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## Polynitroxyl Albumin and Albumin Therapy After Pediatric Asphyxial Cardia Arrest: Effects on Cerebral Blood Flow and Neurologic Outcome

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# Polynitroxyl albumin and albumin therapy after pediatric asphyxial cardiac arrest: effects on cerebral blood flow and neurologic outcome

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**Postresuscitation cerebral blood flow (CBF) disturbances and generation of reactive oxygen species likely contribute to impaired neurologic outcome after pediatric cardiac arrest (CA). Hence, we determined the effects of the antioxidant colloid polynitroxyl albumin (PNA) versus albumin or normal saline (NS) on CBF and neurologic outcome after asphyxial CA in immature rats. We induced asphyxia for 9 minutes in male and female postnatal day 16 to 18 rats randomized to receive PNA, albumin, or NS at resuscitation from CA or sham surgery. Regional CBF was measured serially from 5 to 150 minutes after resuscitation by arterial spin-labeled magnetic resonance imaging. We assessed motor function (beam balance and inclined plane), spatial memory retention (water maze), and hippocampal neuronal survival. Polynitroxyl albumin reduced early hyperemia seen 5 minutes after CA. In contrast, albumin markedly increased and prolonged hyperemia. In the delayed period after resuscitation (90 to 150 minutes), CBF was comparable among groups. Both PNA- and albumin-treated rats performed better in the water maze versus NS after CA. This benefit was observed only in males. Hippocampal neuron survival was similar between injury groups. Treatment of immature rats with PNA or albumin resulted in divergent acute changes in CBF, but both improved spatial memory retention in males after asphyxial CA.**

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## Introduction

Pediatric cardiac arrest (CA) results from asphyxia in 80% of cases. There are no therapies for improving neurologic outcome in children after asphyxial CA, with the possible exception of hypothermia, although it remains unproven. Outcome from pedia-

tric asphyxial CA is poor: of children who sustain out-of-hospital CA, 30% have return of spontaneous circulation (ROSC), 12% survive to hospital discharge, and only 4% have favorable neurologic outcome (Donoghue *et al*, 2005).

Reperfusion and reoxygenation, indispensable to restore viability during cardiopulmonary resuscitation and after CA, may also have undesirable consequences. Although it is indisputable that reperfusion is essential for neuronal survival, the ideal reperfusion pattern after CA remains undefined. After 9 minutes of asphyxial CA in immature rats, hyperemia is seen from 5 to 15 minutes after ROSC in subcortical structures, whereas cortical hypoperfusion appears early and is sustained for up to 3 hours (Manole *et al*, 2009). It is postulated that cerebral hyperemia is beneficial, and hypoperfusion is detrimental for neuronal survival

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after CA (Snyder *et al*, 1975); however, a recent study in experimental cerebral ischemia–reperfusion has suggested that early postresuscitation hyperemia may be deleterious (Pignataro *et al*, 2008).

Reactive oxygen and nitrogen species generated during CA and reperfusion influence postresuscitation cerebral blood flow (CBF). Reactive oxygen and nitrogen species produce vascular damage and loss of autoregulation after brain injury (Nelson *et al*, 1992). Superoxide production increases with reperfusion after global ischemia in the brain (Kofler *et al*, 2005). Superoxide itself has limited reactivity; however, it reacts with nitric oxide to form peroxynitrite in a diffusion-limited reaction, leading to decreased nitric oxide availability, which can alter CBF after CA (Bayir, 2005). Treatment with a superoxide dismutase (SOD) mimetic early after CA has been shown to improve neurologic outcome (Cerchiari *et al*, 1987).

Polynitroxyl albumin (PNA) is an intravascular antioxidant that is synthesized by covalent addition of high molar ratio nitroxide (55:1 on average) to albumin. Polynitroxyl albumin has been shown to exert SOD-mimetic properties and to reduce infarct size after focal ischemia in rats (Beaulieu *et al*, 1998; Kuppusamy *et al*, 1996; Sugawara *et al*, 2001). In our pediatric asphyxial CA model, PNA decreased oxidative and nitrative stress induced by resuscitation with 100% oxygen (Walson *et al*, 2011). Albumin, the parent compound of PNA, has also been shown to improve perfusion and functional outcome in models of focal cerebral ischemia (Belayev *et al*, 1997, 1998, 2002; Liu *et al*, 2001). Besides colloidal properties, albumin binds redox-active transition metals, fatty acids, and heme and has some antioxidant properties due to a free cysteine residue (Gutteridge *et al*, 1984; Rowley *et al*, 1984). Given their large size, PNA and albumin are generally restricted to the intravascular space if the blood–brain barrier is intact, which is the case early after injury in our CA model (Manole *et al*, 2009). Accordingly, we hypothesized that PNA will ameliorate ischemia-induced CBF dysfunction and improve neurologic outcome versus treatment with albumin or normal saline (NS) after pediatric asphyxial CA in immature rats.

## Materials and methods

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and were performed in accordance with these guidelines and regulations. We used postnatal day 16 to 18 Sprague–Dawley rats of both sexes for CBF analysis ( $n = 37$ , 6 to 7 per group) and for neurologic outcome ( $n = 42$ , 10 to 12 per group). Rats were randomized to asphyxial CA (9-minute asphyxia) or sham, and further randomized to receive one of the following solutions at resuscitation or after sham surgery: PNA (20 mL/kg, 10%, SynZyme Technologies, Irvine, CA, USA), albumin (20 mL/kg, 10%, SynZyme

Technologies), or NS (20 mL/kg). Ten percent of human serum albumin was prepared by dilution of 25% human albumin (Baxter, Deerfield, IL, USA; USP) with NS, and was supplied by SynZyme Technologies.

## Anesthesia and Surgery

We used our established protocol (Manole *et al*, 2009) in accordance with institutional guidelines. Rats underwent tracheal intubation and central venous and arterial catheterization. Intravenous analgesia and neuromuscular blockade were achieved using fentanyl (50  $\mu\text{g}/\text{kg}$  per h) and vecuronium (5 mg/kg per h). Ventilatory parameters were adjusted to a target PaCO<sub>2</sub> of 35 to 45 mm Hg. Nine minutes of asphyxia was produced by disconnecting the ventilator. Rats were resuscitated by reconnecting the ventilator with FiO<sub>2</sub> = 1.0, administration of epinephrine (0.005 mg/kg) and sodium bicarbonate (1 mEq/kg), and manual chest compressions until ROSC. At resuscitation, we administered PNA, albumin, or NS. After ROSC, anesthesia and neuromuscular blockade were restarted. A FiO<sub>2</sub> of 1.0 was maintained until 15 minutes after ROSC, and decreased to 0.5 for the remainder of the experiment. Shams underwent all procedures except asphyxia or resuscitation.

## Cerebral Blood Flow Measurement

Arterial spin-labeled magnetic resonance imaging perfusion maps were obtained at baseline and at 5, 10, 15, 30, 60, 90, 120, and 150 minutes after ROSC from CA or sham surgery. Arterial spin-labeled magnetic resonance imaging measurements were performed on a 7-T, 21-cm-bore Bruker Biospec system (Bruker Biospec, Billerica, MA, USA) as described previously (Manole *et al*, 2009). In brief, pixel-by-pixel perfusion maps were generated by  $(M_C - M_L)/M_C$ , where  $M_C$  and  $M_L$  are pixel intensities from the control and labeling image, respectively. Cerebral blood flow was determined by  $\text{CBF} = \lambda \cdot (T_{1\text{obs}} \cdot 2\alpha)^{-1} \cdot (M_C - M_L) \cdot M_C^{-1}$  where  $\lambda$  is the blood–brain partition coefficient of water, assuming a spatially constant value of 0.9 mL/g. The spin-labeling efficiency  $\alpha$ , and apparent brain tissue  $T_1$ ,  $T_{1\text{obs}}$ , were measured at baseline for all rats.

## Assessment of Neurologic Outcome

Gross vestibulomotor function was assessed on days 1 to 5 after asphyxia or sham. We assessed the ability of rats to balance on a suspended, narrow wooden beam for 60 seconds (beam balance test). We measured the maximum angle (45 to 85°) at which the rat maintained its position on an inclined plane for 10 seconds (inclined plane test) (Fink *et al*, 2005). We tested spatial memory acquisition and retention using the Morris water maze on days 7 to 14 after asphyxia or sham surgery. Rats were acclimated in the maze on days 7 to 9 after injury using a visible escape platform. We then used the hidden platform test on days 10 to 13 to assess spatial memory acquisition using external visual cues to find the submerged escape platform. We used probe trials on day 14 to assess spatial

memory retention with the escape platform removed and the time spent in the target quadrant recorded (maximum 60 seconds).

### Histologic Assessment: Unbiased Stereology

At 35 days after asphyxia or sham injury, rats were anesthetized with 3% isoflurane and perfused with 250 mL ice-cold heparinized saline and 250 mL 2% paraformaldehyde. The brains were removed, postfixed in 2% paraformaldehyde for 1 hour, placed in 30% sucrose, and frozen with liquid nitrogen. Coronal sections were cut at 35  $\mu$ m using a cryotome, and 10 to 12 systematic random sections were obtained throughout the dorsal hippocampus. Sections were stained with Cresyl violet. A subset of sections was immunostained using anti-NeuN antibody to label neurons. The two-stage combination of optical disector and Cavalieri volume-point counting methods was used to quantify the total number of neurons in the CA<sub>1</sub> and CA<sub>3</sub> subregions of the dorsal hippocampus, as detailed previously (Sterio, 1984); for review of stereology procedures used in this study, see Mouton (2011). To generate an unbiased sample of 10 to 11 sections per animal, every fifth section was sampled in a systematic-random manner from the total number of sections containing the dorsal hippocampus (range 50 to 70 total sections). With assistance from a computerized stereology system (Stereologer, Stereology Resource Center, Chester, MD, USA), the reference space was outlined at low power ( $\times 4$ ), and neurons with a neuronal phenotype, including clear nuclear membrane and nucleolus, were counted at high magnification ( $\times 60$ ). According to the disector principle, thin focal-plane optical scanning was carried out using a virtual three-dimensional disector of height 11  $\mu$ m through the mean section thickness of 13.5  $\mu$ m.

A guard volume of 1  $\mu$ m was used to avoid artifacts at the tissue surface, e.g., lost caps. Neurons intersecting the unbiased counting frame without touching the 'forbidden lines' were counted. Sampling was continued to a coefficient of error < 0.10 (coefficient of error < 10%).

### Statistical Analysis

Data were analyzed using SPSS version 15 (SPSS, Chicago, IL, USA), and expressed as mean  $\pm$  s.e.m. We used repeated-measures ANOVA (analysis of variance) with Fisher's LSD *post hoc* test for physiologic parameters, CBF, motor function tests, and visible and hidden platforms, and one-way ANOVA with Fisher's Least Significant Difference (LSD) *post hoc* test for neuronal counts and probe trial. Hemispheric CBF, functional outcome parameters, and neuronal counts were similar for NS- and PNA- or albumin-treated shams; thus, we combined data for sham groups for analysis.  $P < 0.05$  was considered significant.

## Results

### Physiologic Data

The duration of CA was similar in the CA groups. Table 1 presents physiologic data (mean arterial pressure (MAP), PaCO<sub>2</sub>, arterial pH, and PaO<sub>2</sub>). Both NS- and albumin-treated groups had increased MAP versus shams at 10 minutes after resuscitation. Shams, NS-, and PNA-treated rats had MAP comparable with baseline, whereas albumin-treated rats had increased MAP versus baseline at 10 minutes after resuscitation. pH was lower in shams 120 minutes after resuscitation. This may

**Table 1** MAP, PaO<sub>2</sub>, PaCO<sub>2</sub>, and pH at baseline and after asphyxial CA

		Baseline	5 minutes	10 minutes	30 minutes	120 minutes	150 minutes
MAP	CA-PNA	69 $\pm$ 4	71 $\pm$ 7	77 $\pm$ 8	56 $\pm$ 5	67 $\pm$ 13	69 $\pm$ 6
	CA-albumin	69 $\pm$ 4	77 $\pm$ 5	90 $\pm$ 4*	70 $\pm$ 4	67 $\pm$ 11	70 $\pm$ 4
	CA-NS	80 $\pm$ 3	82 $\pm$ 4	93 $\pm$ 6*	75 $\pm$ 4	72 $\pm$ 14	75 $\pm$ 4
	Sham	72 $\pm$ 4	75 $\pm$ 4	72 $\pm$ 4	71 $\pm$ 4	73 $\pm$ 15	73 $\pm$ 4
PCO <sub>2</sub>	CA-PNA	33 $\pm$ 2			43 $\pm$ 6	39 $\pm$ 3	41 $\pm$ 3
	CA-albumin	35 $\pm$ 2			51 $\pm$ 5	36 $\pm$ 3	32 $\pm$ 2
	CA-NS	38 $\pm$ 3			44 $\pm$ 6	36 $\pm$ 3	36 $\pm$ 6
	Sham	36 $\pm$ 1			39 $\pm$ 2	41 $\pm$ 3	42 $\pm$ 2
pH	CA-PNA	7.23 $\pm$ 0.02			7.20 $\pm$ 0.05	7.33 $\pm$ 0.03*	7.31 $\pm$ 0.03*
	CA-albumin	7.24 $\pm$ 0.03			7.15 $\pm$ 0.04	7.37 $\pm$ 0.02*	7.37 $\pm$ 0.03*
	CA-NS	7.31 $\pm$ 0.04			7.26 $\pm$ 0.07	7.40 $\pm$ 0.03*	7.38 $\pm$ 0.04*
	Sham	7.24 $\pm$ 0.02			7.26 $\pm$ 0.02	7.21 $\pm$ 0.02 <sup>†</sup>	7.20 $\pm$ 0.02 <sup>†</sup>
PaO <sub>2</sub>	CA-PNA	203 $\pm$ 11			176 $\pm$ 20	266 $\pm$ 18*	275 $\pm$ 40*
	CA-albumin	175 $\pm$ 19			178 $\pm$ 29	258 $\pm$ 17*	256 $\pm$ 14
	CA-NS	216 $\pm$ 9			217 $\pm$ 27	249 $\pm$ 17*	269 $\pm$ 25
	Sham	212 $\pm$ 11			198 $\pm$ 12	189 $\pm$ 10 <sup>†</sup>	182 $\pm$ 12

CA, cardiac arrest; NS, normal saline; PNA, polynitroxyl albumin.

\* $P < 0.05$  versus sham, <sup>†</sup> $P < 0.05$  versus CA-NS.

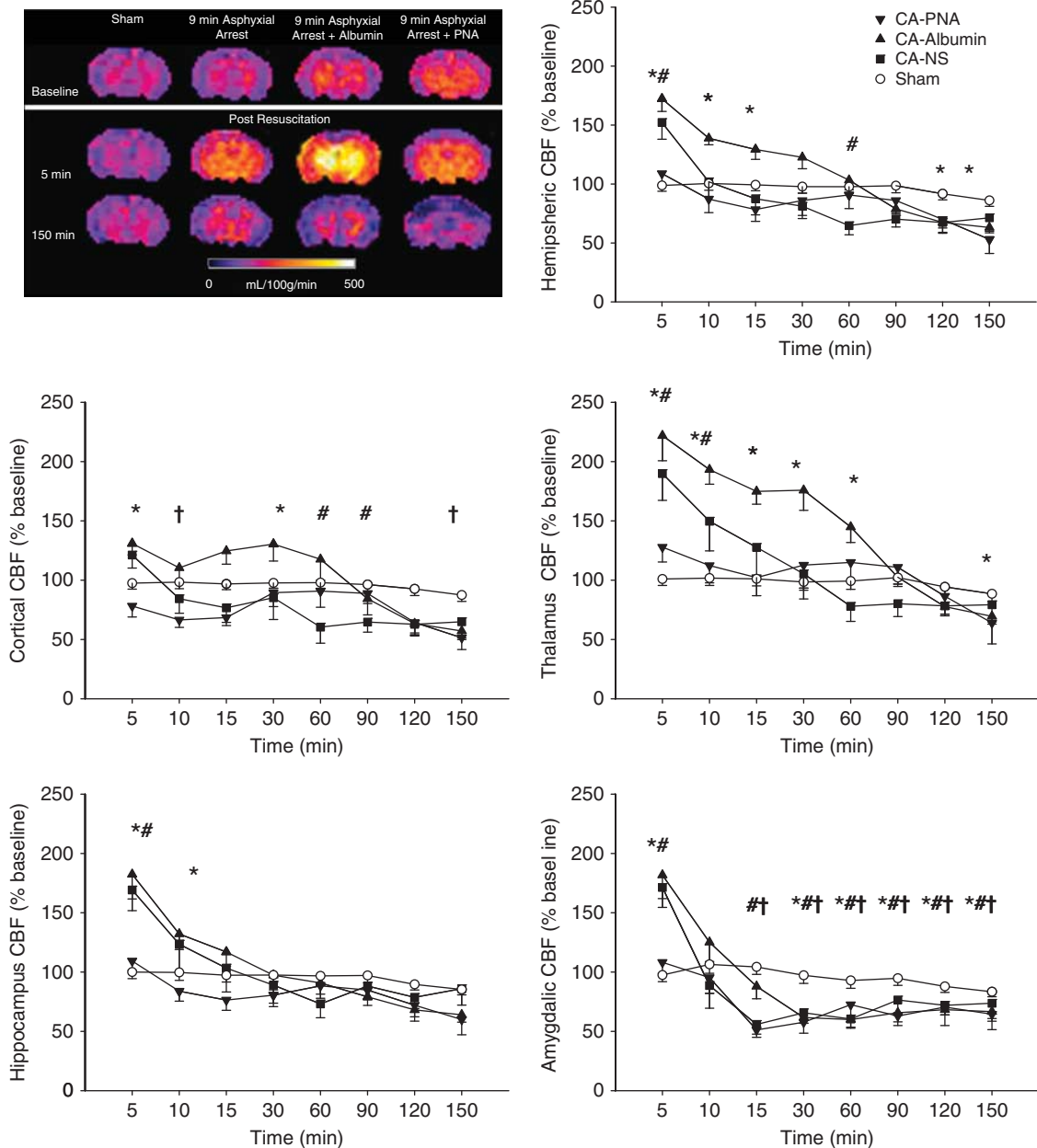
result from the CA groups receiving sodium bicarbonate at resuscitation. PaO<sub>2</sub> was statistically lower in shams versus CA; however, this difference does not seem physiologically meaningful.

### Cerebral Blood Flow

We report postresuscitation CBF for each rat normalized to its respective baseline (percentage baseline of CBF, Figure 1). Postresuscitation CBF displayed marked regional and temporal differences in the three treatment groups.

### Hemispheric Cerebral Blood Flow

Baseline hemispheric CBF was 183 ± 7 mL/100g per min. Normal saline-treated rats had increased hemispheric CBF versus shams at 5 minutes after resuscitation (54%, *P* < 0.001). Cerebral blood flow returned to values comparable to shams after 10 minutes, except for 60 minutes when hemispheric CBF was lower than shams (34%, *P* < 0.05). Polynitroxyl albumin-treated rats had hemispheric CBF similar to shams at all time points. Albumin-treated rats had prolonged increase in hemispheric CBF versus shams from 5 to 15 minutes (74%, *P* < 0.001).



**Figure 1** Regional postresuscitation CBF, percentage change from baseline. Color flow map of CBF at baseline, 5 and 150 minutes after CA. (\**P* < 0.05 CA-albumin versus shams; #*P* < 0.05 CA-NS versus shams; †*P* < 0.05 CA-PNA versus shams). CA, cardiac arrest; CBF, cerebral blood flow; NS, normal saline; PNA, polynitroxyl albumin.

Hemispheric CBF decreased to values comparable to shams at 30 minutes, and below shams from 120 to 150 minutes (26%,  $P < 0.001$ ).

Hemispheric CBF differed between the CA groups. Albumin-treated rats had increased hemispheric CBF versus NS-treated rats from 10 to 60 minutes ( $P < 0.05$ ). Polynitroxyl albumin-treated rats had similar CBF with NS-treated rats with the exception of decreased CBF at 5 minutes ( $P < 0.05$ ).

### Cortical Cerebral Blood Flow

Baseline cortical CBF was  $178 \pm 6$  mL/100 g per min. Cortical CBF of NS-treated rats was comparable to shams in the first 30 minutes after resuscitation and lower than shams 60 to 90 minutes (38%,  $P < 0.05$ ). Polynitroxyl albumin-treated rats had cortical CBF similar to shams, except for 10 and 150 minutes when CBF was lower than shams (42%,  $P < 0.05$ ). Albumin-treated rats had increased cortical CBF versus shams at 5 and 30 minutes (34%,  $P < 0.05$ ), reduced cortical CBF versus shams at 120 minutes (33%,  $P < 0.05$ ).

Cortical CBF was different between the CA groups. Albumin-treated rats had increased cortical CBF versus NS-treated rats from 15 to 60 minutes after resuscitation ( $P < 0.05$ ). Polynitroxyl albumin-treated rats had similar CBF as NS-treated rats with the exception of decreased CBF at 5 minutes ( $P < 0.05$ ).

### Thalamic Cerebral Blood Flow

Baseline thalamic CBF was  $228 \pm 11$  mL/100 g per min. In NS-treated rats, thalamic CBF was increased versus shams at 5 to 10 minutes (88%,  $P < 0.001$ ) and similar to shams from 15 to 150 minutes. In PNA-treated rats, thalamic CBF was similar to shams at all time points. In albumin-treated rats, thalamic CBF was markedly increased versus shams from 5 to 60 minutes (120% at 5 minutes,  $P < 0.05$ ). Cerebral blood flow decreased to values comparable to shams at 90 minutes and was lower than shams at 150 minutes (21%,  $P < 0.05$ ).

Thalamic CBF was different between CA groups. Albumin-treated rats had increased thalamic CBF versus NS-treated rats at 30 to 60 minutes after resuscitation ( $P < 0.05$ ). Cerebral blood flow in PNA-treated rats was similar to NS-treated rats with the exception of decreased CBF at 5 minutes ( $P < 0.05$ ).

### Hippocampal Cerebral Blood Flow

Baseline hippocampal CBF was  $211 \pm 10$  mL/100 g per min. Normal saline-treated rats had increased hippocampal CBF versus shams at 5 minutes (70%,  $P < 0.001$ ), with no difference afterwards. Cerebral blood flow in PNA-treated rats was similar to shams

at all time points. Albumin-treated rats had hippocampal CBF increased versus shams at 5 to 10 minutes (83%,  $P < 0.001$ ) and similar to shams after 15 minutes.

Hippocampal CBF was similar in the CA groups, except for decreased CBF at 5 minutes in PNA-versus NS-treated rats ( $P < 0.05$ ).

### Amygdalic Cerebral Blood Flow

Baseline amygdalic CBF was  $134 \pm 6$  mL/100 g per min. Normal saline-treated rats had increased amygdalic CBF versus shams (75%,  $P < 0.001$ ) at 5 minutes, followed by decreased CBF versus shams from 15 to 60 minutes (46% at 15 minutes,  $P < 0.05$ ). Polynitroxyl albumin-treated rats had CBF similar to shams until 10 minutes and lower than shams from 15 to 60 minutes (50% at 15 minutes,  $P < 0.05$ ). Albumin-treated rats had increased CBF at 5 minutes versus shams (86%,  $P < 0.001$ ), and then decreased CBF versus shams from 30 minutes (35%,  $P < 0.05$ ).

Amygdalic CBF was similar in the CA groups, except for decreased CBF at 5 minutes in PNA-versus NS-treated rats ( $P < 0.05$ ).

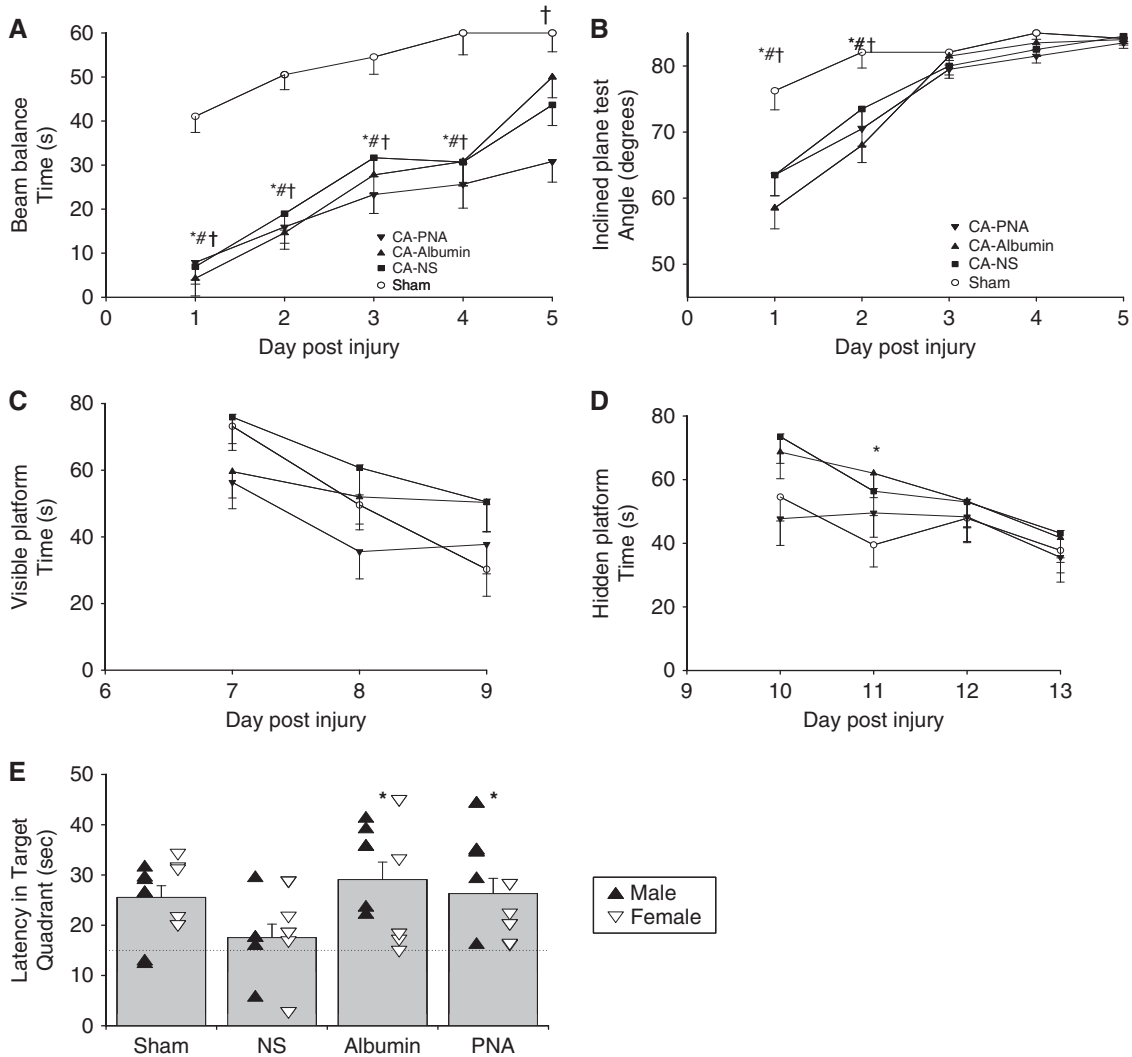
### Postresuscitation Cerebral Blood Flow in Male and Female Rats

There were no hemispheric sex-dependent CBF differences in our study ( $n = 3$  to 4 per sex per group, data not shown). There were no regional sex-dependent CBF differences except for two time points. At 5 and 10 minutes, albumin-treated female rats had higher thalamic CBF than did NS-treated females.

### Functional Outcome

**Beam Balance and Inclined Plane Test:** There were no differences between treatments after CA in either beam balance or inclined plane performance (Figures 2A and 2B). All CA groups had decreased beam balance performance versus shams from days 1 to 4 ( $P < 0.05$ , Figure 2A). On the final day of beam balance testing, albumin and NS-treated rats performed similar to shams, whereas PNA-treated rats performed worse than shams ( $P < 0.05$ ). On the inclined plane, the CA groups performed worse than shams on the first 2 days of testing ( $P < 0.05$ ). There were no differences between groups on days 3 to 5 (Figure 2B). There were no sex-dependent differences in beam balance or inclined plane test performance between treatment groups ( $n = 5$  to 6 per sex per group).

**Morris Water Maze: Visible Platform.** On the first and third days of acclimatization in the water maze, there was no difference in performance between groups. On the second day, PNA-treated rats had reduced latency to finding the platform versus NS-treated rats ( $P < 0.05$ ). All groups except for albumin-treated rats



**Figure 2** (A) Beam balance, (B) inclined plane test, (C) hidden platform test (\* $P < 0.05$  CA-albumin versus shams, # $P < 0.05$  CA-NS versus shams, † $P < 0.05$  CA-PNA versus shams). (D) Probe trial. Triangles represent individual rats in each group, males (▲) or females (△), (\* $P < 0.05$  versus CA-NS). CA, cardiac arrest; NS, normal saline; PNA, polynitroxyl albumin.

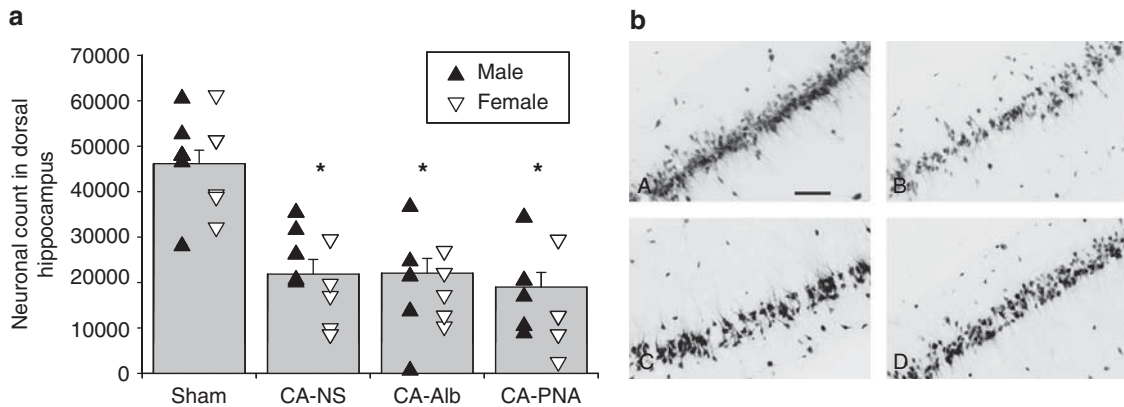
improved the ability to find the platform from the first to the third day of acclimatization ( $P < 0.05$  for shams, PNA- and NS-treated rats;  $P = 0.3$  for albumin-treated rats). There was an overall effect of time ( $P < 0.001$ ). The overall performance of the four groups was similar ( $P = 0.2$ ). There were no sex-dependent differences between groups, except for the first day of acclimatization: NS-treated female rats performed better than both PNA- and albumin-treated female rats ( $n = 5$  to 6 per sex per group).

**Hidden Platform.** There was an overall effect of time ( $P < 0.001$ ). On the first day of testing, PNA-treated rats had reduced latency to finding the hidden platform versus NS-treated rats ( $P < 0.05$ ), performing similar to shams (Figure 2C). During the subsequent days, all groups except for PNA-treated rats improved the ability to find the hidden platform from the first to the fourth day, showing the ability to learn the task ( $P < 0.05$  for shams, albumin- and NS-

treated rats;  $P = 0.1$  for PNA-treated rats). There was no difference among groups on the final day of the hidden platform test. The overall performance of the four groups was similar ( $P = 0.3$ ). There were no sex-dependent differences between groups ( $n = 5$  to 6 per sex per group).

**Probe Trial.** After hidden platform testing, we measured the time spent in the quadrant where the platform was previously located. Random swimming in all four quadrants would yield a time in the target quadrant of 15 seconds of the total 60-second testing period. Albumin- and PNA-treated rats spent more time in the target quadrant versus NS-treated rats ( $P < 0.05$ ). On *post hoc* analysis, this difference was attributable to benefit in males ( $n = 5$  to 6 per sex per group, Figure 2D). The power to detect a difference for groups and genders in the probe trial was 0.145. This gender difference in performance could not be explained by different durations of CA in males and





**Figure 3** (a) Neuronal counts in the CA<sub>1</sub> hippocampus. Triangles represent individual rats in each group, males (▲) or females (△). (\* $P < 0.05$  versus shams). (b) Representative photomicrographs of the CA<sub>1</sub> hippocampus stained with Neu-N from a representative shams (A), CA-NS (B), CA-PNA (C), and CA-albumin (D) rat. Size bar = 60  $\mu\text{m}$ . CA, cardiac arrest; NS, normal saline; PNA, polynitroxyl albumin.

females. The durations of CA in minutes were  $5.5 \pm 0.4$  versus  $6.2 \pm 0.3$  for PNA,  $5.6 \pm 0.5$  versus  $5.6 \pm 0.5$  for albumin, and  $5.5 \pm 0.6$  versus  $4.7 \pm 0.6$  for NS in male and female rats, respectively.

### Neuropathological Outcome

The CA groups had decreased CA<sub>1</sub> hippocampal neuronal survival versus shams ( $P < 0.05$ ). There were no differences in cell counts among CA treatment groups and no difference between males and females ( $n = 5$  to 6 per sex per group). Hippocampal neuronal counts in dorsal CA<sub>1</sub> were  $46,160 \pm 2,933$  for shams, and  $18,989 \pm 3,951$ ,  $22,053 \pm 2,853$ , and  $21,848 \pm 2,803$  for the CA groups treated with PNA, albumin, and NS, respectively ( $P < 0.05$  CA groups versus shams) (Figure 3). There were no differences in CA<sub>3</sub> hippocampal neuronal survival between CA groups and shams. Hippocampal neuronal counts in dorsal CA<sub>3</sub> were  $25,635 \pm 1,869$  for shams, and  $26,929 \pm 3,479$ ,  $24,822 \pm 3,078$ , and  $25,635 \pm 1,869$  for CA groups treated with PNA, albumin, and NS, respectively ( $P = 0.8, 0.6,$  and  $0.3$  versus shams).

### Discussion

To our knowledge, this is the first study exploring the effects of the intravascular antioxidant colloid PNA or albumin on either CBF or functional outcome after asphyxial CA in immature animals. Polynitroxyl albumin and albumin produced divergent CBF changes after asphyxial CA. Polynitroxyl albumin prevented early hyperemia, whereas albumin exacerbated hyperemia in all brain regions, including the cortex, a region where hypoperfusion is seen. Remarkably, despite divergent CBF changes, both PNA and albumin improved functional outcome assessed by the probe trial test compared with NS. The decrease in postresuscitation CBF after PNA

suggests that reactive oxygen and nitrogen species contribute to hyperemia after CA. The improved functional outcome after PNA and albumin despite divergent early CBF changes suggests four possibilities: (1) either blunting or exacerbating early hyperemia is beneficial, (2) alterations of CBF do not influence functional outcome, (3) PNA improves functional outcome independent of effects on CBF, or (4) albumin improves functional outcome independent of effects on CBF in our pediatric asphyxial CA model.

Polynitroxyl albumin is a colloid that has nitroxide moieties with potent antioxidant properties (Kuppusamy *et al*, 1996). Nitroxides act as electron scavengers and have SOD-mimetic activity. Polynitroxyl albumin has been shown to attenuate superoxide-induced vascular adhesion of leukocytes to endothelial cells versus albumin (Russell *et al*, 1998). The dismutation of superoxide yields hydrogen peroxide (Yost and Fridovich, 1976). Hydrogen peroxide is a relatively stable molecule; however, it can produce hydroxyl radical, which is a potent reactive oxidant, in the presence of transition metals in their reduced states (Rowley and Halliwell, 1983). Hydrogen peroxide is removed primarily by glutathione peroxidases. We have shown that CA leads to depletion of glutathione, protein nitration, and lipid peroxidation, which were prevented by PNA (Walson *et al*, 2011). Polynitroxyl albumin has beneficial effects in models of focal ischemia–reperfusion (Steinbauer *et al*, 2000; Zhang *et al*, 2000) and focal brain ischemia (Beaulieu *et al*, 1998; Sugawara *et al*, 2001).

Albumin is a colloid that increases MAP in hypovolemia. A beneficial effect of albumin in neurologic pathologies is suggested by studies in stroke and traumatic brain injury in which lower serum albumin levels are associated with poor outcome (Bernard *et al*, 2008; Idicula *et al*, 2009). Albumin has beneficial effects in animal models of focal ischemia (Belayev *et al*, 1997, 2002; Liu *et al*, 2001); however, it was not found to be neuroprotective.

tive either in a neonatal model of stroke or after clinical traumatic brain injury in adults (Myburgh *et al*, 2007; Wang *et al*, 2007). Albumin's beneficial effects for focal ischemia–reperfusion are attributed to antioxidant properties, inhibition of copper-induced peroxidation, fatty acid oxidation (Gutteridge *et al*, 1984; Rowley *et al*, 1984; Yao *et al*, 2010), binding of fatty acids and heme, and early upregulation of vascular endothelial growth factor (Yao *et al*, 2010). Albumin contains one free cysteine moiety, which is believed to be the key scavenger of reactive oxygen and nitrogen species generated in the intravascular compartment (O'Neill *et al*, 1993). However, it has been shown that human serum albumin preparations commonly used in the clinic are predominantly oxidized at the free cysteine, which was resistant to reduction (Lang *et al*, 2004).

Postresuscitation CBF was divergently affected by administration of PNA and albumin versus NS in our model. The blood–brain barrier is intact to gadoteridol (a molecule smaller than albumin) in the first 3 hours after resuscitation in our model (Manole *et al*, 2009), and consequently, we expect that PNA and albumin exert their effects in the intravascular space. Similar to our findings with PNA, SOD administration reduced early postresuscitation hyperemia in an asphyxial CA model in dogs (Cerchiari *et al*, 1987), and administration of SOD-albumin complex improved blood flow and neurologic outcome in a global ischemia–reperfusion model (Takeda *et al*, 1993). Similar to our study, albumin was shown to improve cortical perfusion after permanent focal cerebral ischemia (Liu *et al*, 2001) and improved perfusion in the microcirculation during the postischemic reperfusion period (Belayev *et al*, 2002). One possible explanation for our findings is mitigation of superoxide-induced vascular dysfunction by PNA versus albumin. Superoxide causes microvascular abnormalities such as loss of autoregulation after ischemia, and treatment with scavengers of superoxide can prevent the loss of autoregulation (Nelson *et al*, 1992). Intravascular antioxidants such as PNA may mitigate the effect of superoxide, attenuate postischemic vascular injury, and prevent the loss of blood pressure autoregulation of CBF after CA. By preventing the loss of autoregulation, PNA may prevent hyperemia early after ROSC, whereas albumin, by increasing MAP and/or cardiac output in the absence of such vascular protection, further promotes cerebral hyperemia. MAP increases early postresuscitation in our rats, but would be expected to remain in the autoregulatory range (Pryds *et al*, 2005). This possibility is supported by data showing that PNA, but not albumin, inhibits the superoxide-induced adherence of polymorphonuclear cells to the endothelium (Russell *et al*, 1998). Another mechanism by which albumin produces hyperemia may be attributed to formation of S-nitroso-albumin. It was shown that albumin binds nitric oxide at the free cysteine and forms S-nitroso-albumin, a compound with endothe-

lium-derived relaxing factor-like properties, vasodilatory effects on coronary vessels, and a half-life substantially longer than nitric oxide (Ignarro, 1989; Keaney *et al*, 1993). Additional mechanistic studies would be required to test these hypotheses.

We serially quantified CBF using baseline measurement for  $\alpha$  and T1obs because repeated measurement of these parameters would preclude serial measurement of perfusion maps. We recognize that injection of nitroxides can reduce blood and tissue T<sub>1</sub> (Hyodo *et al*, 2008). To validate our approach, we serially measured these parameters after injection of PNA or albumin in separate rats. We noted a small decrease in T1obs with both agents. However, both PNA and albumin are large molecules that are restricted to the blood pool, and we attribute this effect to a change in the blood pool T<sub>1</sub>. We believe that the baseline measurement provides a better representation of the tissue T<sub>1</sub> during the study. The PNA used in the study has a free tempol concentration of ~1 mmol/L. A global effect of T<sub>1</sub> is seen in rapid T<sub>1</sub>-weighted images; however, the free nitroxide is reduced quickly and does not affect serial CBF. This was confirmed by repeating experiments with tempol-free PNA in several rats. After albumin administration,  $\alpha$  decreased slightly. This can be attributed to an additional magnetization transfer effect in the arterial blood as a result of the larger concentration of macromolecules. We did not correct for this magnetization transfer effect, but the result leads to an underestimate of CBF by ~8% for the albumin-treated group. No difference in  $\alpha$  is seen after the administration of PNA. The bound nitroxides efficiently relax the macromolecular nuclear spins blunting the additional magnetization transfer effect in the arterial blood.

Morris water maze performance was improved by both PNA and albumin after CA in male rats. Similarly, PNA or SOD confer neuroprotection after global ischemia, and thus, amelioration of vascular injury by PNA may improve outcome (Cerchiari *et al*, 1987; Kofler *et al*, 2005; Schleien *et al*, 1994). The improved functional outcome after albumin administration in our study was not surprising because albumin is also beneficial in animal models of focal ischemia: decreased infarct area, reduced brain edema, and improved functional outcome (Belayev *et al*, 1997, 1998). Albumin improved neurologic outcome in a pilot study in humans after stroke (Ginsberg *et al*, 2006) and a multicenter, randomized controlled trial of albumin in acute ischemic stroke is underway.

Neither PNA nor albumin attenuated CA<sub>1</sub> hippocampal neuronal death versus NS. Thus, the degree of hippocampal neuronal death cannot be directly linked to the degree of early postresuscitation CBF derangements in our model. Further studies are required before a definitive conclusion can be drawn regarding the influence of blunting or augmenting early hyperemia on neuronal death. We selected the CA<sub>1</sub> hippocampus given its vulnerability in this

model and the capacity to quantify neuronal counts through stereology (Fink *et al*, 2005). Quantification of neuronal death in other anatomic areas after CA is warranted. Thalamic areas in particular have early hyperemia that intensified after treatment with albumin. Systematic characterization of thalamic pathology in different thalamic nuclei, along with quantification of gliosis and axonal injury in our model are important and the object of further study (Shoykhet *et al*, 2011).

Our data suggest that males treated with PNA or albumin have improved spatial memory retention after CA. We found no significant sex-dependent difference in postresuscitation CBF, gross vestibulomotor function, or neuronal loss. Our findings might relate to the evidence of a greater role for oxidative stress in males versus females (Du *et al*, 2004). At the time of testing, rats were 31-days old; thus, in addition to possible effects of innate gender differences at the time of injury, hormonal influences in males may contribute to the gender effects seen on functional outcome. However, because our study was not initially designed for sex-dependent analysis, the power to detect a sex difference was low and prospective evaluation of sex-dependent effect is warranted. Our work does support evaluation of sex-dependent treatment effects, even in prepubertal neuronal injury models.

Administration of PNA or albumin at resuscitation is mimicking the clinical scenarios of in-hospital CA or administration at resuscitation by first responders (emergency medical personnel) in an out-of-hospital CA. Assessment of delayed administration of PNA or albumin on neurologic outcome and the time window of administration of PNA and albumin to optimize cerebrovascular-targeted therapies is also valuable and will be evaluated in future studies.

## Summary

Despite divergent effects on hyperemia early after CA in postnatal day 16 to 18 rats, both PNA and albumin modestly improved neurologic outcome in our model in a sex-dependent manner.

## Disclosure/conflict of interest

Carleton JC Hsia, PhD, is CEO of SynZyme Technologies, Irvine, CA (developer of the nitroxide-based solutions), synthesized, and provided the polynitroxyl albumin (PNA) for these studies.

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