after cardiac arrest in a porcine model

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Abstract

Introduction: Swine exhibit cerebral cortex mitochondrial dysfunction and neuropathologic injury after hypoxic cardiac arrest treated with hemodynamic-directed CPR (HD-CPR) despite normal Cerebral Performance Category scores. We analyzed the temporal evolution of plasma protein biomarkers of brain injury and inflammatory cytokines, as well as cerebral cortical mitochondrial injury and neuropathology for five days following pediatric asphyxia-associated cardiac arrest treated with HD-CPR.

Methods: One-month-old swine underwent asphyxia associated cardiac arrest, 10–20 min of HD-CPR (goal SBP 90 mmHg, coronary perfusion pressure 20 mmHg), and randomization to post-ROSC survival duration (24, 48, 72, 96, 120 h; n = 3 per group) with standardized post-resuscitation care. Plasma neurofilament light chain (NfL), glial fibrillary acidic protein (GFAP), and cytokine levels were collected pre-injury and 1, 6, 24, 48, 72, 96, and 120 h post-ROSC. Cerebral cortical tissue was assessed for: mitochondrial respirometry, mass, and dynamic proteins; oxidative injury; and neuropathology.

Results: Relative to pre-arrest baseline (9.4 pg/ml [6.7–12.6]), plasma NfL was increased at all post-ROSC time points. Each sequential NfL measurement through 48 h was greater than the previous value {1 h (12.7 pg/ml [8.4–14.6], p = 0.01), 6 h (30.9 pg/ml [17.7–44.0], p = 0.0004), 24 h (59.4 pg/ml [50.8–96.1], p = 0.0003) and 48 h (85.7 pg/ml [61.9–118.7], p = 0.046)}. Plasma GFAP, inflammatory cytokines or cerebral cortical tissue measurements were not demonstrably different between time points.

Conclusions: In a swine model of pediatric cardiac arrest, plasma NfL had an upward trajectory until 48 h post-ROSC after which it remained elevated through five days, suggesting it may be a sensitive marker of neurologic injury following pediatric cardiac arrest.

Keywords: Cardiac arrest, Neurologic injury, Biomarkers, Pediatrics

Introduction

Pediatric in-hospital cardiac arrests affect more than 15,000 children in the US each year and many more worldwide.[1–3] Greater than half of children do not survive to hospital discharge and many survivors have lasting neurologic morbidity, including impaired ability to perform activities of daily living, attention deficits, chronic symptoms of fatigue and headache, and need for special education.[4–6] Studies of neurologic injury after pediatric cardiac arrest provide tools for prognosticating severe neurologic dysfunction, but do not give guidance to personalize neuroprotective care plans based on risk stratification.[7,8] Understanding the time course of brain injury immediately following cardiac arrest could inform clinical decision making for trial enrollment, duration and intensity of post-arrest therapies, determination of candidacy for neuroprotective therapies, and identification of predictive variables for longer term neuroprognostication of cognitive function, physical function, and daily life skills.[8]

Studies of physiology-directed cardiopulmonary resuscitation strategies demonstrate improved rates of survival and favorable neurologic outcomes in preclinical models of pediatric in-hospital cardiac arrest.[9,10] In our group's work, animals that undergo 10 min of

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hemodynamic-directed CPR (HD-CPR) have improved rates of survival and preservation of gross neurofunctional status as measured by cerebral performance category (CPC) in contrast to animals treated with standard CPR.[11–13] Yet the animals treated with HD-CPR have cerebral cortex mitochondrial bioenergetic dysfunction at both 4 and 24 h after return of spontaneous circulation (ROSC), and have neurologic injury determined by neuropathology.[11,12,14] Therefore, this model of hypoxic cardiac arrest and resuscitation with a HD-CPR protocol results in a bioenergetic and neuropathologic cortical injury that is not discernible by gross CPC assessments. The objective of this exploratory study was to describe the temporal evolution of neurologic injury in a porcine model of pediatric in-hospital cardiac arrest. Accordingly, this study includes the measurement of plasma protein biomarkers of brain injury, plasma inflammatory cytokine levels, cerebral cortical mitochondrial bioenergetic dysfunction, mitochondrial mass, mitochondrial dynamics, oxidative injury, and neuropathology for five days post-arrest.

Methods

Model justification

This study used an established porcine model of cardiac arrest because of similarities in neurodevelopmental and chest compression characteristic between swine and humans.[15,16] The experimental model included asphyxia prior to cardiac arrest, as most children with cardiac arrest have underlying respiratory insufficiency and many of these arrests are due to a respiratory etiology.[2,17,18]

Animal preparation

All animal procedures were approved by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia (IAC 22–001327), were conducted in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and in concordance with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Healthy one-month-old, female swine (*Sus scrofa domesticus*, 8–11 kg) were anesthetized and mechanically ventilated using room air and titrated isoflurane (1.0–2.5% inspired concentration) with a tidal volume of 10–12 mL/kg and positive end-expiratory pressure of 6 cm H2O; respiratory rate was titrated to maintain end-tidal carbon dioxide (ETCO2) of 38–42 mmHg.[11,13,19]

Experimental protocol

Prior to the experiment, animals were randomized to post-ROSC survival durations (24, 48, 72, 96, 120 h, n = 3 per group) or to an anesthetized sham group (120 h, n = 5). Animals that did not achieve ROSC were excluded and the randomization scheme was reset in order to achieve an equal number of survivors per group. Anesthetized animals were invasively ventilated in room air, then underwent 7 min of asphyxia produced by endotracheal tube clamping followed by induction of ventricular fibrillation (VF) to ensure a standardized minimum period of cardiac arrest that would only be terminated by defibrillation (Fig. 1). More detail about the experimental protocol has been reported in previous publications.[11,19,20] Manual chest compressions were provided with a target rate of 100 per minute with guidance from a metronome and a CPR quality monitoring defibrillator (Zoll R-series, Zoll Medical, Chelmsford, MA) and ventilations were provided 10 times per minute with 100% oxygen. In HD-CPR, chest compression force was adjusted to target a systolic blood pressure (SBP) of 90 mmHg and vasopressors were administered as needed to maintain coronary perfusion pressure (CoPP) of at least 20 mmHg. Beginning two minutes into CPR, vasopressors were administered if CoPP < 20 mmHg as follows: epinephrine (0.02 mg/kg), epinephrine (0.02 mg/kg), and vasopressin (0.4 U/kg) then repeated in this order, with minimal dosing interval of 1 min following epinephrine doses, and 2 min following vasopressin administration. Beginning 10 min after the start of CPR, defibrillation was attempted every two minutes as necessary until ROSC or through a maximum of 20 min of CPR. After ROSC, animals remained anesthetized and received standardized post-arrest care including intravenous crystalloid and epinephrine (infusion and bolus dosing) to meet MAP goals (45–60 mmHg), ventilator titration to achieve ETCO2 38–42 mmHg, supplemental oxygen titration to SpO2 94–98% and PaO2 less than 100 mmHg and normothermia for 4–6 h. Following emergence from anesthesia, all surviving animals were extubated and monitored for the *a priori* randomized dura-

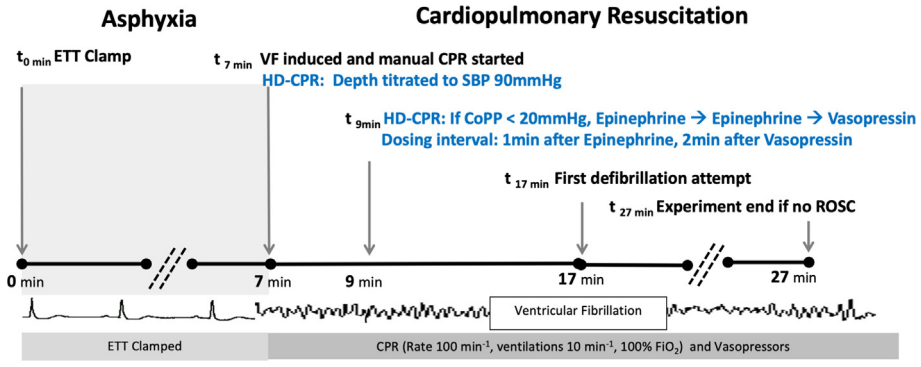

Fig. 1 – Schematic of experimental protocol. Definitions of abbreviations: CPR = cardiopulmonary resuscitation; ETT = endotracheal tube; VF = ventricular fibrillation; CPR = cardiopulmonary resuscitation; HD = hemodynamic-directed; SBP = systolic blood pressure; CoPP = coronary perfusion pressure; ROSC = return of spontaneous circulation.

tion of survival. Shams were anesthetized and instrumented with central venous and arterial catheters, underwent similar, protocolized anesthetic exposure with inhaled isoflurane (≈1.0%), emerged from anesthesia and were monitored for a total of 120 h. Swine Cerebral Performance Category (CPC) scores were independently determined by two trained investigators at 6 and 24 h post-ROSC after complete recovery from anesthesia, and then every 24 h based on randomization group. The scoring system was: 1 is normal (no difficulty standing, walking, etc.); 2 is mild disability (can stand, but unsteady, slow to respond, drinking, not eating); 3 is severe disability (awake but not responding, cannot stand, walk, eat, drink); 4 is in a vegetative state; and 5 is dead.[21,22]

Plasma biomarker, cytokine sample acquisition and laboratory analysis

Plasma was collected prior to injury, then 1 h, 6 h, 24 h, and every subsequent 24 h following ROSC for neurofilament light chain (NfL), glial fibrillary acid protein (GFAP), interleukins 6 and 8, and TNF-α analysis.

Neurofilament Light Chain and Glial Fibrillary Acid Protein Immunoassay: Samples were centrifuged at 4400g for 5 min and plasma was isolated and stored at −80 °C until analysis. Immunoassays were run using the Human Immunology Core service at the University of Pennsylvania using Simoa HD-1 Analyzer.[23]

Cytokine Level Immunoassay: Interleukins 6, 8 and TNF- α quantification was conducted using Q-Plex Porcine Cytokine HS kit (Quansys Biosciences, USA) per manufacturer's instructions. Sample solutions, comprising a mixture of protease inhibitor and plasma were incubated, washed, imaging substrate kit was added, and imaging performed using Quansys Imager LS.

Cerebral cortex sample acquisition and lab analysis

At the predetermined survival endpoint, animals were anesthetized, intubated and placed under general anesthesia. A bilateral craniectomy was performed to expose the brain. Animals were humanely euthanized and cortical tissue was rapidly extracted. Standardized areas were sampled and: 1) preserved in mitochondrial isolation buffer for fresh *ex vivo* mitochondrial function analysis; 2) snap frozen in liquid nitrogen for downstream protein analysis; and 3) coronally sectioned and plate frozen for neuropathologic assessment.[14]

Sample Preparation and Assessment of Mitochondrial Function: Fresh cerebral cortex was weighed and then gently homogenized in ice-cold MiR05 buffer (110 mM of sucrose, 0.5 mM of EGTA, 3.0 mM of $MgCl_2$, 60 mM of K-lactobionate, 10 mM of KH_2PO_4, 20 mM of taurine, 20 mM of HEPES, and 1.0 g/L of fatty acid–free bovine serum albumin) with a Potter-Elvehjem Teflon-glass homogenizer to obtain a final concentration of 100 mg of wet weight tissue/1 mL of MiR05 buffer. High-resolution respirometry (Oxygraph-2 k and DatLab software; Oroboros Instruments, Innsbruck, Austria) measured oxygen consumption (pmol/[s x mg]). Samples were analyzed using a substrate, uncoupler, inhibitor titration (SUIT) protocol for in-depth characterization of the oxidative phosphorylation system (OXPHOS).[24,25]

Assessment of Mitochondrial Content: Preserved chamber contents from high-resolution respirometry were stored at −80 °C and analyzed for citrate synthase (CS) activity quantification (Citrate Synthase Assay Kit, CS0720; Sigma). CS activity (μmol/mL/min) was used as an index of mitochondrial content and used for normalization of respirometry data.[11,25,26].

Sample Preparation and Western Blot for Mitochondrial Dynamics: Frozen cerebral cortex tissue was homogenized (5% wt:vol) with 4 rounds of 5 S in a Potter-Elvehjem teflon-glass homogenizer in RIPA buffer (#89901, Pierce, Rockford, IL, USA) with protease inhibitor (AEBSF, Aprotinin, Bestatin hydrochloride, E-64, Leupeptin, Pepstatin A) (#P8340, Sigma Aldrich, St. Louis, MO, USA) and 5 mM NaF (#201154, Sigma Aldrich, St. Louis, MO, USA) as a phosphatase inhibitor. The homogenate was centrifuged at 12,000g for 10 min, the supernatant whole cell lysate was preserved, and the pellet discarded. Total protein was quantified using the BCA Protein Assay Kit (#23228; Pierce, Rockford, IL, USA).

Equal amounts of lysate were denatured, separated using SDS-polyacrylamide gel electrophoresis (12% Bis-Tris, ThermoFisher, Carlsbad, CA) in a Mini gel tank (ThermoFisher Scientific, Waltham, MA) and then transferred to polyvinyliodene difluoride membrane using iBlot 2 (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's protocols. Membranes were incubated with antibodies against Opa1 (1:1000, NB110-55290, Novus Biologicals, Littleton CO, USA), Mfn2 (1:2000, ab124773, Abcam, Cambridge, MA, USA), Drp1 (1:1000, 8570S, Cell Signaling Technology, Boston, MA), Fis1 (1:2000, 109561AP, ProteinTech, Rosemont, IL, USA), and GAPDH (1:5000, NB300-327, Novus Biologicals, Centennial, CO, USA) at room temperature for 4 h using iBind technology (ThermoFisher Scientific, Waltham, MA). Membranes were then incubated with a 1:1 chemiluminescent substrate solution for five minutes before being imaged on the iBright imager (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's protocols. Images were analyzed on the iBright analysis software (ThermoFisher Scientific, Waltham, MA) and normalized to GAPDH to assess relative pixel densitometry.

Sample Preparation and Assessment of ELISA for Oxidative Injury: Snap frozen cortex was homogenized in phosphate-buffered saline pH 7.4 (50 mg/ml), containing protease inhibitor, phosphatase inhibitor and antioxidant dibutylhydroxytoluene using Potter-Elvehjem type Teflon-glass tissue homogenizers. Homogenized samples were centrifuged at 16,000g for 10 min at 4 °C and the supernatant was collected. For protein carbonyl measurements, supernatant was treated with streptomycin (1% v/v) to remove nucleic acids, incubated for 30 min at 4 °C, centrifuged again at 16,000g for 10 min at 4 °C, and remaining supernatant was used per manufacturer recommendations (OxiSelect Protein Carbonyl ELISA Kit #STA-310, San Diego CA, USA). For 4-HNE investigation, samples and standards were combined with 50ul of biotinylated antibody, incubated for 45 min at 37 °C and then thoroughly washed with diluted wash solution. Streptavidin conjugate was added for 30-minute incubation period at 37 °C. Substrate solution was added to each well, then stop solution added after visual appearance of a color gradient for standards was observed. The plate was imaged for endpoint absorbance at 450 nm using a SpectraMax iD3 (Molecular Devices, San Jose, USA, 4-Hydroxynonenal ELISA #ab287803, Abcam, Cambridge, UK).

Sample Preparation and Neuropathologic Assessment: Immunofluorescence was performed using a modified version of the previously described protocol.[27] Briefly, slides were covered with GFAP (1:1000; aveslabs.com) and Iba1 (1:1000; VWR) antibodies then incubated with Alexa Fluor 488 (1:1000; ab150077) and Alexa Fluor 647 (1:1000; A32933 thermofisher.com) then diluted in Trueblack (Biotium) and Vectashield Plus Antifade mounting medium with DAPI (vectorlabs.com).

Image statistics were calculated for confluence, defined as the number of pixels in the image that have an intensity between the minimum and the maximum threshold divided by the total number of pixels in the image. Confluence was averaged across all 3 ROIs per case.

Outcomes and statistical analysis

Outcomes of this exploratory study include plasma biomarkers of neurologic injury and inflammatory cytokine levels at serial timepoints and cerebral cortical mitochondrial bioenergetic dysfunction, mitochondrial mass, mitochondrial dynamics, oxidative injury and neuropathology at sequential time points after ROSC. For serially measured variables (i.e., plasma biomarkers and cytokines), the timing of sample acquisition was used to classify the measurements regardless of the subsequent survival duration. For endpoint-related mitochondrial and pathologic data, each post-ROSC survival duration was considered an independent group (n = 3 per group). All outcomes except for plasma biomarkers were additionally compared between animals that received HD-CPR and survived to 120 h post-ROSC (n = 3) and sham animals 120 h post-anesthetic exposure (n = 5).

Statistical analysis was performed using GraphPad Prism v7 (GraphPad, La Jolla, CA) and Stata Version 14 (StataCorp, College Station, TX). Time was considered a categorical variable for both endpoint comparisons and longitudinal measurements. Plasma biomarkers and cytokines were compared using mixed effects analysis because of differing number of values per each time point, followed by multiple comparisons between time points using Fishers least significant difference test without adjusting for multiple comparisons, as time point comparisons were planned *a priori* and because of the exploratory nature of this analysis. Homoscedasticity plots were used to ensure mixed effects analysis assumption of equal variance. For all other variables, parametric analyses between multiple groups were performed using one-way ANOVA followed by multiple comparisons using Tukey HSD. Comparisons between 120 h post-ROSC animals and 120 h uninjured shams were performed via unpaired two-tailed *t*-test. All results are expressed as medians with interquartile ranges.

Results

Overview

Seventeen animals underwent cardiac arrest and CPR. Two failed to achieve ROSC and were excluded from analyses. Fifteen achieved ROSC and all 15 survived to their respective timepoints. Plasma neurologic injury biomarkers and cytokines were measured serially, and therefore comparisons were made based on sample availability (24 h n = 15, 48 h n = 12, etc.). For all other variables, comparisons were made between cardiac arrest time points groups (n = 3), and between 120 h injured animals (n = 3) and sham uninjured animals (n = 5). Fourteen animals had CPC of 1 at 24-hour post-ROSC and one animal had a CPC of 2, which improved to 1 by 48 h. All animals continued to have a CPC of 1 through all subsequent time points.

Plasma neurologic injury biomarkers and cytokines

Plasma NfL was elevated from pre-injury baseline at one-hour post-ROSC (9.4 pg/ml [6.7–12.6] vs. 12.7 pg/ml [8.5–14.6], p = 0.010). Each sequential time point out to 48 h was greater than the previous value {1 h (12.7 pg/ml [8.4–14.6]), 6 h (30.9 pg/ml [17.7–44.0], p = 0.0004), 24 h (59.4 pg/ml [50.8–96.1], p = 0.0003) and 48 h (85.7 pg/ml [61.9–118.7], p = 0.046)}. After 48 h, no differences were measured between subsequent time points. In contrast, plasma GFAP, interleukins 6 and 8, and TNF- α were not demonstrably different from baseline at any post-arrest timepoint, and not demonstrably different over time (Fig. 2).

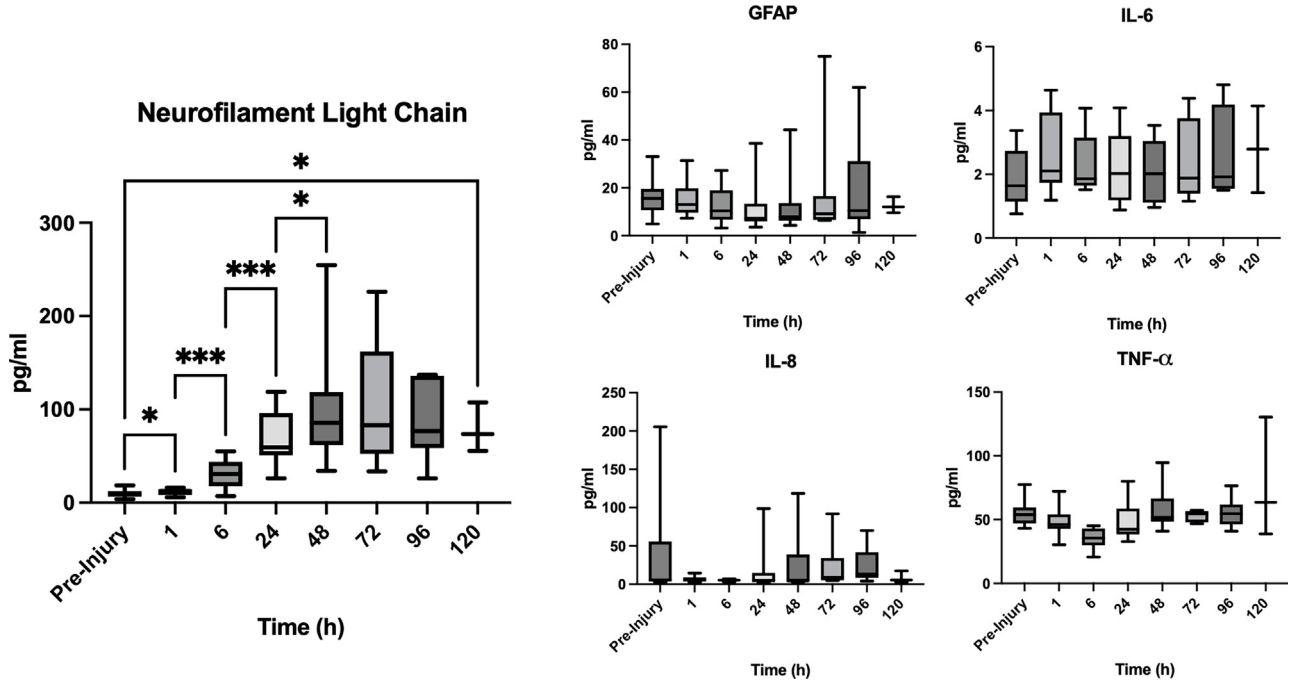

Fig. 2 – Plasma neurologic injury biomarkers and cytokines. Neurofilament light chain (NfL) is elevated from pre-injury baseline by 1 h, at each sequential point until 48 h and remains elevated at 120 h post CA. Inherent to study design, pre-injury n = 15, 24 h n = 13, 48 h n = 11, 72 h n = 7, 96 h n = 6, 120 h n = 3. No differences are seen between time points for plasma GFAP or cytokine measures.

Mitochondrial bioenergetic dysfunction and mitochondrial mass

Complex 1-linked respiration, complex 1 + 2-linked respiration, leak respiration, and respiratory control ratios did not differ between endpoints among the HD-CPR animals or between sham animals and HD-CPR cardiac arrest animals at 120hr. Citrate synthase activity as a marker of mitochondrial mass was not different among the HD-CPR animals between time points or in comparison to shams. (Fig. 3).

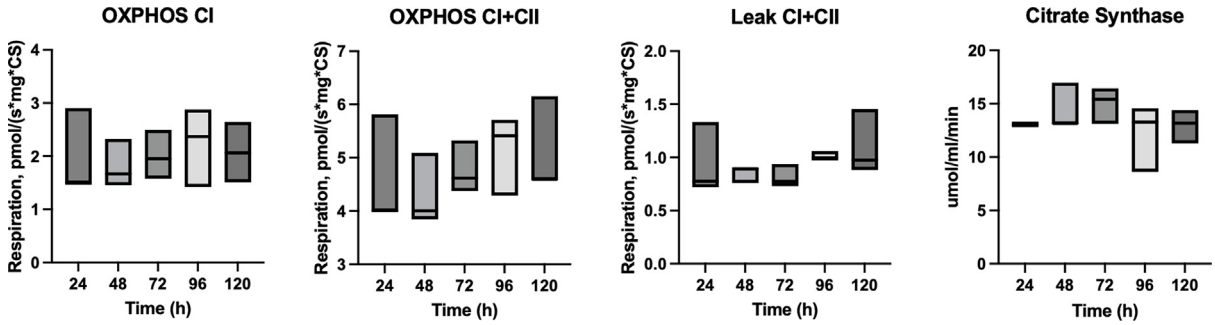

Fig. 3 – Mitochondrial bioenergetic function and mass. There is no significant difference in cerebral cortical mitochondrial function or mass between time points after ROSC.

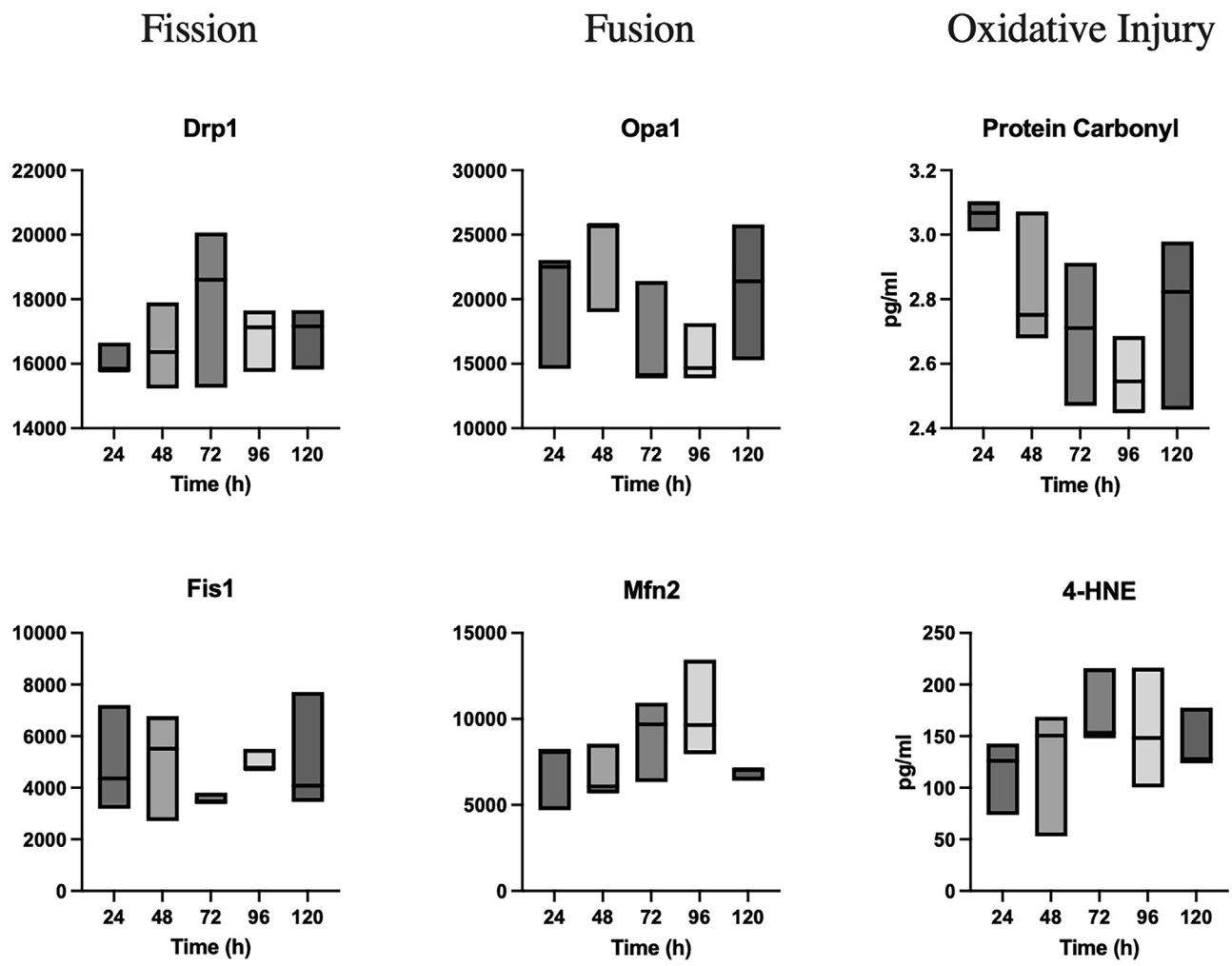

Fig. 4 – Mitochondrial dynamics protein expression and oxidative injury. There is no significant difference in cerebral cortical mitochondrial dynamics via measures of fission (Drp1, Fis1) or fusion (Opa1, Mfn2) relative to GAPDH, or oxidative injury (protein carbonyl, 4-HNE). Western blot protein quantification normalized to GAPDH and presented as relative units.

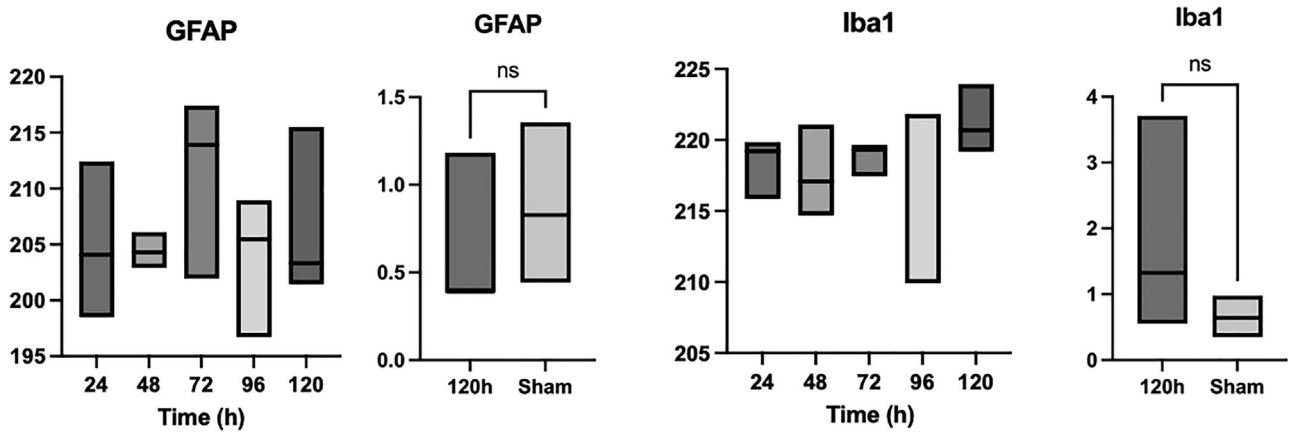

Fig. 5 – Neuropathology quantification of GFAP and Iba1. There is no demonstrable difference in cortical neuropathologic quantification of GFAP (astrocyte reaction) or Iba1 (microglial activation) between time points or relative to sham animals. Values presented as relative units compared to total pixels per image.

Mitochondrial dynamics protein expression and oxidative injury

There were no differences between time points among HD-CPR animals or in comparison to sham animals for expression of fission proteins (Drp1, Fis1) or fusion proteins (Opa1, Mfn2) to evaluate mitochondrial dynamics, nor were there any differences measured in oxidative injury markers (protein carbonyl, 4-HNE) in cerebral cortex. (Fig. 4).

Neuropathology

Neuropathologically quantified cortical GFAP, a marker of astrocyte reaction, and Iba1, a marker of microglial activation did not differ between time points among the HD-CPR animals or in relation to sham animals. (Fig. 5).

Discussion

This study expands on previous work by our group analyzing the effect of a hemodynamic-directed CPR strategy after asphyxia-associated cardiac arrest by describing a time course of neurologic injury and biomarkers after 7 min of asphyxia and 10 min of HD-CPR, intended to simulate in-hospital cardiac arrest in the pediatric ICU.[28] Although nearly all animals had achieved and maintained grossly normal neurofunctional status by 24 h after cardiac arrest, we identified elevations in plasma NfL as early as one hour post-ROSC that persisted until at least 120 h after injury, with incremental increases over the first 48 h. We were not able to identify demonstrable differences between time points among the HD-CPR animals or compared to control animals without cardiac arrest in other important markers of cerebral injury, including mitochondrial bioenergetic dysfunction, mitochondrial mass, mitochondrial dynamic protein expression, oxidative injury or cerebral neuropathologic expression of immunologic response, though we are limited by small sample sizes. Overall, these findings suggest that NfL may be an early and persistent marker out to five days after cardiac arrest of post-arrest neurologic injury that is not discernible by gross neurocognitive assessment or other cortical tissue analyses. To our knowledge, these findings are the first reported post-ROSC time course analysis of plasma biomarkers, plasma cytokines and cerebral cortical tissue in a porcine model of pediatric cardiac arrest.

Following pediatric cardiac arrest, higher values of NfL, a neuronal protein that is preferentially expressed in the axons of neurons, are associated with unfavorable neurologic outcome.[29,30] In a single center study of patients with acute respiratory distress syndrome who suffered a cardiac arrest, elevated NfL within 48 h of CPR was associated with death.[30] Our NfL timecourse parallels those of a multi-center prospective study of blood-based biomarkers in children who suffered a cardiac arrest, where blood levels of NfL increased in the first 3 days after cardiac arrest and elevated NfL was associated with unfavorable 1-year outcomes.[29] In an adult swine model of myocardial infarction associated cardiac arrest with moderate post-arrest injury, NfL levels were elevated at 48 h to 63 [35,232] pg/ml compared to 29 [21,34] pg/ml in controls, which are comparable to values reported in our study.[31] In our study, NfL levels show a clear upward trajectory following ROSC despite these animals being grossly neuro-functionally normal. This suggests that a neurologic insult that is not otherwise evident in other measured plasma or cortical tissue assessments can be detected by NfL. This finding warrants further understanding of the clinical significance of NfL elevation after ROSC, as well as the potential to study a low NfL value as a reassuring clinical finding after pediatric cardiac arrest.[32–34] Additionally, current clinical practice relies on early neurologic examination and other clinical characteristics to define neuroprotective therapies. Brain injury biomarkers, such as NfL, may be valuable adjuncts to incorporate into risk-stratification to determine eligibility, duration, and intensity of post-cardiac arrest neuroprotective interventions, as well as for determining interventional trial eligibility.

In contrast to prior clinical studies, we did not find a significant change in plasma GFAP in our animals after cardiac arrest.[29] GFAP, a component of the astrocyte cytoskeleton, is upregulated during astrocyte activation. The absence of changes in GFAP may reflect the relatively mild neurologic injury observed after successful treatment with HD-CPR. We also did not find any changes in cortical GFAP expression, indicating a lack of astrocyte activation in the cortex rather than inadequate GFAP spillage into plasma, which is also a topic of ongoing inquiry.[35] An increase in neuronal injury marker

NfL without a change in GFAP or any other markers of systemic inflammation may be related to the type or degree of neurologic injury present in this study and showcases the complexity of the brain response to ischemia and reperfusion after cardiac arrest.

We also did not identify differences in microglial activation marker Iba1 in the cortex between time points, or at 120 h post-ROSC compared to sham animals. The role of neuroinflammation by way of microglial activation after cardiac arrest continues to be an exciting field of study, and future study should incorporate biomarkers of neuronal, axonal and astroglial damage in conjunction with local and systemic markers of neuroinflammation.

We did not detect any temporal trajectories of mitochondrial bioenergetic dysfunction, mitochondrial mass, mitochondrial dynamics or oxidative injury after cardiac arrest. Although we have previously shown cerebral cortex and hippocampus mitochondrial respiratory dysfunction and elevation of cortical Opa1 expression 24 h after cardiac arrest treated with HD-CPR in swine of this age, we did not observe temporal changes in cerebral cortex mitochondrial respiration or mitochondrial fusion or fission protein expression. Our inability to detect differences across time in other outcome measures is likely due to relative adequacy of cerebral perfusion during HD-CPR and power limitations of the comparisons.[11,12,14,36,37] However, we believe the clinical scenario and injury severity are clinically relevant to simulate an in-hospital cardiac arrest in the pediatric ICU.[28] Temporal analysis of hippocampus or other deep brain structures sensitive to hypoxic ischemic injury were not performed for this study. Additionally, we were not able to identify cortical mitochondrial dysfunction or mitochondrial dynamics protein expression at 120 h after cardiac arrest compared to shams. This suggests that these changes may have resolved by 120 h, though we are limited by the low statistical power of those comparisons.

Future directions

Further investigation of the clinical relevance of low-grade elevation in plasma NfL in animals and patients with grossly normal, mildly dysfunctional, or severely dysfunctional neurologic status after cardiac arrest is warranted and could be accomplished by assessing NfL levels after depth-guided standard of care CPR or a longer duration of CPR prior to defibrillation, both of which we expect would yield a more severe neurologic insult. This could help to define plasma NfL as not only a marker of death or severe neurologic injury, but also as a marker of milder neurologic insult that could be used clinically for risk stratification and to guide duration and intensity of neuroprotective therapies. Future investigation should consider integration of additional promising biomarkers of neurologic injury, including neuron specific enolase (NSE), ubiquitin carboxyl-terminal esterase L1 (UCH-L1) and tau.

Limitations

A limitation of this study is that HD-CPR preserved gross neurologic function – a more severe hypoxic-ischemic injury may have yielded discernible changes in markers of cerebral injury beyond NfL alone and a similar study with a more substantial neurologic injury is certainly warranted. However, there is value in defining the time course of sensitive and specific markers of brain injury, as demonstrated with plasma NfL levels. Our small sample size for each time point in this pilot study increases the chance of type II error. We cannot definitively conclude that temporal changes in biomarkers would not have been evident with larger samples. Finally, we did not acquire pre-injury data for any of the tissue analyses, nor did we evaluate brain injury biomarkers and cytokines in sham animals, as the primary goal was to compare post-ROSC trends instead of measuring deviations from baseline.

Conclusion

In this prospective pilot study of plasma biomarkers and cortical endpoints in pediatric swine after cardiac arrest, we found that despite returning to a grossly normal neurofunctional status by 24 h, animals had an upward trajectory of plasma neurofilament light chain up until 48 h, with ongoing elevation in values up to 120 h from pre-injury baseline. This suggests that plasma NfL may be a sensitive marker of neurologic injury and warrants further prospective correlation with more discerning markers of neurologic injury. We were not able to identify any temporal differences in neuroinflammation, bioenergetic dysfunction, mitochondrial mass, dynamics or oxidative injury. However, this exploratory study was practically designed to detect differences to be corroborated with further dedicated study and was not powered to rule out temporal changes.

CRediT authorship contribution statement

Kumaran Senthil: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Abhay Ranganathan:** Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sarah Piel:** Supervision, Investigation, Data curation. **Marco M. Hefti:** Writing – review & editing, Investigation, Formal analysis. **Ron W. Reeder:** Writing – review & editing, Methodology, Formal analysis. **Matthew P. Kirschen:** Writing – review & editing, Conceptualization. **Jonathan Starr:** Supervision, Investigation, Data curation. **Sarah Morton:** Supervision, Investigation, Data curation. **Hunter A. Gaudio:** Visualization, Investigation, Formal analysis, Data curation. **Julia C. Slovis:** Methodology, Investigation, Conceptualization. **Jeremy R. Herrmann:** Writing – review & editing, Conceptualization. **Robert A. Berg:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Todd J. Kilbaugh:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Ryan W. Morgan:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Conflict of interest

This work was supported by the Children's Hospital of Philadelphia Resuscitation Science Center, NIH National Heart, Lung, and Blood Institute K23HL148541 (Dr. Morgan), NIH National Heart, Lung, and Blood Institute R01HL141386 (Dr. Kilbaugh).

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. Julia Slovis currently works for GalaxoSmithKline, but her employment began after completion of her contribution to this manuscript.

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Appendix A. Supplementary material

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