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Short paper

# Early cytotoxic lymphocyte localization to the brain following resuscitation in a porcine model of asphyxial cardiac arrest: A pilot study

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## Abstract

**Introduction:** Out-of-hospital cardiac arrest (OHCA) is a major cause of morbidity and mortality in the US. Of major concern is a lack of therapies to mitigate associated brain injury. Immune cell infiltration (ICI) into the brain, which may exacerbate injury post-resuscitation, is one possible therapeutic target, although the post-OHCA immune response has not been fully characterized.

**Objective:** In this pilot study, we aimed to detect early post-resuscitation cytotoxic lymphocyte ICI in porcine brain using a model of opioid-mediated asphyxial OHCA.

**Methods:** Ten young, healthy swine (26.7+/-3.4 kg) were sedated, anaesthetized and paralyzed. In eight of the animals, this was followed by induction of asphyxial OHCA via fentanyl bolus and concurrent airway occlusion. The remaining two 'sham' animals were instrumented but did not undergo asphyxia. After nine minutes of asphyxia, mechanical CPR and manual ventilations were started, in an initial BLS followed by ALS configuration. At termination of resuscitation or euthanasia, the whole brain was removed. Immune cells were extracted and analyzed via flow cytometry.

**Results:** 304 +/− 62.2 cells/g were discovered to be CD8 single positive cells in animals that achieved ROSC, 481 +/− 274.4 cells/g in animals that did not achieve ROSC, and 40 +/− 11.31 cells/g in sham animals. CD8 single positive cells made up 0.473 +/− 0.24% of detected cells in animals that achieved ROSC, 0.395 +/− 0.062% in animals that did not achieve ROSC, and 0.19 +/− 0.014% in sham animals (No ROSC vs Sham, $p = 0.012$).

**Conclusions:** These data suggest that cytotoxic lymphocytes may be localizing to the brain during cardiac arrest resuscitation.

**Keywords:** Post cardiac arrest syndrome, Cardiac arrest, Resuscitation, Lymphocyte, Cytotoxic, T-lymphocyte, Out of hospital cardiac arrest, Immune, Brain, CD8, CD8+, Asphyxia, Immunology

## Introduction

Cardiac arrest is a major cause of morbidity and mortality in the US, with over 350,000 patients suffering from this deadly medical emergency every year. At this time, the prognosis following out-of-hospital cardiac arrest (OHCA) is poor, and the survival rate to hospital discharge is approximately 10%.[1] Given that the cause of death of 68% of all cardiac arrest patients successfully resuscitated in the field and admitted to the ICU is related to anoxic brain injury,[2] and that a significant proportion of survivors suffer from neurological sequalae incurred as a result of brain damage sustained during ischemia and reperfusion, it is clear that the development of neuroprotective therapies is paramount to improving outcomes following OHCA.

After resuscitation, patients are known to suffer from a 'post-cardiac arrest syndrome' (PCAS) characterized by the dysfunction of multiple organ systems, including the cardiovascular and immune systems.[3,4] It has been established that diffuse endothelial damage and activation occurs following cardiac arrest, and that this is correlated with mortality.[5–9] The observed effect on survival may be due to the role of the endothelium in the maintenance of physiologic

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compartments within the body such as the gastrointestinal tract and the blood-brain-barrier (BBB).

Previous work has demonstrated that failure of the BBB after ischemia allows the infiltration of various immune subsets into the brain, including cytotoxic lymphocytes.[10] Murine studies of ischemic stroke have suggested that these lymphocytes begin localizing to the brain between 24 and 48 h following an ischemic insult.[11] In contrast, previous murine studies of global ischemia detected lymphocyte infiltration as early as three hours following resuscitation.[12] We attempted to determine if increased lymphocyte localization would be observed immediately following resuscitation in our porcine model, which provides an excellent analog to human patients both in terms of the biophysics of resuscitation and the similarity of the human and porcine immune system.

## Methods

This study was conducted as a post-mortem ancillary analysis of an experiment comparing adrenaline and vasopressin for resuscitation following asphyxial cardiac arrest in our laboratory. Prior to conducting any work, the experimental protocol was approved the University of Pittsburgh Institutional Animal Care and Use Committee. No exclusion criteria were set for enrollment in our pilot study.

### *Resuscitation*

Ten young, healthy, mixed-breed domestic swine (mean: 26.99 +/− 3.42 kg, 1:1 male to female ratio) were sedated with a combination of ketamine (10 mg/kg) and xylazine (4 mg/kg) delivered via intramuscular injection. Intravenous (IV) access was achieved via an ear vein, and animals were instrumented with continuous electrocardiographic and pulse oximetry monitoring. Fentanyl was used to induce and maintain anesthesia using an initial bolus dose (50 mcg/kg) followed by continuous infusion (30−100 mcg/kg/h, titrated to effect). Animals were then paralyzed using IV vecuronium and intubated via direct laryngoscopy using 5.0 endotracheal tubes. Central arterial and venous pressures were measured using micro-manometer-tipped transducers (Millar, Inc.) placed via femoral cutdown. In eight animals, asphyxial cardiac arrest was induced beginning with a vecuronium bolus (10 mg), and a fentanyl bolus (30 mcg/kg) delivered over 2 min. The other two animals were utilized as shams, and were anaesthetized and monitored without asphyxia or resuscitation prior to euthanasia. In the eight experimental animals, the ventilator circuit was disconnected and the endotracheal tube was occluded. After 9 min, resuscitation began as detailed by Fig. 1.

### *Tissue processing*

At the termination of resuscitation or euthanasia, the whole brain was removed and rinsed in distilled water to remove surface blood. Segments of basal ganglia, thalami, and hippocampi were isolated and weighed prior to being washed again in distilled water. The tissue segments were then mechanically dissociated by sequential passage through 100-um and 45-um cell strainers. Myelin was removed from the sample using density gradient centrifugation. Three milliliters of HBSS containing the dissociated tissue was layered above 10 mL s of a 0.9 M sucrose solution and centrifuged at 300*g* for 20 min. The myelin layer that appeared following centrifugation was aspirated using a pipette. Cells were isolated from the remaining sucrose

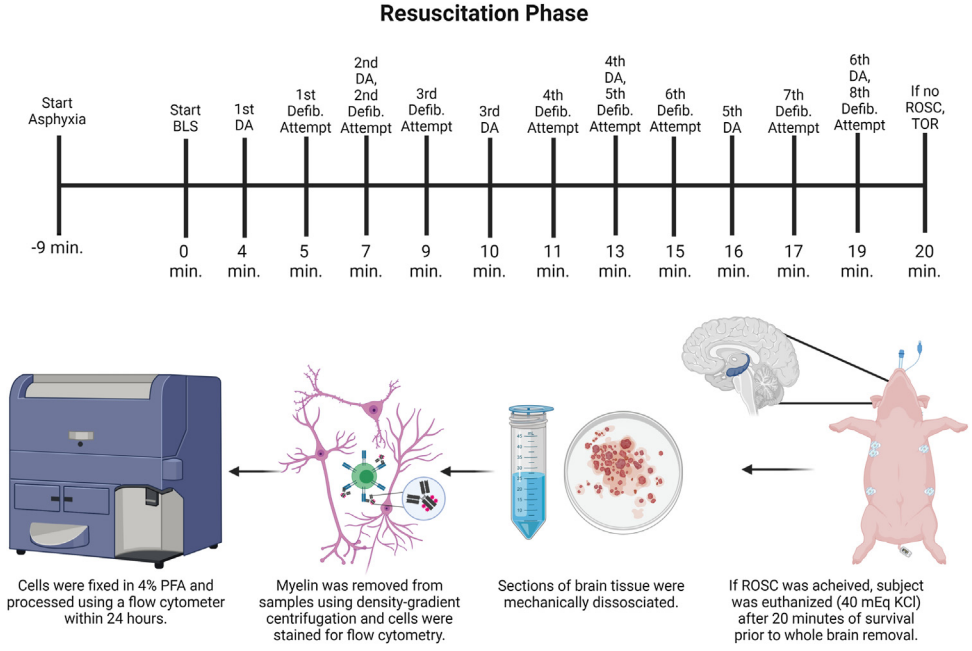


**Fig. 1 – Experimental design.**
**Graphical representation of our methods. The agents delivered at the first drug administration were high dose adrenaline (0.1 mg/kg) or vasopressin (1.6 IU/kg) and sodium bicarbonate (1den mEq/kg). Subsequent drug administrations were standard ACLS dose adrenaline (0.015 mg/kg), intended to imitate the current standard of care for these patients. At each defibrillation attempt that coincided with drug administration, a shock was delivered (if subject presented in a shockable rhythm) prior to drug delivery. BLS = basic life support, DA = drug administration, ROSC = return of spontaneous circulation, TOR = termination of resuscitation.**

solution, resuspended in FACS buffer (Phosphate buffered saline + 10% fetal bovine serum) and stained with fluorophore-conjugated antibodies for twenty minutes (CD8, PE; CD4, FITC; CD3, PerCP-Cy5.5). After staining, cells were resuspended in 4% paraformaldehyde and analyzed via flow cytometry within 24 h.

### *Analysis*

One animal was excluded from cell number analyses due to abnormally low cell numbers caused by the loss of a cell pellet following a centrifugation step.

During flow cytometry, the material contained within our samples was suspended in a fluidic stream and moved past sensors that quantified size, granularity, and the presence of fluorophore conjugated antibodies for each discrete particle. Each detected piece of material or cell within the sample was classified as an event. The FlowJo_V10 software platform (FlowJo_LLC, Becton, Dickinson, and Company) was used to analyze these data. Detected events classified as single cells expressing CD8 were included in the final analysis. We chose to focus on CD8 in this analysis because it is a marker specific to cytotoxic lymphocytes (cytotoxic T-cells and natural killer cells). CD8 single positive events were also found to yield the most well-defined cell populations during analysis, possibly due to non-specific binding of the other fluorophore-conjugated antibodies used.

Independent *t*-tests of means and Tukey's multiple comparison tests were performed using GraphPad Prism 7.03. We considered a p-value $<0.05$ to be statistically significant.

## Results

No significant relationship was discovered between anatomical location in the brain and the number of cytotoxic lymphocytes present per gram of tissue.

Animals that achieved ROSC and survived for twenty minutes were found to have an average of 7.6 times the number of cells per unit mass of brain tissue (304 +/− 62.2 cells/g) in comparison to sham animals (40 +/− 11.31 cells/g). Animals that did not achieve ROSC, and were sampled immediately following the cessation of 20 min of CPR, had an average of 12.0 times the number of cells per unit mass of brain tissue (481 +/− 274.4 cells/g) in comparison to sham animals (Fig. 2). Statistical significance was not achieved for this measure for resuscitated animals in comparison to sham animals and between groups.

The percentage of the total isolated cells determined to be CD8+ was compared by anatomical region (basal ganglia, thalamus, hippocampus) and circulatory status prior to euthanasia (ROSC vs No ROSC) (Fig. 3). Animals that achieved ROSC had a mean percentage of 0.473 +/− 0.24% CD8+ cells. Animals that did not achieve ROSC were found to have a mean percentage of 0.395 +/− 0.062%. Sham animals had a mean percentage of 0.19 +/− 0.014%. The proportion of CD8+ lymphocytes in the brain was significantly higher at euthanasia in the brain tissue of animals that did not achieve ROSC ($p = 0.012$) when compared to sham animals. No other significant relationships were discerned in relation to percent composition of CD8+ cells between groups or in comparison to the sham group ($\alpha = 0.05$) (Table 1).

## Discussion

These data suggest that CD8+ lymphocytes are localizing to the brain in the acute phase of cardiac arrest resuscitation. Importantly, these cells have been suggested to play a causative role in neurological damage following resuscitation by an experimental series in a murine cardiac arrest model.[12] Cytotoxic lymphocytes may directly damage neurons and associated cells, or stimulate a harmful proinflammatory microenvironment through interactions with resident immune subsets such as microglia.

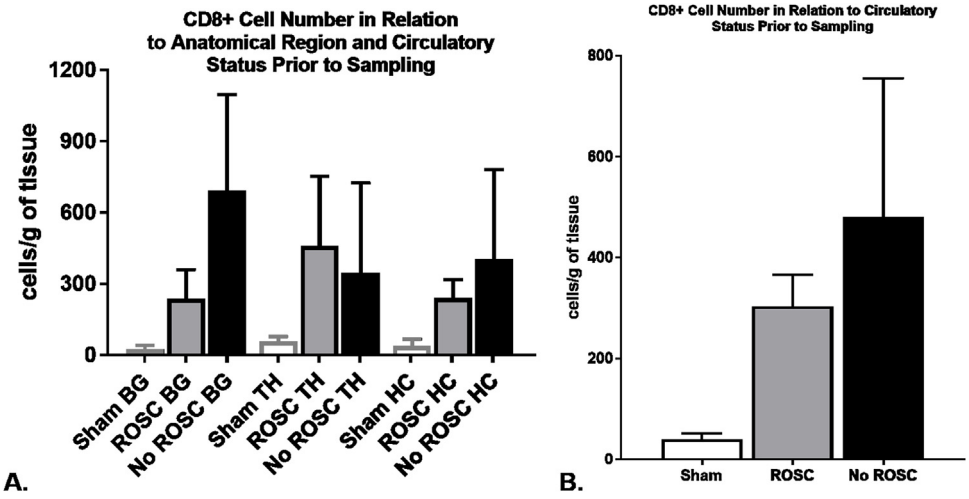


**Fig. 2 – Number of CD8+ cells per unit mass.**
**The concentrations of cytotoxic lymphocytes (CD8+ cells/gram of tissue) detected in each sampled anatomical region (basal ganglia, thalamus, hippocampus) are displayed in Panel A. These data are subdivided by circulatory status prior to sampling (ROSC, no ROSC, sham). The overall average concentrations of detected cytotoxic lymphocytes (CD8+ cells/gram averaged across the three sampled anatomical regions for each subject) are displayed in Panel B. These data are also subdivided by circulatory status prior to sampling. No statistically significant differences were discerned between groups ($\alpha = 0.05$). ROSC = return of spontaneous circulation, BG = basal ganglia, TH = thalamus, HC = hippocampus.**

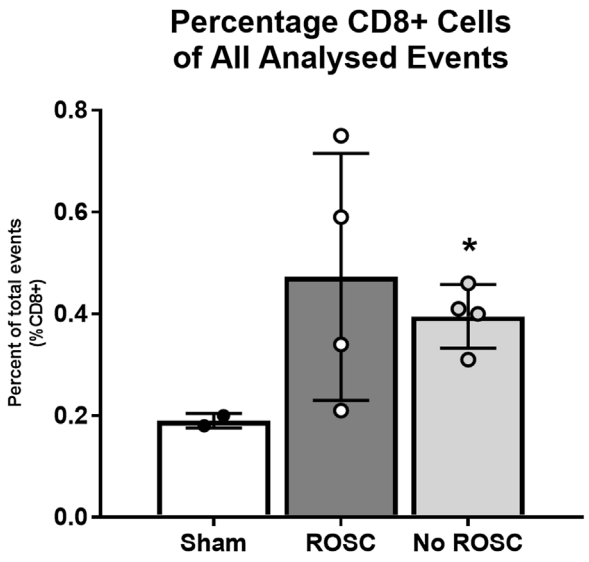


**Fig. 3 – Percentage CD8+ cells of all analysed events. For each individual, the average CD8+ cell percentage was calculated from an average of the percentage of detected events (see Section "Methods") classified as CD8+ in each of the three sampled anatomical regions (basal ganglia, thalamus, and hippocampus). These data are displayed subdivided by circulatory status prior to sampling (ROSC, no ROSC, sham). The difference in the mean percentage between the Sham group and the No ROSC group was found to be significant ($p = 0.012$, $\alpha = 0.05$). It is likely that the decreased variability observed in the 'No ROSC' group in comparison to the 'ROSC' group is due to the uniform insult suffered by the "No ROSC" group, whereas animals that achieved ROSC experienced different durations of CPR. * = significant difference from 'Sham' group. ROSC = return of spontaneous circulation.**

We observed greater variability in the proportion of CD8+ cells present in the brains of animals that did achieve ROSC than in the brains of animals in the 'No ROSC' group (Fig. 3). It is likely that the decreased variability observed in the 'No ROSC' group in comparison to the 'ROSC' group is due to the uniform insult suffered by the "No ROSC" animals (20 continuous minutes of CPR), whereas animals that achieved ROSC suffered different durations of CPR (4.5 −8.7 min). This suggests that the localization of these cells to the brain following cardiac arrest may be related to the severity of the global insult.

Our study has several limitations. Non-specific binding of antibodies to myelin and myeloid cells in our samples could have led to false positives in terms of cytotoxic lymphocyte identification. Due to the preliminary nature of this study, we did not perform immunohistochemical staining on brain sections, which would allow us to determine whether these cells are crossing the BBB and moving into the brain parenchyma, or if these cells are merely gathering on the inside of the vascular endothelium at these early timepoints. Furthermore, we did not measure the number of cytotoxic lymphocytes in the systemic circulation at the time brain tissue was sampled. It is possible that the increases observed in this study are due to global increases in the number of circulating CD8+ cells following resuscitation. Finally, due to the preliminary nature of this analysis and the variability observed, our study may have been underpowered to detect relevant relationships between immune cell infiltration, brain region, and perfusion status prior to sampling.

## Conclusions

These data suggest that cytotoxic lymphocytes may be localizing to the brain during cardiac arrest resuscitation. This could indicate that these cells are entering the brain across a dysfunctional BBB and beginning to cause damage at this acute timepoint. Future steps include the experimental manipulation of lymphocyte movement into the brain following cardiac arrest resuscitation in a murine model to determine if the contribution of these immune cells to neurological damage is modifiable via pharmacological means.

## Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## CRediT authorship contribution statement

**Tanner Smida:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Allison C. Koller:** Investigation, Writing - review & editing. **James J. Menegazzi:** Resources, Methodology, Investigation, Writing - review & editing. **David D. Salcido:** Supervision, Methodology, Investigation, Writing - review & editing.

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**Table 1 – Subject demographics.**

| | ROSC (n = 4) | No ROSC (n = 4) | Sham (n = 2) |
|---|---|---|---|
| Duration of CPR | 7.38 +/− 2.71 min | 20 min (until termination of resuscitation timepoint) | N/A |
| Mass | 28.2 +/− 3.59 kg | 27.55 +/− 2.76 kg | 9.95 +/− 0.07 kg |
| Baseline heart rate | 92 +/− 18.13 beats per minute | 86.25 +/− 17.35 beats per minute | 97.5 +/− 20.51 beats per minute |
| Baseline mean arterial pressure | 87.58 +/− 15.07 mmHg | 79.83 +/− 5.32 mmHg | 77.4 +/− 1.98 mmHg |
| Sex (% female) | 25% | 50% | 100% |

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