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Oximetry-Guided Normoxic Resuscitation Following Canine Cardiac Arrest Reduces Cerebellar Purkinje Neuronal Damage

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Abstract

Background: Animal studies indicate that maintaining physiologic O₂ levels (normoxia) immediately after restoration of spontaneous circulation (ROSC) from cardiac arrest (CA) results in less hippocampal neuronal death compared to animals ventilated with 100% O₂. This study tested the hypothesis that beneficial effects of avoiding hyperoxia following CA are apparent in the cerebellum and therefore not limited to one brain region.

Methods: Adult beagles were anesthetized and mechanically ventilated. Ventricular fibrillation CA was induced by electrical myocardial stimulation and cessation of ventilation. Ten min later, dogs were ventilated with 100% O₂ and resuscitated using 3 min of open chest CPR followed by defibrillation. Dogs were ventilated for one hr with either 100% O₂ or with O₂ titrated rapidly to maintain hemoglobin O₂ saturation at 94 to 96%. FiO₂ was adjusted in both groups between one and 24 hr post-arrest to maintain normoxic PaO₂ of 80 -120 mm Hg. Following 24 hr critical care, dogs were euthanized and cerebellum analyzed for histochemical measures of neuronal damage and inflammation.

Results and Conclusions: Hyperoxic resuscitation increased the number of injured Purkinje cells by 278% and the number of activated microglial/macrophages by 18% compared to normoxic resuscitation. These results indicate that normoxic resuscitation promotes favorable histopathologic outcomes in the cerebellum (in addition to hippocampus) following CA/ROSC. These findings emphasize the importance of avoiding unnecessary hyperoxia following CA/ROSC.

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Conflicts of Interest: none

Keywords

Cerebellum; cardiac arrest; restoration of spontaneous circulation; normoxia; hyperoxia; Purkinje neuron; canine; reperfusion injury; microglia; macrophage; inflammation; calbindin; Iba-1; pulse oximetry; ventilation

Introduction

Prior to 2010, the American Heart Association advanced cardiac life support guidelines stressed the need to provide high levels of supplemental O₂ for resuscitation and critical care following ventricular fibrillation (VFib) cardiac arrest (CA) in adults. However, two studies demonstrated that gerbils breathing 100% O₂ for 3-6 hr following 15 min global cerebral ischemia exhibited greater brain damage and mortality compared to gerbils not receiving O₂ [1,2]. Using a canine VFib CA model, we found that ventilation on 100% O₂ for one hr following 10 min CA results in greater cerebral cortex lipid oxidation and worse short-term (24 hr) neurologic outcome compared to dogs receiving 21 – 30% O₂ following ROSC [3]. We also found that early hyperoxia following global cerebral ischemia in rats resulted in greater hippocampal neuronal death and worse long-term (30 day) neurologic outcome than rats that were maintained as normoxic [4].

These preclinical studies utilized young, healthy animals requiring little supplemental O₂ to maintain normoxia. Since the typical VFib CA victim is elderly, often with impaired pulmonary gas exchange, we compared post-ROSC results between animals ventilated with 100% O₂ for 1 hr (hyperoxia) to animals reaching physiologic O₂ levels (normoxia) by pulse oximetry. This paradigm could be a much safer approach to oxygen titration than simply ventilating with room air. Using the canine CA model, we found that oximetry-guided normoxic resuscitation significantly reduces short-term neurologic injury and hippocampal neuronal death compared to reperfusion under hyperoxic conditions, i.e., PaO₂ > 300 mm Hg during the first hr of ROSC [5].

The neuropathology performed in these resuscitation studies focused on hippocampal neuronal death; benefits of normoxic reperfusion seen in hippocampus may not be reproduced in other brain regions. Moreover, there could be regions, e.g., cortex and cerebellum that undergo greater post-ischemic hypoperfusion and may therefore profit from exposure to hyperoxia. This study tested the hypothesis that, when compared to hyperoxic reperfusion, pulse-oximetry guided normoxic resuscitation decreases short-term cerebellar neuronal injury and inflammatory microglial activation following VFib CA.

Methods

Canine Model of Cardiac Arrest and Return of Spontaneous Circulation (ROSC):

Female pure-bred beagles 3 to 5 years of age and 7.5-13.0 Kg were obtained from Covance Research Products Inc. All animal protocols were performed as approved by the University of Maryland Baltimore Institutional Animal Care and Use Committee. Dogs were group housed in large runs within the institutions fully accredited animal facility with free access to food and water. The nine CA dogs used in this study were a subset of those used

previously to compare the effects of normoxic and hyperoxic resuscitation on hippocampal neuronal death and neurologic injury following CA [5]. The three Sham dogs used in this study were new and not used in a previous study. There were no baseline differences in age or weight between groups.

Surgical preparation was performed in a dedicated large animal operating suite. On the morning of the experiment, initial sedation then long-term anesthesia was provided by intravenous sodium pentobarbital (12.5 mg/kg) followed by two doses of alpha chloralose (37.5 mg/kg) administered 30 minutes apart. Following endotracheal intubation, mechanical ventilation was set at 14-18 breaths per min and 15-18 mL/kg tidal volume to maintain pO_2 and pCO_2 within normal physiologic ranges of 80 to 120 mm Hg and 25 to 35 mm Hg, respectively. Saline was infused intravenously at a constant rate of 2-4 ml/kg/hr throughout the entire surgical and recovery periods. Electrocardiogram (ECG), hemoglobin O_2 saturation (sO_2) arterial blood pressure, and core temperature were monitored continuously. A femoral artery catheter was placed for the monitoring of blood pressure and collection of arterial blood gas samples. A femoral venous catheter advanced to the level of the inferior vena cava was placed for anesthetic and resuscitative drug delivery. A left lateral thoracotomy was performed in preparation for induction of CA.

Cardiac arrest was induced by applying brief current directly to the epicardium using a Grass electrical stimulator and the cessation of mechanical ventilation for 10 minutes. All animals exhibited Vfib CA within 30 sec after current application. Resuscitation consisted of simultaneous drug delivery (epinephrine 2 μ g/kg plus sodium bicarbonate 1 mEq/kg), resumption of mechanical ventilation, and open chest CPR for 3 min under 100% O_2 , followed by internal defibrillation at 8-10 Joules.

Experimental Groups

Following surgical preparation, dogs that underwent CA were randomized to one of two groups: CA/Oximetry (n=5) or CA/Hyperoxic (n=4). We also included a new Sham/ Normoxic group (n=3), which was not randomized. Following CA and defibrillation, dogs were ventilated under either an oximetry-guided normoxic procedure or a hyperoxic procedure consisting of ventilation on 100% O_2 for one hr. The CA/Oximetry procedure used pulse oximetry to rapidly lower the FiO_2 from 100 to 50% and then further lowering the FiO_2 by 5% every 2 min until the sO_2 reached 96%. This level was reached within 12 min of ROSC. ABGs were then measured and used to guide respiratory parameters. At 1 hr ROSC, the mean pO_2 values for the Sham, CA/Oximetry, and CA/Hyperoxic groups were 107 ± 4 sem, 82 ± 2 and 488 ± 61 mm Hg, respectively [5]. Following this measurement, the pO_2 and pCO_2 were maintained within normal physiologic ranges via ABG-guided ventilatory adjustments. Sham animals underwent the same anesthesia and surgical procedures without CA or resuscitative drug delivery.

Post-resuscitative Care

All dogs received constant critical care for 24 hours after ROSC. Analgesia during this period was maintained by a morphine drip ($0.1 \text{ mg kg}^{-1} \text{ hr}^{-1}$), with additional boluses as needed. Pancuronium (0.1 mg/kg) was used, as needed, to facilitate ventilation and only

after verification that the animal was adequately sedated. Ventilation was adjusted to maintain a PaCO₂ of 30 to 35 and a PaO₂ of 80 to 100 torr. At hr 20 the animals were weaned from controlled ventilation. At the completion of the experimental period (24 hr), the thoracotomy was reopened, the thoracic aorta cross-clamped, and the right atrial appendage clamped and incised. Simultaneous with release of the atrial appendage clamp, transcardial perfusion was initiated with 1% paraformaldehyde (2 minutes at 600 mL/min) followed with 4% paraformaldehyde (20 minutes at 400 mL/min).

Tissue Preparation

Brains were removed, blocked and immersion fixed in 4% paraformaldehyde for 2 weeks and then immersed in 30% sucrose for an additional week or until brains sank to the bottom of the container. 40 µm sections were collected and stored in a 30% ethylene glycol/30% sucrose/30% PBS buffered polyvinylchloride solution until further processing was initiated.

Immunohistochemistry

For single-labeling immunohistochemistry, sections were removed from cryoprotectant and rinsed thoroughly in 1× Tris-buffered saline (TBS) buffer before and after a 10 minute exposure to a 1% sodium borohydride solution. Tissue used for Purkinje cell and microglial identification were placed into their respective primary antibodies diluted in TBS + 0.4% triton-X100 for 1 hr at room temperature, followed by 48 hours at 4°C (Anti-Calbindin, 1:1,000K, Sigma-Aldrich, St. Louis, MO, USA; anti-Iba1, 1:20K, Wako, Osaka, Japan). Sections were rinsed in TBS buffer before and after a 1 hr incubation in biotinylated anti-mouse or anti-rabbit secondary antibody (1:600, Vector Laboratories, Burlingame, CA, USA) and then in Vectastain ABC solution. Sections were then washed 3 times in a 0.175 M sodium acetate solution and exposed to a nickel diaminobenzidine solution (Ni-DAB) for 15 min. Staining was terminated by washing the sections in sodium acetate solution and then in TBS three times each.

Following tissue staining, all sections were mounted onto gelatin-coated glass slides and dried overnight. For Iba1-stained slides only, counterstain was performed using neutral red solution. Slides were dehydrated in ethanol then xylene and cover-slipped with DPX Mountant.

Stereologic Quantification of Neuronal Injury and Microglial/Macrophage Activation

A total of 5-7 sections from a 1 in 16 series throughout the cerebellum were processed separately for Calbindin and Iba1 immunoreactivity. Quantification was performed by a blinded investigator using the optical fractionator method (Stereoinvestigator). The area of interest was defined as the lateral edge of the simplex lobule located lateral to the vermis and primary fissure of the cerebellum. Boundaries of the Purkinje cell layer were outlined under low magnification (4×) then quantified under 10× (Calbindin) or 20× (Iba-1) magnification. Randomly placed counting frames (Calbindin: 55×55 µm, Iba1: 100×100 µm) within the Purkinje cell layer were used to count cells in optical dissectors 7-10 µm in depth [6].

Calbindin-stained Purkinje cells were morphologically classified into PC type I, PC type II, and PC type III neurons based upon a paradigm previously described [7]. Purkinje neurons

(PC type I) were characterized as homogeneously intact morphology, with darkly staining nuclei (Fig. 1A). Injured neurons were characterized as having shrunken or fragmented morphology (PC type II) or as “dark neurons” (PC type III) intensely stained cytoplasm (Fig. 1B, 1C). PC type II and PC type III neurons were grouped together and classified as “injured neurons” while PC type I neurons were classified as “healthy” neurons. Iba-1 positive cells demonstrating relatively small cell bodies with many finely-branched processes were classified as “resting” (Fig. 3A) whereas those with a hypertrophic cell bodies with few or no processes were classified as “activated” (Fig. 3B, 3C) as previously described [8].

Data Analysis

Statistical analyses were performed using SigmaPlot 14.0. PC type II and PC type III neurons were grouped together as “injured neurons” while PC type I neurons were classified as “healthy” neurons. Raw data including both the number of healthy and injured neurons and the number of resting and activated microglia was analyzed with a separate one-way ANOVA. Assumptions for normality (Shapiro-Wilk) and equal variance (Brown-Forsythe) were met with treatment differences identified post-hoc using the Fisher’s Least Significant Difference (LSD) method.

Results

Previous studies have shown that cerebellar Purkinje neurons are relatively vulnerable to death caused by cerebral ischemia/reperfusion associated with cardiac arrest (CA) and resuscitation [9–11]. This study tested the hypothesis that systemic hyperoxia during the first hr following CA results in greater cerebellar Purkinje cell death and inflammatory activation of microglia/macrophages than when systemic normoxia is maintained.

The majority of Purkinje neurons from sham animal brains appeared healthy, with prominent nuclear staining and lighter staining throughout cell bodies and dendrites (Fig. 1D). The appearance of calbindin-stained tissue obtained at 24 hr of ROSC was considerably different than that seen in Sham animals. In the brains from animals that underwent oximetry-guided resuscitation, the frequency of healthy neurons was reduced (Fig. 1E). Moreover, there were immunostained cell morphologies that were rarely observed in Shams, including those classified as injured dark neurons and those which lacked well-defined dendritic arborization.

The appearance of the cerebellum from dogs subjected to 10 min CA/ROSC and hyperoxic ventilation under 100% O₂ for one hr was strikingly different from either the Shams or the dogs that were resuscitated using the pulse-oximetry guided oxygenation. The hyperoxic resuscitation dogs exhibited very few normal neurons. Most of the immunostained structures appeared to represent fragments of cells that had undergone autolysis.

The apparent shift from healthy to injured Purkinje neurons following cardiac arrest was quantified using a stereologic approach. Figure 2 provides the relative numbers of healthy and injured Purkinje cells per mm³ present at 24 hr following Sham surgery and following CA followed by either oximetry-guided or hyperoxic resuscitation. The total number of

Purkinje neurons (healthy + injured) did not differ between groups. Greater than 95% of the Purkinje cells present in Shams were healthy, as expected. In contrast, while greater than 75% of Purkinje neurons present in hyperoxic resuscitated dogs were injured, only 25% from oximetry resuscitated dogs were classified as injured. Thus there were significantly less healthy neurons in hyperoxic animals compared to either oximetry-guided animals or Shams. The number of healthy neurons was not significantly different between Shams and oximetry animals. These injured neurons were much greater in the hyperoxic group compared to the normoxic group or Shams. Nevertheless, there were still significantly more injured neurons in the oximetry group than in Shams.

Neuro-inflammation is present following almost all forms of acute and chronic neurodegeneration, often exacerbating neuronal injury and death. Neuroinflammatory microglial/macrophage activation was monitored by assessing morphologic changes in Iba-1 immunostained glia. The majority of Iba-1 positive microglia from Sham (Fig. 3D) animals appear evenly distributed and exhibit “resting” morphology. Following cardiac arrest and oximetry-guided resuscitation, Iba-1 immunostaining was less evenly distributed and exhibited evidence for hypertrophic activation (Fig. 3E). Following hyperoxic resuscitation (Fig. 3F), the majority of microglia appeared to be moderately or highly activated with very few resting cells present. In addition, immunostaining was much more localized to the immediate area where Purkinje cells devoid of Nissl substance appeared as “ghost” cells.

The apparent shift from a resting to activated microglial phenotypes following cardiac arrest was quantified using a stereologic approach. The total number of Iba-1 positive microglia (resting + activated) present at 24 hours after sham or cardiac arrest did not differ between groups. At least 80% of the Iba-1 immunopositive microglia present in Shams exhibited a “resting” morphology. In contrast, approximately 80% of the cerebellar microglia present in hyperoxic dogs displayed morphology classified as either moderately or highly activated. The number of activated microglia present in either hyperoxic or oximetry resuscitated animals was significantly greater than that of Shams but there was no difference in the number of activated microglia present in hyperoxic compared to normoxic resuscitation. Compared to the number of resting microglia in Shams, the number present following resuscitation was 50% less in the oximetry group and 78% less in the hyperoxic animals. There were also significantly fewer resting microglia in the hyperoxic compared to oximetry resuscitated animals.

Discussion

It has been demonstrated that cerebellar Purkinje cell density is severely reduced compared to sham-injured controls following CA/ROSC in rats [11]. The most important new observation made in this study is that oximetry-guided normoxic resuscitation after CA reduces the number of injured Purkinje neurons present in dog cerebellum by over 70%. This effect size is actually greater than the 50% reduction in hippocampal CA1 pyramidal neuron injury we observed in the same animals following CA and oximetry-guided normoxic resuscitation [5]. The fact that the beneficial effect of normoxic reperfusion is observed in more than one highly vulnerable brain region increases the clinical relevance for avoiding unnecessary hyperoxia following CA. The clinical applicability of maintaining

normoxia after resuscitation is supported by our previous finding that the mean neurologic deficit score (NDS; 0% = normal, 100% = brain dead) for dogs in the normoxic group (NDS=41) was significantly lower than the mean score measured following hyperoxic resuscitation (NDS=61%) [5]. Additionally, a recent prospective trial in humans demonstrated that early hyperoxia exposure after CA/ROSC was independently associated with poor neurological function at discharge [12].

There was also a greater loss of “resting” microglia/macrophages in the cerebellum of hyperoxic resuscitated dogs compared to those observed after normoxic reperfusion. Nevertheless, the microglial activation was similar between the two CA groups. Therefore, while normoxic resuscitation is very effective at reducing cell death, it only exerts a minor anti-inflammatory response. This same conclusion was made in a study comparing normoxic to hyperoxic reperfusion in a rat global cerebral ischemia model [4]. At 7 days post-ischemia, the number of non-injured hippocampal CA1 neurons following normoxic reperfusion was almost twice as great as those observed after hyperoxic reperfusion. At 7 days, the number of activated microglia/macrophages was significantly lower (25%) in the hippocampus CA1 region of normoxic rats compared to the hyperoxic group. Nevertheless, the number of activated microglia in the normoxic animals was more than ten-fold greater than those in Shams. In the canine CA study, there was a six-fold increase in activated microglia in the normoxic animals at 24 hr post-arrest. Therefore, in both the canine CA model and the rat global cerebral ischemia model, normoxic reperfusion is much more effective at reducing neuronal death than microglial/macrophage activation. Considering the fact that the short-term neurological deficit score for the normoxic-resuscitated dogs of 43% is far from optimal, additional interventions beyond normoxic reperfusion are needed. Based on the high level of inflammation observed with both animal models following either normoxic or hyperoxic reperfusion, development of therapeutically effective anti-inflammatory strategies is of high priority and in progress.

The current study is limited, however, by the use of only female dogs of “middle age” compared to the elderly age of both men and women who represent the typical CA population. Another limitation is that the histopathology was only performed at a short-term outcome time of 24 hr, which does not necessarily represent outcomes at later times.

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Fig. 1. Purkinje cell injury in the cerebellum at 24 hr following cardiac arrest. Calbindin immunostained Purkinje neurons were classified as healthy (A), or injured (B or C) morphotypes. Purkinje neurons of Sham animals appeared healthy with an intact nucleus and soma (D). Following CA and oximetry resuscitation, a combination of healthy and injured neurons were present (E). The cerebellum obtained from the brains of hyperoxic-resuscitated dogs exhibited primarily injured Purkinje neurons.

Fig. 2. Stereologic quantification of healthy and injured cerebellar Purkinje neurons 24 hr following cardiac arrest.

Fewer neurons are injured using oximetry (5386 ± 1343) as compared to hyperoxic (17360 ± 1779) resuscitation.. Values represent the means $\pm$ sem for $n=3-5$ per group. * $p<0.05$; *** $p<0.001$

Fig.3. Microglial/macrophage activation in the cerebellum at 24 hours following cardiac arrest. Iba-1 immunostained microglia were classified as resting (A) or activated (B, C) based on morphologic criteria. The majority of microglia of sham (D) animals are resting whereas following CA, activated microglia far outnumber resting phenotypes (E, F).

Fig. 4. Stereologic quantification of resting and activated cerebellar microglia following cardiac arrest.

Increase in activation following CA is not different between oximetry (14302 ± 1038) and hyperoxic groups (16625 ± 1882). Values represent the means $\pm$ sem for $n = 3-5$ per group.

* $p < 0.05$; *** $p < 0.001$