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The state of systemic circulation, collapsed or preserved defines the need for hyperoxic or normoxic resuscitation in neonatal mice with hypoxia-ischemia

Dzmitry Matsiukevich[#], Tara M. Randis[#], Irina Utkina-Sosunova, Richard A. Polin, and Vadim S. Ten

Department of Pediatrics, Division of Neonatology, 3959 Broadway, BHS1-115, Columbia University Medical Center, New York, NY, 10032 USA

Abstract

Background—The return of spontaneous circulation (ROSC) is a primary goal of resuscitation. For neonatal resuscitation the International Liaison Committee on Resuscitation (ILCOR) recommends oxygen concentrations ranging from 21% to 100%.

Aims and methods—This study (a) compared the efficacy of resuscitation with room air (RA) or 100% O₂ in achieving ROSC in 46 neonatal mice with circulatory collapse induced by lethal hypoxia-ischemia (HI) and (b) determined whether re-oxygenation with RA or 100% O₂ alters the extent of HI cerebral injury in mice with preserved systemic circulation (n = 31). We also compared changes in generation of reactive oxygen species (ROS) in cerebral mitochondria in response to re-oxygenation with RA or 100% O₂.

Result—In HI-mice with collapsed circulation re-oxygenation with 100% O₂ versus RA resulted in significantly greater rate of ROSC. In HI-mice with preserved systemic circulation and regional (unilateral) cerebral ischemia the restoration of cerebral blood flow was significantly faster upon re-oxygenation with 100% O₂, than RA. However, no difference in the extent of brain injury was detected. Regardless of the mode of re-oxygenation, reperfusion in these mice was associated with markedly accelerated ROS production in brain mitochondria.

Conclusion—In murine HI associated with circulatory collapse the resuscitation limited to re-oxygenation with 100% O₂ is superior to the use of RA in achievement of the ROSC. However, in HI-mice with preserved systemic circulation hyperoxic re-oxygenation has no benefit over the normoxic brain recovery.

Introduction

Infants with perinatal depression often require cardio-pulmonary resuscitation (CPR). Resuscitation of asphyxiated infants has traditionally been initiated only with 100% oxygen. Although, an ischemic tissue dies without oxygen, an excessive O₂ delivery during

*Corresponding Author: Vadim S. Ten M.D., Ph.D., Division of Neonatology, Department of Pediatrics, Columbia University, NY, USA. 3959 Broadway CHN 1201, New York, NY 10032. Phone: 212-305-5827, vt82@columbia.edu, Fax: 212-305-7086.

[#]Drs. Tara Randis and Dzmitry Matsiukevich contributed equally to this work.

Conflict of Interest.

Authors have no conflict of interest to disclose.

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resuscitation can exacerbate an oxidative stress initiated by reactive oxygen species (ROS). Recently the International Liaison Committee on Resuscitation (ILCOR) revised their guidelines recommending initiation of neonatal resuscitation with concentrations of O₂ between 21% and 100% [1]. This wide range of O₂ concentrations recommended for resuscitation reflects (a) the lack of evidence supporting either 21% or 100% O₂ and (b) our limitations in understanding the mechanisms responsible for cellular salvage and oxidative damage in the ischemic tissue during resuscitation-induced reperfusion. The main goal of resuscitation is the return of spontaneous circulation (ROSC) evidenced by a fleeting pulse, normalization of the heart rate and respiratory efforts. Most of the studies comparing re-oxygenation strategies used models of HI brain injury in which cerebral circulation was artificially compromised (occluded carotid arteries) however, the systemic circulation was preserved [2,3,4]. The end-point of resuscitation in these studies was shifted from the ROSC toward neurological outcome. This may explain inconsistency in experimental data reported to date. For example, in asphyxiated piglets re-oxygenation with 100% O₂ versus room air (RA) resulted in a significantly faster restoration of the cerebral blood flow (CBF) and mean arterial blood pressure. This was associated with an attenuated surge of excitotoxic amino acids in the brain during reperfusion [5]. The same group reported that resuscitation with 100% O₂ versus RA exacerbates cerebral injury in similarly asphyxiated piglets [6]. It also has been shown that following repetitive brief asphyctic episodes re-oxygenation with 100% O₂, compared to RA was associated with a better neuronal survival in piglets [7].

This study tested the hypothesis that the resuscitation limited to re-oxygenation with 100% O₂ versus RA is superior in achieving ROSC in neonatal mice exposed to lethal HI insult associated with systemic circulatory collapse. However, in HI-animals with a preserved systemic circulation, hyperoxic re-oxygenation offers no benefit for postischemic brain recovery.

Materials and Methods

Induction of HI and resuscitation

Unilateral cerebral HI was produced in nine-ten day old mice (C57/Bl/6J) by permanent ligation of the right carotid artery followed by exposure to 8% O₂/92% N₂ at 37 °C [4]. Changes in the cerebral and peripheral blood flows (CBF and PBF) were recorded prior to, during HI and reperfusion. Two study groups were formed. In one group the model was modified to mimic a lethal HI insult associated with collapsed systemic circulation.

Study groups—In the first group, the HI was produced until CBF and PBF had collapsed (Fig. 1A). At five seconds following a collapse of cerebral and peripheral circulation, mice were resuscitated by the re-oxygenation achieved by flushing hypoxic chamber (6 liters) either with 100% O₂ (n = 23) or RA (n = 23) at a gas-flow rate 8 liters/minute. In the mice re-oxygenated with 100% O₂ the gas was switched to RA once CBF returned to the pre-hypoxic level (Fig. 1A). The hypoxic-exposure time to circulatory collapse, the rate of ROSC and immediate (30 minutes) survival rates were recorded and compared between groups.

In the second group of mice the HI was limited to 20 minutes followed by the re-oxygenation with either 100% (n = 21) oxygen or RA (n = 22) as described above (Fig. 1B). Once CBF in the hemisphere ipsilateral to the carotid artery ligation side returned to the pre-hypoxic level, 100% O₂ was switched to RA. At five minutes of reperfusion with CBF restored to the pre-hypoxic level, in randomly selected mice (n = 6 in each group) ROS generation in cerebral mitochondria was assessed (Fig. 2B). These data were compared to that in naïve (n = 6) and HI-mice sacrificed at the end of HI, at 0 minutes of reperfusion (n = 6). In the main cohort the extent of cerebral brain injury was compared between RA and O₂ re-oxygenated mice at 24 hours of reperfusion. Mice that died prior to 24 hrs of reperfusion were not included in this

analysis. The protocol was approved by the Institutional Animal Care and Use Committee of Columbia University.

Measurement of cerebral and peripheral blood flow

In all mice, CBF was measured using laser Doppler flowmetry (PeriFlux 5000, Perimed) [8]. Briefly, prior to the hypoxic exposure, under isoflurane anesthesia, Doppler probes were placed on the skull (2 mm posterior and 3 mm lateral to the bregma) using 15 cm fiberoptic extensions. To measure changes in the peripheral blood flow a Doppler probe was placed on the rear paw in the projection of the medial marginal artery. Changes in CBF and PBF were continuously recorded starting from 2 minutes prior to, during, and 10 minutes after hypoxic exposure. CBF and PBF values were expressed as % of the pre-hypoxic level. Changes in CBF and PBF in response to carotid artery ligation and 90 minutes of recovery from anesthesia (Fig. 1C) were recorded in separate cohort of mice (n = 4). CBF and PBF values at the end of recording in these mice were used to approximate pre-hypoxic level of CBF in the ipsilateral hemisphere in all experimental animals.

Blood Gas Analysis was done to verify that HI limited to regional brain ischemia causes metabolic changes consistent with perinatal depression requiring resuscitation. In a separate cohort of mice, blood gases and lactate levels were measured immediately following HI using i-STAT blood gas analyzer (Abbott, USA). Mixed arterial and venous blood samples were obtained from the neck stump in HI animals (n = 5) and naïve littermates (n = 5) immediately upon removal from the hypoxic chamber (before reperfusion could occur). It has been shown, that there are only minimal differences between venous and arterial blood samples in pH, Base Excess and pCO₂ values [9]

Mitochondrial isolation assay

Brain nonsynaptosomal mitochondria were isolated as described [10] with minor modification: the tissue was homogenized manually using a dounce homogenizer (Wheaton Ind., NJ) with 0.2 mm differential (10 strokes) followed by 0.1 mm differential (10 strokes) and the final mitochondrial pellet was re-suspended in 0.07 ml of albumin-free sucrose buffer.

Measurement of mitochondrial H₂O₂ production—The H₂O₂ was estimated by Amplex Ultrared fluorescence assay using Hitachi 7000 spectrofluorimeter (Ex. 555 nm, Em. 581 nm) [11]. Briefly, mitochondria (0.05 mg/ml) were suspended in respiratory buffer [10] containing 5 mM succinate, 10 μM Amplex® Ultrared (Invitrogen, USA) and 4 U/ml of horse radish peroxidase (HRP). A succinate (FAD-linked substrate) was used because (a) it supports the highest ROS generation rate in brain mitochondria [11] and (b) succinate markedly (~300%) accumulates in the cerebral cortex during ischaemia, while other Creb's cycle intermediates are reduced [12]. The changes in fluorescence recorded for 400 seconds following supplementation of mitochondria represents a total rate of ROS emission. To estimate the contribution of C-I to mitochondrial ROS generation samples were supplemented with 1 μM rotenone (inhibitor of C-I) and the rate recorded for 200 sec after rotenone supplementation was subtracted from the total ROS production rate [13]. The calibration curve was obtained by adding five 100 nmol aliquots of H₂O₂ to the same respiration buffer containing Amplex® Ultrared and HRP. The rate of H₂O₂ emission was expressed in pmoles of H₂O₂ per mg of mitochondrial protein per min.

Neuroanatomical assessment of HI brain injury

At 24 hours of reperfusion in HI-mice (group 2) brains were sectioned into six 1 mm thick coronal slices and stained with 2% triphenyl-tetrazolium chloride (TTC). TTC is oxidized only by active mitochondrial dehydrogenases converting TTC into a red colored formazan [14]. Digital images of infarcted (pale-white) and viable (red) areas of brains were traced (Adobe

Photoshop 4.0.1) and analyzed (NIH image 1.62) by an investigator (D.M) “blinded” to a study groups. The extent of brain injury (direct infarct volume) was expressed as a percentage of the hemisphere ipsilateral to the carotid artery ligation side.

Statistical analysis

Data were expressed as mean $\pm$ SEM. To compare mortality and ROSC rates χ^2 analysis was used. A comparison between O₂ and RA re-oxygenated and naïve littermates was done using one-way ANOVA with Fisher’s post-hoc analysis. To analyze changes in CBF during hypoxia and re-oxygenation, ANOVA for repeated measures followed by t-test analysis of differences between groups was used. Data were considered significantly different if $p \leq 0.05$.

Results

The first group all mice subjected to lethal HI exhibited complete collapse of CBF in both hemispheres and PBF at the end of hypoxic exposure (Fig 1A, 2A and B). Resuscitation initiated by re-oxygenation with 100% O₂ resulted in sustained ROSC and immediate survival in ten out of 23 animals (Fig. 2A). In contrast, when resuscitation was initiated with re-oxygenation using RA, only three out of 23 mice demonstrated a sustained ROSC (χ^2 $p = 0.02$). The duration of hypoxic exposure causing circulatory arrest did not differ between O₂ and RA re-oxygenated animals (Fig. 2B).

In the second group in which the HI was limited to 20 minutes, blood flow in the ipsilateral hemisphere declined significantly at the end of an index event. However, the same HI did not cause a collapse of the peripheral circulation (Fig. 2C). At the end of HI, mice exhibited metabolic lactic-acidosis consistent with metabolic changes seen in infants with birth depression (pH 7.28 ± 0.03 , BE -9.6 ± 1.6 , lactate 15.4 ± 0.8 mmol/l, $p < 0.01$ compared to naïves, pH 7.41 ± 0.06 , BE 2.4 ± 0.51 , lactate 2.4 ± 0.51 mmol/l), hypoxemia (pO₂ 27.6 ± 9.4 mmHg versus 58.2 ± 2.7 mmHg in controls, $p < 0.01$), mild hypocapnia (33.6 ± 1.4 mmHg versus 43.1 ± 2.7 mmHg, $p < 0.02$). Re-oxygenation initiated with 100% O₂ resulted in significantly faster (2.31 ± 0.23 versus 5.67 ± 1.62 minutes, $p = 0.01$) restoration of CBF in the ipsilateral hemisphere compared to that in mice re-oxygenated in RA (Fig. 2C). A brisk rise of CBF in the ipsilateral hemisphere in response to hyperoxic re-oxygenation was blunted following a switch from 100% O₂ to RA (Fig. 2C). However, in spite of significantly faster restoration of CBF in the ischemic hemisphere, the extent of HI brain injury in mice re-oxygenated with 100% O₂ was similar to that in RA re-oxygenated counterparts (Fig. 2D).

Brain mitochondria at 0 minutes of reperfusion exhibited significantly decreased net ROS emission rate compared to naïve counterparts. Similarly, the rate of ROS release from C-I was markedly decreased at 0 minutes of reperfusion compared to naïve mice. However, at five minutes of reperfusion, regardless of the mode of re-oxygenation ROS emission rates (total and C-I dependent) were significantly accelerated compared to naïve mice and that assessed prior to reperfusion (Fig. 3A, B and D). Following rotenone supplementation mitochondria isolated from HI-mice at 0 minutes of reperfusion and naïve mice exhibited similar rates of ROS emission (Fig. 3C).

Discussion

In the neonatal mouse model of unilateral HI a regional ischemic brain injury (hemisphere ipsilateral to the ligation side) serves as a primary research focus. A simple modification of this model by extending the duration of hypoxic exposure until a collapse of systemic circulation separates two clinically relevant outcomes of perinatal HI and resuscitation: (1) the effect of hyperoxic or normoxic re-oxygenation on the rate of ROSC in animals with arrested circulation and (2) the effect of 100% O₂ or RA on recovery of regional cerebral blood flow

and the extent of ischemic brain injury in mice with preserved systemic circulation. When mice were subjected to HI insult associated with circulatory arrest, the resuscitation initiated by hyperoxic re-oxygenation resulted in a significantly greater rate of ROSC and immediate survival than resuscitation limited to the re-oxygenation with RA. In contrast, a report by Campbell et al demonstrated that in asphyxiated newborn rabbits resuscitation by positive pressure ventilation (PPV) supplemented with 100% O₂ or RA resulted in similar rates of immediate survival. The same authors reported that resuscitation limited to re-oxygenation resulted in significantly greater survival rate if asphyxiated rabbits were exposed to a hyperoxic hyperbaric (400% O₂) versus normobaric (100% O₂) environment [15]. In our experiments resuscitation was limited to the re-oxygenation and the ventilation was dependent upon spontaneous residual (gasping) respiratory efforts. It is very likely that introduction of PPV in our experimental design would have eliminated the difference in the survival rate between 100% O₂ and RA re-oxygenated animals. Of note, however, after prolonged (25 minutes) cardiopulmonary arrest in mature pigs, CPR with the use of mechanical ventricular assisting device and PPV significantly improved the rate of sustained ROSC, myocardial wall compliance and cardiac output only if resuscitation was supplemented with hyperbaric (400% O₂) re-oxygenation [16]. In contrast, following a brief (one or six minutes) cardiac arrest CPR with RA or 100% O₂ resulted in similar rates of ROSC in immature and mature animals [17, 18]. This suggests that the length of circulatory arrest determines whether the use of PPV supplemented with higher O₂ concentration enhances the rate of ROSC. In our study the time of circulatory arrest was intentionally shortened to five seconds in order to avoid a complete cessation of respiratory efforts (secondary apnea) and assess the effect of a single variable, the inspiratory oxygen concentration.

In mice with a preserved systemic circulation when HI insult was limited to unilateral cerebral ischaemia, re-oxygenation with 100% O₂ versus RA resulted in accelerated recovery of cerebral blood flow in the “ligated” hemisphere. Although, this accelerating effect of hyperoxic re-oxygenation on CBF restoration has been suggested to be beneficial for post-ischemic brain recovery [5], in our study it was not associated with either attenuation or exacerbation of brain injury. In contrast to our finding, hyperoxic re-oxygenation for 30 minutes and longer has been associated with markedly exacerbated brain injury in a similar mouse model of unilateral HI [19]. It is critical to note, that in this and many other reports [19,20,21] the duration (30–60 minutes) of hyperoxic re-oxygenation was chosen without defining a therapeutic goal targeted by hyperoxia, which resulted in supraphysiological hyperoxemia extended into post-resuscitation stage of disease. In our experiments the use 100% O₂ was limited to the recovery of cerebral perfusion induced by re-oxygenation minimizing oxygen toxicity. This may explain the similar extent of brain injury found in hyperoxia and normoxia re-oxygenated mice.

Concern regarding the use of 100% oxygen for resuscitation in asphyxiated neonates arises from the potential exacerbation of oxidative injury. A rapidly expanding body of evidence demonstrates that post-ischemic mitochondria serve as the major source of oxygen radicals causing oxidative tissue damage during reperfusion [reviewed in 22,23]. We found that mitochondria (respiring on succinate) isolated from the ischemic hemisphere (a) prior to reperfusion exhibited markedly reduced rate of ROS emission compared to intact organelles, (b) at five minutes of reperfusion dramatically increased ROS generation, exceeding that in naïve mice. The inhibition of ROS generation observed prior to reperfusion could be explained by the depressed activity of respiratory chain, in particular, C-I. Indeed, the C-I dependent rate of ROS generation was markedly decreased compared to naïve mice indicating an inhibition of the C-I during HI. However, once reperfusion was established the contribution of C-I to ROS release from the respiratory chain increased dramatically compared to that prior to reperfusion. This suggests that at five minutes of reperfusion the activity of C-I is restored contributing to an excessive generation of ROS. Importantly, in RA and 100% O₂ re-oxygenated mice mitochondrial ROS emission rates (total, and C-I dependent) were

accelerated to a similar degree. This suggests that hyperoxic re-oxygenation limited to a recovery of perfusion in the ischemic brain does not contribute to an excessive generation of mitochondrial ROS known to cause an oxidative stress during reperfusion. Notably, this was associated with comparable extent of brain injury in both groups.

Conclusion

Our data suggest that when HI-insult is associated with preserved systemic circulation, initiation of re-oxygenation with 100% O₂ versus RA offers no benefit for brain recovery. However, if HI insult causes a collapse of systemic circulation the re-oxygenation initiated with 100% O₂ is superior to the RA in achieving a sustained ROSC. These results provide an important translational message: the assessment of clinical value of re-oxygenation strategy during CPR should be restricted to a single end-point, the ROSC.

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Fig. 1.

A, representative tracing of changes in the ipsilateral (I) and contralateral (C) hemispheres and peripheral (P) blood flows in response to lethal HI and re-oxygenation with 100% O₂.

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B, CBF in the ipsilateral hemisphere during HI and re-oxygenation with RA or 100% O₂ with time-points (arrows) of assessment of mitochondrial ROS emission rates. **C**, CBF (C contra and I ipsilateral hemispheres) and PBF (P) tracing during ligation of the right carotid artery and 90 minutes of recovery.

Fig. 2.

A, PBF tracing during lethal HI and resuscitation in mice resuscitated with RA (closed dots, n = 21) and 100% O₂ (open dots, n = 22). B, duration of hypoxia which induced circulatory collapse in mice re-oxygenated with RA (black bar, n = 23) or 100% O₂ (striped bar, n = 23). C, Changes in CBF and PBF during HI limited to 20 minutes and re-oxygenation with RA (closed dots, n = 16) or 100% O₂ (open dots, n = 15). Squared are values significantly different compared to pre-hypoxic level (p < 0.01). * p < 0.05 compared to that recorded at the end of HI. D, TTC stained cerebral sections and infarct volume in HI-mice (group 2) re-oxygenated with RA (black bar, n = 16) or 100% O₂ (striped bar, n = 15).

Fig. 3.

A, tracing of H_2O_2 fluorescence in mitochondria (Mito) isolated from the ipsilateral hemisphere in naïve, and HI-mice at 0 and 5 minutes of reperfusion. Arrows indicate rotenone (Rot) supplementation. **B**, **C** and **D**, H_2O_2 emission rates [**B** is total rate, **C** is rotenone-inhibited rate and **D** is C-I dependent rate (see methods)] from mitochondria isolated from naïve (open bar, $n = 6$), HI-mice at the 0 minutes of reperfusion (dotted bar, $n = 6$), re-oxygenated with 100% O_2 (black bar, $n = 6$) and RA (striped bar, $n = 6$). * $p \leq 0.03$ compared to naïves, # $p < 0.0001$ (in Fig. 3C $p \leq 0.02$) compared to that prior to reperfusion. All data are mean $\pm$ SEM.