Hydrogen Peroxide Accumulation in the Choroid During Intermittent Hypoxia Increases Risk of Severe Oxygen-Induced Retinopathy in Neonatal Rats

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PURPOSE. Extremely low gestational age neonates (ELGANs) requiring oxygen therapy often experience frequent episodes of intermittent hypoxia (IH) and are at high risk for severe retinopathy of prematurity (ROP). Using an established model for oxygen-induced retinopathy (OIR), we examined the hypothesis that there is a critical number of daily brief IH episodes which will result in irreversible retinal oxidative damage.

METHODS. Newborn rats were exposed to increasing daily clustered IH episodes (12% O₂ with 50% O₂) from postnatal day (P) 0 to P7 or P0 to P14, or placed in room air (RA) until P21 following 7- or 14-day IH. RA littermates at P7, P14, and P21 served as controls. A group exposed to constant 50% O₂ (CH) served as a second control. Blood gases, eye opening at P14, retinal, and choroidal oxidative stress and lipid peroxidation (8-isoPGF_{2α}), oxidants (H₂O₂) and antioxidants (catalase and SOD), retinal pathology (adenosine diphosphatase (ADPase)-stained retinal flatmounts), and mitochondria-related genes were assessed.

RESULTS. pO₂ levels were higher with increasing IH episodes and remained elevated during the reoxygenation period. High SO₂ levels were associated with most severe OIR. Levels of all measured biomarkers peaked with six IH episodes and decreased with 8 to 12 episodes. H₂O₂ accumulated in the choroid during the reoxygenation period with irreversible retinal damage.

CONCLUSIONS. Our data suggest that six is the maximum number of IH episodes that the retina can sustain. Accumulation of H₂O₂ in the choroid may result in high levels being delivered to the entire retina, ultimately resulting in irreversible retinal oxidative damage.

Keywords: antioxidants, choroid, hyperoxia, intermittent hypoxia, oxidative stress, oxygen-induced retinopathy, retina

Retinopathy of prematurity (ROP) is a leading cause of blindness in extremely low gestational age neonates (ELGANs) requiring oxygen therapy. Immature retinas of ELGANs have an impaired ability to limit oxygen delivery, which—in combination with immature antioxidant systems,^{1,2} an abundance of polyunsaturated fatty acids, and high metabolic rate—make the retina particularly susceptible to lipid peroxidation and free radical damage.³ The incidence and severity of ROP increase with decreasing birth weight and gestational age, and immature respiratory control. Seventy to ninety percent of all ELGANs experience recurrent, clinically significant apnea during the first weeks of life. These apneic episodes last several seconds and result in arterial O₂ desaturations.⁴ The relationship between fluctuating arterial O₂ saturation and the risk for severe ROP has been demonstrated in humans,^{5–10} and in animal models of oxygen-induced retinopathy.^{11–13} Infants experiencing the greatest PaO₂ fluctuations are at highest risk for developing threshold ROP.^{5–10} We have previously shown that the pattern of O₂ fluctuations may exacerbate the severity of retinal disease in rats.^{12,13} This was recently confirmed in ELGANs.⁸

Intermittent hypoxia followed by reoxygenation can lead to accumulation of reactive oxygen species (ROS) and oxidative stress.^{14,15} The retina has several enzymatic antioxidant defense mechanisms for the elimination of ROS.¹⁶ However, due to their immature and inadequate antioxidant defenses, ELGANs have a limited ability to produce antioxidants in response to O₂.¹⁷ In the retina, the first line of defense against oxidative stress is superoxide dismutase (SOD), which scavenges superoxide anion, converting it to H₂O₂ and O₂.^{18,19} Overexpression of SODs in response to high ROS production exacerbates oxidative damage and accelerates retinal degeneration,²⁰ particularly in conditions of low catalase and/or glutathione peroxidase (GPx), thereby leading to accumulation of H₂O₂.

The rat has similar retinal oxygen supply and consumption to that of primates²¹ and neovascularization occurs similar to ELGANs.²² During the vasoobliteration phase of ROP, the choroid is the only source of retinal oxygenation, and since there are no autoregulatory mechanisms, excessive amounts of oxygen (and ROS) will be supplied to the inner retina when the percentage of inspired O₂ is increased.^{23–25} Although choroidal O₂ delivery appears to participate in the pathogenesis of ROP,

FIGURE 1. Flow chart of experimental design and groups studied. The IH groups were exposed to 2, 4, 6, 8, 10, or 12 IH cycling episodes from: P0 to P7; P0 to P14; P0 to P7, followed by reoxygenation in RA for 14 days from P7 to P21; or P0 to P14, followed by reoxygenation in RA for 7 days from P14 to P21. The 50% O₂ groups were exposed for: 7 days; 14 days; 7 days with 14 days of reoxygenation in RA; or 14 days with 7 days of reoxygenation in RA. These served as O₂ controls. The RA controls were raised in RA from birth to P7, P14, or P21, with all conditions identical except for atmospheric oxygen.

there is a paucity of studies examining and comparing the effects of IH on the two vascular beds. Therefore, we embarked on a series of studies to understand the underlying mechanisms associated with oxidative stress resulting from exposure to hyperoxia and intermittent hypoxia (IH) in the immature retina and choroid. It is well-documented that ELGAns who experience frequent arterial O₂ desaturations are at high risk for severe ROP. However, the number of daily IH episodes that the retina can sustain is not known. We hypothesized that there is a critical number of daily brief IH episodes that will result irreparable retinal oxidative damage. To test our hypothesis, we determined the effects of increasing IH episodes on O₂ saturation; eye opening (as a measure of retinal neural maturation); retinal and choroidal biomarkers for oxidative stress; and retinal and choroidal mitochondria-related genes responsible for oxidative phosphorylation (OXPHOS) for 7 and 14 days.

MATERIALS AND METHODS

All experiments were approved by the State University of New York, Downstate Medical Center Institutional Animal Care and Use Committee (Brooklyn, NY). Animals were managed according to the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. Animals were treated humanely, according to the guidelines outlined by the United States Department of Agriculture and the Guide for the Care and Use of Laboratory Animals.

Experimental Design

Certified infection-free, timed-pregnant Sprague-Dawley rats were purchased from Charles River Laboratories, Inc. (Wilmington, MA) at 17 days gestation. The animals were housed in an animal facility with a 12-hour-day/12-hour-night cycle and provided a standard laboratory diet and water ad libitum until delivery. Within 2 to 3 hours of birth, newborn rat pups delivering on the same day were pooled and randomly assigned to expanded litters of 18 pups/litter (nine males, nine females). Sex was determined by the anogenital distance. The expanded litter size was used to simulate relative postnatal malnutrition of ELGAns who are at increased risk for ROP. Each pup was weighed and measured for linear growth (crown to rump length in centimeters). A total of 31 groups of 18 rat pups (nine males, nine females) were studied according to the experimental design (Fig. 1). The groups are described as follows: groups 1 through 6 were exposed to 2, 4, 6, 8, 10, or 12 IH cycling episodes from postnatal day (P)0 to P7; groups 7 through 12 were exposed to 2, 4, 6, 8, 10, or 12 IH cycling episodes from P0 to P14; groups 13 through 18 were exposed to 2, 4, 6, 8, 10, or 12 IH cycling episodes from P0 to P7, followed by reoxygenation in room air (RA) for 14 days from P7 to P21; groups 18 through 24 were exposed to 2, 4, 6, 8, 10, or 12 IH cycling episodes from P0 to P14, followed by reoxygenation in RA for 7 days from P14 to P21; groups 25 through 28 were exposed to 50% O₂ only for 7 days, 14 days, 7 days with 14 days of reoxygenation in RA, or 14 days with 7 days of reoxygenation in RA (these served as O₂ controls); and groups 29 through 31

TABLE 1. Blood Gas Parameters

	RA, 21%	50% O ₂	2 IH Cycles/d	4 IH Cycles/d	6 IH Cycles/d	8 IH Cycles/d	10 IH Cycles/d	12 IH Cycles/d
7 d O ₂								
pH	7.5 ± 0.02	7.6 ± 0.01*	7.7 ± 0.03**	7.6 ± 0.02*	7.7 ± 0.04**	7.6 ± 0.01*	7.5 ± 0.03	7.7 ± 0.03**
pCO ₂	40.1 ± 1.7	41.8 ± 1.0	36.5 ± 2.2	35.9 ± 3.4	32.5 ± 2.4	38.9 ± 1.3	43.3 ± 2.8	31.9 ± 1.8
pO ₂	91.7 ± 6.4	146.6 ± 6.6*	141.2 ± 9.7	147.7 ± 3.2*	148.1 ± 6.4**	134.8 ± 4.8	149.4 ± 3.7*	131.1 ± 7.1
SO ₂	97.3 ± 0.5	99.8 ± 0.5	99.6 ± 0.1	93.3 ± 2.8**	99.7 ± 0.2*	99.4 ± 0.2	99.6 ± 0.2	99.4 ± 0.2
P21–7 d O ₂								
pH	7.4 ± 0.01	7.4 ± 0.009	7.5 ± 0.01	7.7 ± 0.01**	7.4 ± 0.02	7.4 ± 0.02	7.4 ± 0.007	7.4 ± 0.02
pCO ₂	50.2 ± 1.0	51.1 ± 0.6	41.7 ± 1.0	38.6 ± 1.1	42.3 ± 1.9	45.7 ± 1.8	48.2 ± 0.9	48.0 ± 1.8
pO ₂	109.5 ± 13.3	144.4 ± 10.3**	147.0 ± 10.0**	170.5 ± 7.6**	168.5 ± 0.7**	174.4 ± 4.0**	102.5 ± 13.0	189.3 ± 11.23**
SO ₂	96.4 ± 1.2	98.8 ± 0.3	94.2 ± 1.0	93.3 ± 1.3	99.7 ± 0.2	99.8 ± 0.2	96.8 ± 0.8	99.9 ± 0.1
14 d O ₂								
pH	7.5 ± 0.02	7.5 ± 0.02	7.5 ± 0.006	7.5 ± 0.09	7.5 ± 0.01	7.5 ± 0.02	7.6 ± 0.03	7.5 ± 0.03
pCO ₂	38.3 ± 1.2	46.9 ± 1.7*	42.6 ± 0.8	45.0 ± 0.8	38.9 ± 0.8	39.8 ± 1.7	33.7 ± 2.6	33.9 ± 2.5
pO ₂	132.1 ± 6.6	189.8 ± 7.2**	150.1 ± 3.4	152.3 ± 8.3	140.0 ± 2.8	174.6 ± 6.7**	178.0 ± 6.2**	153.5 ± 0.4
SO ₂	99.0 ± 0.2	99.9 ± 0.1	95.1 ± 2.5**	99.4 ± 0.3	99.0 ± 0.1	99.9 ± 0.1	100.0 ± 0.000	99.5 ± 0.2
P21–14 d O ₂								
pH	7.4 ± 0.01	7.4 ± 0.02	7.5 ± 0.008	7.5 ± 0.02*	7.4 ± 0.007	7.5 ± 0.02**	7.3 ± 0.06	7.4 ± 0.007
pCO ₂	50.2 ± 1.0	42.5 ± 1.8	39.2 ± 1.0	40.4 ± 1.3	47.5 ± 1.4	39.8 ± 1.7	42.3 ± 3.0	44.4 ± 1.1
pO ₂	109.5 ± 13.3	168.6 ± 11.9**	45.1 ± 2.0**	78.6 ± 8.8	167.0 ± 4.6**	174.6 ± 6.7**	178.5 ± 10.7**	187.0 ± 8.0**
SO ₂	96.4 ± 1.2	99.8 ± 0.3*	81.3 ± 3.1**	94.1 ± 1.7	99.3 ± 0.2*	99.9 ± 0.1*	99.3 ± 0.2	99.9 ± 0.1*

Data are mean ± SD.

* $P < 0.05$.** $P < 0.01$ vs. 21%.

were littermates raised in RA from birth to P7, P14, or P21 with all conditions identical except for atmospheric oxygen, and served as RA controls.

IH Cycling

The IH cycles consisted of hyperoxia (50% O₂)/hypoxia (12% O₂) in stepwise increments of brief (1-minute), hypoxia (12%) clusters (3 clusters) during 50% O₂ for a total of 2, 4, 6, 8, 10, or 12 episodes/d. This clustering design has been shown to produce a severe form of OIR in neonatal rats.¹²

Blood Gases

Four males and four females were randomly chosen from each group for blood gas analyses. The rat pups were immediately euthanatized by decapitation and blood samples were collected. Animals exposed to constant hyperoxia or IH were euthanatized while in the O₂ chamber so that there was no room air influence. Mixed arterial-venous blood samples were analyzed for blood gases and oxygen saturation using a portable blood gas analyzer (VetScan i-STAT; Abaxis North America, Union City, CA).

Retinal and Choroidal Sample Collection

Both eyes from nine male and nine female pups in each group were enucleated and rinsed in ice-cold phosphate buffered saline (pH 7.4) on ice. The retinas were then excised and processed as previously described.^{12,13} The choroids were harvested by scraping off the sclera. To obtain enough tissue, retinas and choroids were pooled and a total of six samples (three males, three females) per group were analyzed. Eyes from the remaining 12 rats were used for adenosine diphosphatase (ADPase) staining; mitochondrial energy metabolism PCR arrays; and hematoxylin and eosin staining (data not shown).

ADPase Staining

ADPase staining of the retinas and computer imaging were carried out as previously described using an Olympus BX53 microscope and CellSens digital imaging software, version 1.8 (Olympus America, Center Valley, PA).^{12,13}

Oxidative Stress and Lipid Peroxidation

8-isoprostane, or 8-isoPGF_{2α}, is commonly studied and is abundantly generated in vivo during oxidative stress and lipid peroxidation. To establish ocular oxidative stress, levels of 8-isoPGF_{2α} were determined in the retinal and choroidal homogenates using commercially available enzyme immunoassay kits from Assay Designs, Inc. (Ann Arbor, MI), according to the manufacturer's protocol. Levels in the homogenates were standardized using total cellular protein levels according to the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA).

Antioxidant Activities

SOD, catalase (CAT) activities were determined in the homogenates using commercially available assay kits from Cayman Chemical Company (Ann Arbor, MI) according to the manufacturer's protocol. SOD absorbance was determined at 450 nm and catalase absorbance was read at 540 nm. Levels were standardized using total cellular protein levels.

H₂O₂ Assay

H₂O₂ levels in the retinal and choroidal homogenates were determined using H₂O₂ fluorescent kits available from Cayman Chemical Company according to the manufacturer's protocol. H₂O₂ levels were standardized using total cellular protein levels.

TABLE 2. Eye Opening at P14

	50%	2/d	4/d	6/d	8/d	10/d	12/d
7 d O ₂ left	17/18, 94%	0/18, 0%***	0/18, 0%***	0/18, 0%***	0/18, 0%***	0/18, 0%***	0/18, 0%***
7 d O ₂ right	17/18, 94%	0/18, 0%***	0/18, 0%***	0/18, 0%***	0/18, 0%***	0/18, 0%***	0/18, 0%***
P21-7 d O ₂ left	17/18, 94%	2/18, 11%***	18/18, 100%	11/18, 61%**	0/18, 0%***	9/18, 50%***	6/18, 33%***
P21-7 d O ₂ right	16/18, 89%	2/18, 11%***	16/18, 89%	9/18, 50%***	1/18, 6%***	7/18, 39%***	7/18, 39%***
14 d O ₂ left	13/18, 72%**	0/18, 0%***	6/18, 33%***	8/18, 44%***	1/18, 6%***	8/18, 44%***	4/18, 22%***
14 d O ₂ right	13/18, 72%**	8/18, 44%***	9/18, 50%***	12/18, 67%**	2/18, 11%***	10/18, 56%***	5/18, 28%***
P21-14 d O ₂ left	12/18, 67%**	6/18, 33%***	6/18, 33%***	6/18, 33%***	4/18, 22%***	2/18, 11%***	12/18, 67%**
P21-14 d O ₂ right	13/18, 72%**	7/18, 39%***	5/18, 28%***	8/18, 44%***	1/18, 6%***	1/18, 6%***	12/18, 67%**

All left and right eyes in the RA groups were 100% open.

** $P < 0.01$.

*** $P < 0.001$.

Total Cellular Protein Assay

On the day of the assay, retinal and choroidal homogenates were assayed for total protein levels using a dye-binding protein assay (Bio-Rad Laboratories, Inc.) with bovine serum albumin as a standard.

Mitochondria-Related Genes

Total RNA from the retina and choroid was extracted as previously described.^{13,26} Real-time PCR arrays were carried out in duplicate using the rat mitochondrial energy metabolism

PCR array system (SABiosciences, Frederick, MD) with a real-time instrument (BioRad IQ5; BioRad Laboratories, Inc.) according to the manufacturer's protocol.

Statistical Analysis

One-way and two-way ANOVA were used to determine differences among the groups for normally distributed data, and Kruskal-Wallis test was used for non-normally distributed data following Bartlett's test for equality of variances. Post hoc analysis was performed using the Tukey, Bonferroni, and Student-Newman-Keuls tests for significance. Significance was

FIGURE 2. Effects of incremental IH episodes on retinal (A, C) and choroidal (B, D) 8-isopGF_{2α} in neonatal rats at P7 or P14 (open bar) and P21 (solid bar). P7 animals were exposed to brief clustered IH episodes with 50% O₂ or constant 50% O₂ from P0 to P7. P14 animals were exposed to brief clustered IH episodes with 50% O₂ or constant 50% O₂ from P0 to P14. P21 animals were exposed to constant 50% O₂ or brief clustered IH episodes with 50% O₂ from P0 to P7 or P0 to P14 then placed in RA from P7 to P21 or P14 to P21 for reoxygenation. Data are presented as mean $\pm$ SEM ($n = 6$ samples/groups).

FIGURE 3. Effects of incremental IH episodes on retinal (A, C) and choroidal (B, D) SOD in neonatal rats at P7 or P14 (open bar) and P21 (solid bar). P7 animals were exposed to brief clustered IH episodes with 50% O₂ or constant 50% O₂ from P0 to P7. P14 animals were exposed to brief clustered IH episodes with 50% O₂ or constant 50% O₂ from P0 to P14. P21 animals were exposed to constant 50% O₂ or brief clustered IH episodes with 50% O₂ from P0 to P7 or P0 to P14, then placed in RA from P7 to P21 or P14 to P21 for reoxygenation. Data are presented as mean $\pm$ SEM (n = 6 samples/groups).

set at $P < 0.05$ and data are reported as mean $\pm$ SEM. All analyses were two-tailed and performed using statistical software (SPSS version 20.0; SPSS, Inc., Chicago, IL).

RESULTS

Blood Gases

Mixed arterial-venous blood gases are presented in Table 1. Seven-day exposure resulted in higher pH and PO₂ levels in all groups compared with RA. There were no changes in PCO₂. O₂ saturation was also higher in all groups except the 4/d group. During reoxygenation, pH levels remained higher only in the 4/d group and PO₂ also remained higher in all groups, except the 10/d group. During 14-day exposure, PO₂ levels were higher in the constant 50% O₂ (CH) group, as well as the 8/d and 10/d groups, while SO₂ levels were lower in the 2/d group. During reoxygenation, pH levels were higher in the 4/d and 8/d groups, PO₂ levels were higher in all groups except 4/d, and SO₂ levels were higher in the 8/d group despite exposure to RA for 7 days.

Eye Opening at P14

The cecal period (conception to eye opening)^{27–31} was recorded to determine the effects of IH on retinal neural maturation (Table 2). We examined both eyes to determine

whether one or both eyes are affected. We found that exposure of CH for the first 7 days of life did not significantly influence the cecal period, and this was similar in those rats that underwent 14 days of reoxygenation. However, exposure for a longer period up to the second week of life caused prolongation of the cecal period in about 28% of the rats, and this persisted in the 7-day reoxygenation groups. Conversely, 7-day exposure to IH from 2 to 12 events/d prolonged the cecal period in 100% of the animals, but this was reduced in those animals that underwent 14 days of reoxygenation. In that group, there was a peak at 4 days followed by a decline with higher IH episodes. Animals exposed to IH for 2 weeks showed a sustained reduction in the number of animals that experienced eye opening at P14 through P17, and this was also sustained during the reoxygenation period.

Lipid Peroxidation and Oxidative Stress

8-isoPGF_{2 α} levels were measured as a marker for lipid peroxidation and oxidative stress.^{32,33} Despite higher vascular density in the choroid, the levels of 8-isoPGF_{2 α} at P7 were generally higher compared with the retina (Fig. 2). Exposure to CH increased 8-isoPGF_{2 α} in both vascular systems, although there was a greater response in the retina. This effect was ameliorated in the retina (Fig. 2A), but not in the choroid (Fig. 2B). In the retina, elevations in 8-isoPGF_{2 α} occurred in the groups exposed to IH, which progressively increased at 2 and

FIGURE 4. Effects of incremental IH episodes on retinal (A, C) and choroidal (B, D) catalase in neonatal rats at P7 or P14 (open bar) and P21 (solid bar). P7 animals were exposed to brief clustered IH episodes with 50% O₂ or constant 50% O₂ from P0 to P7. P14 animals were exposed to brief clustered IH episodes with 50% O₂ or constant 50% O₂ from P0 to P14. P21 animals were exposed to constant 50% O₂ or brief clustered IH episodes with 50% O₂ from P0 to P7 or P0 to P14 then placed in RA from P7 to P21 or P14 to P21 for reoxygenation. Data are presented as mean $\pm$ SEM (n = 6 samples/groups).

peaked at 4 (during exposure) and 6 (during recovery/reperfusion) cycles/d. However, from 8 to 12 cycles/d, the levels declined to almost undetectable levels. This pattern persisted during the 14-day recovery period. A different response occurred in the choroid. At 2 and 4 cycles, the levels were comparable with control levels. There was a moderate increase at 6 cycles/d followed by a decline at 8 to 12 cycles/d, but not to the same extent as in the retina. Exposure to 50% O₂ for a longer 2-week period had no effect on retinal and choroidal 8-isoPGF_{2 α} levels. However, there was a similar pattern of increases with 4 and 6 cycles/d followed by reductions with 8 to 12 cycles/d in the retina. In the choroid, there was a marked increase with 6 cycles/d during the reoxygenation period.

SOD Activity

SOD catalyzes the conversion of superoxide anion to oxygen and H₂O₂. Therefore, SOD activity was measured in the retina and choroid to determine the ability of these vascular beds to mount an antioxidative response during IH. The levels of SOD in the retina and choroid were similar in the controls (Fig. 3). With CH exposure, the SOD levels increased in the retina, but decreased in the choroid. Retinal SOD levels increased in response to 2 cycles/d, but decreased with 4 to 12 cycles/d. During the 14-day reoxygenation period, the levels were lower with 2, higher with 4 to 8, and unchanged with 12 cycles/d.

Similarly, choroidal levels initially increased with 2 cycles/d, then declined to undetectable levels at 4 and 6 cycles/d. This was followed by a progressive increase with 8 to 12 cycles/d. During the reoxygenation period, choroidal SOD levels continued to rise from 2 to 12 cycles/d. After 2 weeks of exposure, retinal SOD levels were lower than choroidal levels and remained comparable with controls, except for lower levels with 8 cycles/d. During reoxygenation, the levels were higher with 2, 4, and 12 cycles/d than control levels. In the choroid, there was a biphasic response of higher SOD levels that began with CH to peak with 4 cycles/d. The levels declined with 6 cycles/d, then increased further with 8 to 12 cycles/d. This similar pattern was noted during the reoxygenation period.

Catalase Activity

Catalase is a potent scavenger of H₂O₂ and converts it to H₂O and O₂. Catalase activity was measured in the retina and choroid to determine whether the vascular beds can increase production of catalase to provide additional antioxidative protection during repeated IH. During CH, catalase activity decreased in the retina (Fig. 4). During 7-day exposure, there was a moderate increase with 2 and 4 cycles/d. This was followed by a decrease with 6 to 12 cycles/d. During the reoxygenation period, the levels remained low with 2 IH events, but were higher than RA levels with 4 to 12 IH events.

FIGURE 5. Effects of incremental IH episodes on retinal (A, C) and choroidal (B, D) H₂O₂ in neonatal rats at P7 or P14 (open bar) and P21 (solid bar). P7 animals were exposed to brief clustered IH episodes with 50% O₂ or constant 50% O₂ from P0 to P7. P14 animals were exposed to brief clustered IH episodes with 50% O₂ or constant 50% O₂ from P0 to P14. P21 animals were exposed to constant 50% O₂ or brief clustered IH episodes with 50% O₂ from P0 to P7 or P0 to P14, then placed in RA from P7 to P21 or P14 to P21 for reoxygenation. Data are presented as mean $\pm$ SEM (n = 6 samples/groups).

In the choroid, CH suppressed catalase activity. IH resulted in a biphasic effect of increased levels with 2 and 4, decreased levels at 6, and increased levels at 8 to 12 cycles/d. The activity pattern was similar during reoxygenation. Despite the similarities in patterns of catalase increased and decreased activities, there were distinct differences in the levels with CH, and 2 to 10 IH cycles/d. Longer exposure to IH insults resulted in much lower retinal catalase activity compared with that in the choroid. In the retina, longer CH exposure resulted in increased catalase activity. This was similar for 2 and 6 to 12 IH cycles/d. The recovery period followed a similar pattern. In the choroid, the response was similar to that seen with short-term exposure, with a biphasic response of lower levels with CH, higher levels with 2 and 4, lower levels with 6, and higher levels with 8 and 10 IH cycles/d. The recovery period followed a similar pattern.

H₂O₂ Levels

H₂O₂ is the most abundant ROS and is formed by dismutation of superoxide anion by SOD. We measured H₂O₂ (μM/mg protein) in the retina and choroid to determine the functional activity of SOD in response to repeated IH cycling (Fig. 5). Retinal H₂O₂ was 1° of magnitude lower than that in the choroid. At P7, exposure to CH decreased H₂O₂ levels (0.04 ± 0.0091 , $P < 0.01$) compared with 7-day RA levels ($0.12 \pm$

0.015). No changes in retinal H₂O₂ levels occurred with increasing IH episodes. Interestingly, it was during the recovery period that retinal H₂O₂ levels increased to peak at 6 cycles/d (0.26 ± 0.024 , $P < 0.01$) compared with P21-RA (0.11 ± 0.0017) and 7-day O₂ (0.11 ± 0.0053 , $P < 0.001$), and decreased with 8 to 12 cycles/d. A quite different response pattern occurred in the choroid. At P7, a similar decrease was noted in the 50% O₂ group (0.09 ± 0.019 , $P < 0.01$), but the levels increased with 2 (0.67 ± 0.01 , $P < 0.05$), 4 (0.74 ± 0.02 , $P < 0.01$), and 6 cycles/d (0.81 ± 0.12 , $P < 0.01$) compared with RA (0.42 ± 0.08). The levels declined at 8 to 12 cycles/d. During the recovery period, the levels increased at 4 (2.11 ± 0.068), 6 (3.0 ± 0.27), 8 (2.1 ± 0.23), 10 (1.8 ± 0.18), and 12 cycles/d (0.88 ± 0.19) compared with RA (0.49 ± 0.013 , $P < 0.01$) and compared with the 7-day O₂ groups (0.74 ± 0.02 , $P < 0.001$; 0.81 ± 0.12 , $P < 0.001$; 0.45 ± 0.072 , $P < 0.001$; 0.33 ± 0.01 , $P < 0.001$; 0.24 ± 0.05 , $P < 0.01$) for 4, 6, 8, 10, and 12 cycles/d, respectively. Retinal and choroidal H₂O₂ levels following 14 days of IH with 7 days of RA recovery/reperfusion are presented in Figures 5C and 5D. In the retina, H₂O₂ levels increased only in the 6 cycles/d group following 14 days of IH (0.27 ± 0.055 , $P < 0.05$). In contrast, the levels increased during the reoxygenation period following 50% O₂ (0.23 ± 0.027 , $P < 0.01$), as well as 6 (0.23 ± 0.019 , $P < 0.01$) and 12 (0.21 ± 0.033) cycles/d. In the choroid, 14 days of IH cycling resulted in H₂O₂ increases with 4 ($1.6 \pm$

FIGURE 6. Representative retinal flatmounts showing ADPase stained retinas (optic disc and periphery) from P7 (A–F) and P21 (G–L) rats. (A, B) 7-day rats exposed to RA. (C, D) Rats exposed to constant 50% O₂. (E, F) Rats exposed to 8 IH cycles/d. (G, H) 7-day rats exposed to RA. (I, J) Rats exposed to constant 50% O₂. (K, L) Rats exposed to 8 IH cycles/day. Eight cycles/d was chosen because this is the “critical” number of cycles where the retina did not respond based on the data. Images are $\times 10$ magnification.

0.29), 6 (1.21 ± 0.15), 8 (4.7 ± 0.29), and 10 (1.51 ± 0.17) IH cycles/d compared with RA (0.56 ± 0.11 , $P < 0.01$). During the reoxygenation period, the levels remained elevated with 2 (1.5 ± 0.16), 4 (1.38 ± 0.14), 6 (2.9 ± 0.19), 8 (3.4 ± 0.05), and 10 (2.2 ± 0.32) compared with RA (0.49 ± 0.13 , $P < 0.01$). Only the 8 cycles/d group was lower during reoxygenation than during the IH cycling (3.4 ± 0.05 vs. 4.7 ± 0.29 , $P < 0.01$).

Retinal Flatmounts

Figures 6A through 6L represent the retinal flatmounts for the 7-day groups exposed to RA (6A, 6B), CH (6C, 6D), and 8 IH cycles/d (6E, 6F); and the 21-day groups exposed to RA (6G, 6H), CH for 7 days followed by 14 days of RA recovery (6I, 6J), and 8 IH cycles/d for 7 days followed by 14 days of RA recovery (6K, 6L). The 7-day O₂ groups showed no significant differences in pathology compared with the RA groups. There was a predominance of hyaloid vessels and a large avascular area at the periphery. Reoxygenation at P21 resulted in large hemorrhagic areas predominantly at the periphery in the CH group (arrows), and punctate hemorrhages in the zone 1 area in the group exposed to 8 IH cycles/d. This group also exhibited

hemorrhage, tortuous, and dilated vessels at the periphery (arrow). Figures 7A through 7L represent the retinal flatmounts for the 14-day groups exposed to RA (7A, 7B), 50% O₂ (7C, 7D), and 8 IH cycles/d (7E, 7F); and the 21-day groups exposed to RA (7G, 7H), 50% O₂ for 14 days followed by 7 days of RA recovery (7I, 7J), and 8 IH cycles/d for 14 days followed by 7 days of RA recovery (7K, 7L). Retinas from animals exposed to 14 days of 50% O₂ (Figs. 7C, 7D) had evidence of hemorrhage throughout the retina with a less dense vasculature than the RA controls (Figs. 7A, 7B). Similarly, retinas from animals exposed to 14 days of 8 IH cycles/d had persistence of hyaloid vessels extending to the ciliary body (arrow), and hemorrhage throughout the retina (Figs. 7E, 7F). During the reoxygenation period, the animals exposed to 50% O₂ had retinas with large and punctate hemorrhages (arrows) as well as tortuous, dilated vessels (Figs. 7I, 7J). Retinas from animals exposed to 8 IH cycles/d had extensive hemorrhage throughout the retinas with dilated, tortuous vessels (arrows; Figs. 7K, 7L).

Mitochondria-Related Genes

Tables 3 and 4 represent the changes in fold regulation of mitochondrial-related genes involved in OXPHOS in the retina

FIGURE 7. Representative retinal flatmounts showing ADPase stained retinas (optic disc and periphery) from P14 (A–F) and P21 (G–L) rats. (A, B) 7-day rats exposed to RA. (C, D) Rats exposed to constant 50% O₂. (E, F) Rats exposed to 8 IH cycles/d. (G, H) 7-day rats exposed to RA. (I, J) Rats exposed to constant 50% O₂. (K, L) Rats exposed to 8 IH cycles/d. Eight cycles/d was chosen because this is the critical number of cycles that the retina did not respond based on the data. Images are $\times 10$ magnification.

and choroid. Data are fold-regulated compared with saline controls. Only the CH and 8 IH cycles/d data for the reoxygenation groups are presented in order to demonstrate differences in the 0 group and the number of cycles that was identified as a critical number based on the data in total, and to limit amount of data. For the 7-day exposure to CH resulted in upregulation of most genes involved in complexes I through V in the retina and choroid (data not shown). Conversely, 8 IH cycles/day resulted in downregulation of 22% (complex I), 100% (complex II), 63% (complex III), 27% (complex IV), and 44% (complex V) of genes in the retina, and a 6-fold upregulation of ATP5i gene in complex V. In the choroid, 8 IH cycles/day downregulated 100% of the genes involved in complexes I through V, most notably was the 7-fold downregulation of Sdhc (complex II), Cox15 (complex IV), and ATP5d (complex V). The reoxygenation period following 7 days of CH resulted in mild downregulation of approximately 30% of genes involved in the complexes in the retina, whereas in the choroid, 100% of the genes were downregulated, particularly Ndufa11 (20-fold), Ndufs3 (12-fold), Ndufs4 (13-fold), Cox4il (39-fold), Cox8a (8-fold), Cox17 (17-fold), Atp5h (58-fold), and

Atp5i (31-fold), which are involved in complexes I, IV, and V. The reoxygenation period following 7 days of 8 IH cycles/d resulted in downregulation of 100% of the genes involved in complexes I through V in retina and choroid with 6-fold and 13-fold downregulation of Cox6a (complex IV) and Atp4b (complex IV), respectively, in the retina (Table 3). For the 14-day exposure (P14) to CH, there was downregulation of 100% genes involved in complexes I–V in the retina, specifically Atp5a1 (246-fold) involved in complex V. In the choroid, 100% of the genes involved in all the complexes were upregulated. A similar trend occurred following exposure to 8 IH cycles/d, but to a lesser degree with 66% of genes downregulated in the retina compared with only 10% in the choroid (data not shown). During the reoxygenation period following 14 days of CH, all genes involved in complexes I through V were upregulated in the retina and choroid. In the retina, 70% of genes involved in complexes I through V remained downregulated during the reoxygenation following 14 days of 8 IH cycles/d. However, in the choroid, 100% of the genes were downregulated (Table 4).

TABLE 3. Expression of Mitochondrial Energy Metabolism Genes

Genes of Interest	50% O ₂ Retina	50% O ₂ Choroid	8 Cycles/d Retina	8 Cycles/d Choroid
Complex I				
Ndufa1	1.8	2.8	1.1	-1.4
Ndufa2	1.7	2.6	-1.1	-1.5
Ndufa5	1.5	3.4	-1.1	-1.3
Ndufa6	1.9	2.5	-1.1	-1.5
Ndufa7	2.1	2.7	-1.3	-1.2
Ndufa8	2.8	3.3	-1.2	-1.4
Ndufa9	3.0	2.8	2.0	-1.5
Ndufa10	2.4	2.8	-1.1	-1.5
Ndufa11	2.4	3.0	-1.2	-1.5
Ndufab1	2.2	3.6	1.5	-1.3
Ndufb2	2.2	3.3	-1.1	-1.2
Ndufb3	2.2	3.9	1.2	-1.2
Ndufb5	2.3	3.8	1.2	-1.4
Ndufb6	2.8	3.1	-1.6	-1.2
Ndufb7	2.7	2.9	1.4	-1.5
Ndufb8	2.0	3.2	-1.8	-1.4
Ndufb9	2.0	2.3	-1.1	-1.4
Ndufs1	2.0	3.2	-1.5	-1.4
Ndufs2	2.1	1.8	1.0	-1.6
Ndufs3	1.8	1.8	1.3	-2.1
Ndufs4	2.6	2.3	1.2	-1.5
Ndufs6	1.9	3.2	-1.2	-1.3
Ndufs7	2.1	3.6	1.0	-1.4
Ndufs8	2.8	3.4	1.1	-1.5
Ndufv1	2.2	2.6	-1.4	-1.5
Ndufv2	1.7	3.0	-1.3	-1.4
Complex II				
Sdha	2.3	2.7	-1.2	-1.4
Sdhb	2.0	2.8	-1.1	-1.5
Sdhc	1.9	2.8	-2.5	-1.7
Sdhd	2.5	2.5	-1.3	-1.9
Complex III				
Cyc1	2.1	2.9	-1.5	-1.6
Uqcrb	1.8	2.0	-1.6	-1.4
Uqcrc1	2.1	2.7	-1.4	-1.3
Uqcrc2	1.7	2.8	-1.5	-1.7
Uqcrfs1	2.0	3.5	-1.5	-1.6
Uqcrh	1.7	2.4	-1.2	-1.6
Uqcrq	2.1	2.5	-1.7	-1.8
Complex IV				
Cox4i1	2.7	3.4	2.1	-2.0
Cox4i2	2.6	1.9	-1.0	-1.2
Cox5a	2.3	3.3	-1.0	-1.2
Cox5b	2.3	3.6	1.1	-1.4
Cox6a1	3.0	2.6	-1.1	-1.8
Cox6a2	1.6	7.1	-1.5	-4.1
Cox6c	1.7	3.3	1.1	-1.4
Cox6c-ps1	2.5	1.2	-1.0	-2.5
Cox7a2	1.6	2.7	-1.2	-1.4
Cox7a2l	1.9	1.8	-1.3	-2.3
Cox7b	1.8	3.1	1.1	-1.4
Cox8a	2.9	3.4	1.3	-1.3
Cox8c	1.5	-1.0	-1.9	-3.0
Cox15	1.9	2.2	-1.3	-1.6
Cox17	2.2	2.4	1.9	-1.7

TABLE 3. Continued

Genes of Interest	50% O ₂ Retina	50% O ₂ Choroid	8 Cycles/d Retina	8 Cycles/d Choroid
Complex V				
Atp12a	1.8	1.8	-1.4	-2.9
Atp4a	2.1	2.6	-1.1	-1.3
Atp4b	4.0	2.4	-1.6	-1.8
Atp5a1	2.0	2.9	-1.8	-1.3
Atp5b	1.8	2.1	-1.3	-1.7
Atp5c1	2.1	3.1	-1.2	-1.6
Atp5d	1.9	2.7	-1.3	-2.2
Atp5f1	2.4	5.7	-1.5	-1.4
Atp5g2	2.8	2.6	1.2	-1.8
Atp5g3	3.4	3.4	-1.1	-1.7
Atp5h	2.1	3.7	2.0	-1.6
Atp5i	2.9	2.4	3.1	-2.5
Atp5j	1.9	3.0	-1.1	-1.5
Atp5l	2.1	3.0	1.1	-1.2
Atp5o	2.2	2.7	1.2	-1.3
Atp6v0a2	2.5	1.8	-1.1	-1.6
Atp6v0d2	1.8	5.8	-1.6	-3.8
Atp6v1c2	2.1	2.0	1.1	-2.3
Atp6v1e2	2.2	2.3	-1.5	-1.2
Atp6v1g3	-1.7	2.7	-2.2	-1.3
Lhpb	2.2	2.3	-1.4	-1.5
Ppa1	2.0	2.6	-1.4	-1.1
Uncoupling proteins				
Ucp1	2.1	-1.1	-1.1	-5.2
Ucp2	1.9	2.2	-1.5	-1.8
Ucp3	2.2	4.5	-1.0	-11.4

Data obtained from neonatal rat retina and choroid after 7 days of frequent, brief, intermittent hypoxia, followed by 14 days of recovery in room air. Data are fold change from room air controls.

All data were corrected using five different housekeeping genes. Genes are selected from a profile of 84 genes. Genes of interest are:

1. Complex I: Ndufa, NADH dehydrogenase (ubiquinone) α subcomplex 1-11; Ndufb, NADH dehydrogenase (ubiquinone) β subcomplex 2-9; Ndufab, NADH dehydrogenase (ubiquinone) α/β subcomplex 1; Ndufs, NADH dehydrogenase (ubiquinone) Fe-S protein 1-8; Ndufv, NADH dehydrogenase (ubiquinone) flavoprotein 1,2.

2. Complex II: Sdha, Succinate dehydrogenase complex subunit A, flavoprotein; Sdhb, succinate dehydrogenase complex subunit B, iron sulfur; Sdhc, succinate dehydrogenase complex subunit C, integral membrane protein; Sdhd, succinate dehydrogenase complex subunit D, integral membrane protein.

3. Complex III: Cyc1, cytochrome C-1; Uqcrb, ubiquinol-cytochrome c reductase binding protein; Uqcrc, ubiquinol-cytochrome c reductase core protein I-II; Uqcrfs1, ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1; Uqcrh, ubiquinol-cytochrome c reductase hinge protein; Uqcrq, Ubiquinol-cytochrome c reductase, complex III subunit VII.

4. Complex IV: Cox, cytochrome C oxidase subunits.

5. Complex V: Atp12a, ATPase, H⁺/K⁺ transporting, nongastric, α polypeptide; Atp4a, ATPase, H⁺/K⁺ exchanging, α polypeptide; Atp4b, ATPase, H⁺/K⁺ exchanging, β polypeptide; Atp5, ATP synthase, H⁺ transporting, mitochondrial F1 complex, α subunits; Atp6, ATPase, H⁺ transporting, lysosomal V0 subunits; Lhpb, phospholysine phosphohistidine inorganic pyrophosphate phosphatase; Ppa1, pyrophosphatase (inorganic) 1; Ucp, uncoupling protein 1 (mitochondrial, proton carrier 1-3).

DISCUSSION

This study indicate that repeated, brief IH during hyperoxia, in early life impacts on blood gases, retinal maturation, biomarkers of lipid peroxidation and oxidative stress, and mitochon-

TABLE 4. Expression of Mitochondrial Energy Metabolism Genes

Genes of Interest	50% O ₂ Retina	50% O ₂ Choroid	8 Cycles/d Retina	8 Cycles/d Choroid
Complex I				
Ndufa1	1.2	1.8	2.0	−2.8
Ndufa2	1.2	1.5	1.6	−2.8
Ndufa5	1.1	2.0	−1.1	−2.4
Ndufa6	1.1	1.9	1.5	−2.7
Ndufa7	1.1	2.3	1.3	−2.8
Ndufa8	1.7	2.2	1.3	−3.3
Ndufa9	2.3	2.1	3.5	−2.8
Ndufa10	1.2	1.4	−1.2	−4.1
Ndufa11	1.3	1.7	1.6	−3.0
Ndufab1	2.2	2.5	2.2	−2.7
Ndufb2	1.2	2.2	1.2	−2.6
Ndufb3	1.5	2.1	1.3	−2.3
Ndufb5	1.6	2.5	1.5	−2.4
Ndufb6	1.2	2.3	1.2	−3.8
Ndufb7	1.4	1.8	1.7	−2.6
Ndufb8	1.2	1.7	1.1	−3.2
Ndufb9	1.2	1.4	1.4	−3.0
Ndufs1	1.0	2.0	−1.4	−3.0
Ndufs2	1.3	1.2	1.4	−3.1
Ndufs3	1.3	1.5	3.1	−2.6
Ndufs4	1.3	1.6	2.3	−3.1
Ndufs6	1.2	2.2	1.2	−2.4
Ndufs7	1.7	2.0	−1.2	−4.0
Ndufs8	1.4	1.9	1.8	−2.6
Ndufv1	1.2	1.5	−1.4	−5.1
Ndufv2	1.2	1.6	−1.0	−2.5
Complex II				
Sdha	1.1	1.5	−1.1	−3.0
Sdhb	1.1	1.7	−1.0	−2.7
Sdhc	1.1	1.5	−2.0	−7.0
Sdhd	1.5	1.3	−1.2	−3.0
Complex III				
Cyc1	1.1	1.7	−1.1	−4.0
Uqcrb	−1.3	1.7	1.1	−4.8
Uqcrc1	1.3	1.8	−1.0	−2.4
Uqcrc2	1.0	1.6	1.1	−2.7
Uqcrfs1	1.4	2.0	−1.3	−2.6
Uqcrh	1.1	1.5	−1.2	−2.5
Uqcrq	−1.2	1.8	−1.2	−3.0
Complex IV				
Cox4i1	3.8	2.0	3.1	−5.4
Cox4i2	1.8	1.6	1.2	−3.8
Cox5a	1.2	2.4	1.1	−2.5
Cox5b	1.3	2.3	1.4	−2.6
Cox6a1	1.4	1.7	1.3	−3.9
Cox6a2	1.7	2.9	−1.0	−1.5
Cox6c	1.3	1.9	1.2	−2.7
Cox6c-ps1	1.3	−1.6	1.3	−3.4
Cox7a2	1.1	1.7	1.6	−2.8
Cox7a2l	1.4	1.3	−1.0	−5.0
Cox7b	1.5	2.0	1.6	−2.1
Cox8a	2.2	2.3	2.3	−2.9
Cox8c	1.7	−1.1	−1.5	−1.7
Cox15	1.2	1.5	−1.4	−6.8
Cox17	2.3	1.6	1.3	−4.9

TABLE 4. Continued

Genes of Interest	50% O ₂ Retina	50% O ₂ Choroid	8 Cycles/d Retina	8 Cycles/d Choroid
Complex V				
Atp12a	−1.2	−1.2	−1.8	−3.3
Atp4a	1.1	1.5	−1.3	−3.5
Atp4b	1.2	−1.1	−1.4	−1.8
Atp5a1	−1.2	1.4	−1.1	−3.5
Atp5b	−1.1	1.2	1.5	−3.6
Atp5c1	−1.0	1.8	1.2	−2.6
Atp5d	1.5	1.7	1.0	−6.5
Atp5f1	1.2	3.5	1.1	−2.8
Atp5g2	1.3	1.6	2.0	−3.0
Atp5g3	1.5	−1.1	1.5	−3.7
Atp5h	2.5	2.4	3.1	−3.1
Atp5i	2.6	1.4	6.0	−4.6
Atp5j	−1.3	1.6	1.1	−2.9
Atp5l	1.3	1.8	2.3	−3.0
Atp5o	1.3	1.7	2.1	−2.8
Atp6v0a2	1.1	1.4	1.1	−3.0
Atp6v0d2	1.5	−1.1	−2.0	−2.9
Atp6v1c2	1.0	1.2	2.0	−4.0
Atp6v1e2	1.1	1.9	1.9	−3.6
Atp6v1g3	−1.6	2.5	−1.4	1.5
Lhpp	1.1	1.4	−1.5	−4.0
Ppa1	1.2	2.0	−1.1	−3.1
Uncoupling proteins				
Ucp1	−1.5	−1.7	−1.1	−3.0
Ucp2	1.3	1.6	−2.1	−3.8
Ucp3	−2.6	−1.2	−3.1	−4.0

In the neonatal rat retina and choroid after 14 days of frequent, brief, intermittent hypoxia, followed by 7 days of recovery in room air. Data are fold change from room air controls.

All data were corrected using five different housekeeping genes. Genes are selected from a profile of 84 genes. Genes of interest are as described in Table 3.

drial-related genes involved in OXPHOS. The effects of IH on the retina persist, and may even worsen, during the reoxygenation period as shown in our model that simulates brief desaturations or apneic spells experienced by ELGANs.^{12,13} Animal models using a longer hypoxic period also show a greater degree of retinal neovascularization compared with hyperoxia alone.¹¹ However, critically ill ELGANs experience O₂ fluctuations on the order of minutes, not hours.^{8,9,34,35} In our study, a shorter, one-minute clustered approach was used to establish a dose response of IH on the ocular vascular beds. ELGANs experience increasing clustered IH events several times per day.⁹ These brief increasing IH events can damage the retina and worsen during reoxygenation, as shown by the retinal flatmounts showing contrasting pathologies between hypoxia and reoxygenation, and between IH and CH.

Our data demonstrated five major findings:

1. IH, and not CH, was responsible for prolongation of the cecal period, suggesting delayed retinal neural maturation.^{27–31}
2. pO₂ levels were higher despite increasing IH episodes and these levels remained substantially elevated during the reoxygenation period. This may indicate hyperventilation and carotid body programming.^{36,37} One complexity was the lack of associated changes in pCO₂. Respiratory rate increases mean airway pressure and,

consequently, pO₂ and SO₂. In our rats, mixed arterial-venous blood gas was assessed. This may explain why increased respiratory rate had no effect on pCO₂ levels. High SO₂ levels were associated with the most severe OIR at both phases.

3. A biphasic response to increasing IH episodes with levels increasing from 2 to 6 and decreasing from 8 to 12 IH episodes, suggesting that the maximum number of clustered IH episodes that the retina can sustain is 6.
4. Major differences in the responses to IH between the retina and choroid possibly due to differences in their capacity to autoregulate.²⁵
5. Accumulation of H₂O₂ in the choroid, particularly during the reoxygenation period, with associated reductions in mitochondria-related genes and more severe OIR.

Together, these findings indicate that IH events greater than 6/d causes accumulation of H₂O₂ in the choroid, which may be the only source of oxygen under these conditions. In this context, the choroid may be the main driving force responsible for retinal oxidative damage. The implication is that critically ill ELGANs with frequent, increasing, clustered IH episodes are at a higher risk for long-term, and possibly permanent, retinal damage resulting from H₂O₂ accumulation in the choroid. Our data are in accord with the increasing evidence that premature babies develop repeated hyperoxic-hypoxic adverse events and that these hypoxic events leads to irreparable or irreversible organ damage when they reach a certain number. Whether the critical number of hyperoxic-hypoxic events identified in the newborn rat will ultimately be similar to the preterm newborn infant must be evaluated in future clinical studies. Our data provide the foundation for the design of future clinical trials to answer these questions.

Recurrent and chronic IH initiated by greater than 6 IH episodes/d decreases the recovery time between episodes, disturbs the oxygen homeostasis, and induces pathological hypoxic conditions, leading to damage in the inner retina.³⁸ The optimal oxygen saturation targets for ELGANs remain controversial and undefined.³⁹ Blood gases measurements immediately postexposure and during reoxygenation suggest hyperventilation initiated by chronic IH.^{36,37,40} The data also confirm that higher FiO₂ is associated with more severe OIR, possibly due to increased ROS production. 8-iso-PGF_{2α} is one of the major forms of F-isoprostanes, and is a reliable indicator of oxidative stress and lipid peroxidation.^{41,42} The maximum 8-iso-PGF_{2α} response of the retina to IH was with 6 cycles/day. This critical number may have generated sufficient 8-iso-PGF_{2α} to contribute to retinal vasoconstriction. Therefore, the effect of decreased 8-iso-PGF_{2α} with 8 to 12 cycles may be a consequence of retinal ischemia. Irrespective of reoxygenation, there was no evidence for reciprocity suggesting extensive, and possibly, permanent vascular damage, as demonstrated in the retinal flatmounts. There were subtle dissimilarities in the pattern of responses between the 7- and 14-day retinas and choroids, providing evidence for an ischemic response. One complexity was the increased 8-iso-PGF_{2α} levels in the choroid following reoxygenation from 6 IH cycles/d. A tenable interpretation is that there is a free radical burst due to H₂O₂ accumulation.

The retina has several enzymatic antioxidant defense mechanisms for the elimination of ROS and thereby alleviating oxidative stress.¹⁶ During IH exposure, the maximum SOD response of the retina was 2 IH cycles/d, beyond which the levels were depleted. SOD appeared to be derived predominantly during reoxygenation and this was most evident in the choroid. One possible explanation for the differences in tissues responses during reoxygenation is that they may differ in their

ability to increase activity of endogenous SOD. Since oxygen requirements of the inner retina exceed that of the outer retina, SOD activity may be limited due to severe and rapid degeneration at higher IH cycling episodes. Studies have implied that catalase is more important than GPx when concentrations of H₂O₂ exceed physiologic levels.⁴³ Catalase is a potent scavenger of H₂O₂ and provides a powerful antioxidant defense in the retina.⁴⁴ Consistent with SOD, catalase activity was higher in the choroid than in the retina and the maximum response occurred with 2 cycles/d. In contrast to SOD, we noted a biphasic response with reductions at 6 cycles/d, which may account for the burst of 8-iso-PGF_{2α}. These expression patterns may be due to turnover rates and feedback mechanisms. It seems logical that a longer reoxygenation period between IH episodes (with 2 and 4 cycles/d) will elicit a more intense response. The most important and novel finding of our study is the effect of IH on H₂O₂ production in the retina and choroid. Choroidal H₂O₂ levels were several-fold higher than retinal levels and followed a similar pattern to SOD levels, providing a role for SOD/catalase production during the reoxygenation period in the choroid. Although H₂O₂ is a nonradical oxygen species, it is highly reactive, membrane permeable, and can be converted to ROS, which accounts for its deleterious effects. One fascinating and unexpected finding was the reduction in retinal H₂O₂ levels with CH, elevations during the reoxygenation period, and increases with increasing IH to peak with 6 cycles/d. This suggests that the reoxygenation period following hypoxia may generate ROS that could not be efficiently scavenged by endogenous antioxidant defenses, resulting in injuries to cellular structures.⁴⁵ This excess production of H₂O₂ may contribute to overexpression of SOD in the choroid. In the setting of increasing IH episodes, experienced by ELGANs, the choroid is the only source of oxygen for the entire retina.²⁵ In addition to oxygen, it may provide high levels of H₂O₂ and SOD, which adds to the demise of the immature retina. Accumulation of H₂O₂ leads to upregulation of VEGF⁴⁶ and neovascularization, as well as inhibition of mitochondrial respiration.⁴⁷ This novel finding leads to the provocative speculation that targeting H₂O₂ accumulation in the choroid may be a new therapeutic approach to prevent and/or treat severe ROP. The underlying mechanisms associated with H₂O₂ accumulation in the choroid are of great interest and are currently under investigation in our lab.

Oxidative stress is one of the key mechanisms of reoxygenation injury. This may be due to large reductions in ATP content.⁴⁸ Degradation of ATP increases the formation of hypoxanthine and intracellular calcium. Upon reoxygenation, xanthine oxidase oxidizes the accumulated hypoxanthine to uric acid and release of superoxide. Our data demonstrating reductions in ATP in the choroid during reoxygenation following 8 IH cycles/d are consistent with those previous findings and suggests that the brunt of the damage following IH occurs during reoxygenation. This may explain the high levels of 8-iso-PGF_{2α} and H₂O₂ in the choroid and suggests a less efficient respiratory chain.⁴⁹ The main mechanism for mitochondrial ROS production is OXPHOS, particularly complexes I and III of the respiratory chain.^{50,51} We found that all mitochondria-related genes involved in OXPHOS were down-regulated in the choroid during reoxygenation following 8 IH cycles/d, suggesting defects in OXPHOS. It is noteworthy that if reoxygenation of the hypoxic retina amplifies the injury sustained during oxygen deprivation as demonstrated by our data, then the damage is magnified at each reoxygenation episode following an IH or apneic episode, which occurs several times per day in ELGANs, thus adding to the complexity of the disease.

In summary, our data showed that the maximum number of IH episodes per day that the retina can sustain appears to be six in this rat model. Since oxygen requirements of the retina exceed that of the choroid,²⁵ numerous IH events may result in severe and rapid degeneration. We conclude that accumulation of H₂O₂ in the choroid may provide high levels to the entire retina. As an inherent corollary, there is a deficit in OXPHOS and a lack of antioxidant response, leading to severe OIR. Further studies are underway to examine the role of the choroid and H₂O₂ in the development of ROP as well as possible therapeutic interventions.

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References

- Papp A, Németh I, Karg E, Papp E. Glutathione status in retinopathy of prematurity. *Free Radic Biol Med*. 1999;27:738–743.
- Hardy P, Dumont I, Bhattacharya M, et al. Oxidants, nitric oxide and prostanooids in the developing ocular vasculature: a basis for ischemic retinopathy. *Cardiovasc Res*. 2000;47:489–509.
- SanGiovanni JP, Chew EY. The role of omega-3 long-chain polyunsaturated fatty acids in health and disease of the retina. *Prog Retin Eye Res*. 2005;24:87–138.
- Di Fiore JM, Martin RJ, Gauda EB. Apnea of prematurity—perfect storm. *Respir Physiol Neurobiol*. 2013;189:213–222.
- Cunningham S, Fleck BW, Elton RA, McIntosh N. Transcutaneous oxygen levels in retinopathy of prematurity. *Lancet*. 1995;346:1464–1465.
- Chow LC, Wright KW, Sola A; CSMC Oxygen Administration Study Group. Can changes in clinical practice decrease the incidence of severe retinopathy of prematurity in very low birth weight infants? *Pediatrics*. 2003;111:339–345.
- York JR, Landers S, Kirby RS, Arbogast PG, Penn JS. Arterial oxygen fluctuation and retinopathy of prematurity in very-low-birth-weight infants. *J Perinatol*. 2004;24:82–87.
- Di Fiore JM, Bloom JN, Orge E, et al. A higher incidence of intermittent hypoxemic episodes is associated with severe retinopathy of prematurity. *J Pediatr*. 2010;157:69–73.
- Di Fiore JM, Kaffashi F, Loparo K, et al. The relationship between patterns of intermittent hypoxia and retinopathy of prematurity in preterm infants. *Pediatr Res*. 2012;72:606–612.
- Saito Y, Omoto T, Cho Y, Hatsukawa Y, Fujimura M, Takeuchi T. The progression of retinopathy of prematurity and fluctuation in blood gas tension. *Graefes Arch Clin Exp Ophthalmol*. 1993;231:151–156.
- Penn JS, Henry MM, Tolman, BL. Exposure to alternating hypoxia and hyperoxia causes severe proliferative retinopathy in the newborn rat. *Pediatr Res*. 1994;36:724–731.
- Coleman R, Beharry KD, Brock RS, Abad-Santos P, Abad-Santos M, Modanlou HD. Effects of brief clustered versus dispersed hypoxic episodes on systemic and ocular growth factors in a rat model of oxygen-induced-retinopathy. *Pediatr Res*. 2008;64:50–55.
- Brock RS, Gebrekristos BH, Kuniyoshi KM, Modanlou HD, Falcao MC, Beharry KD. Biomolecular effects of JB1 (an IGF-I peptide analog) in a rat model of oxygen-induced retinopathy. *Pediatr Res*. 2011;69:35–41.
- Li S-Y, Fu ZJ, Lo AC. Hypoxia-induced oxidative stress in ischemic retinopathy. *Oxid Med Cell Longev*. 2012;2012:426769.
- Hartnett ME. The effects of oxygen stresses on the development of features of severe retinopathy of prematurity: knowledge from the 50/10 OIR model. *Doc Ophthalmol*. 2010;120:25–39.
- Agardh CD, Gustavsson C, Hagert P, Nilsson M, Agardh E. Expression of antioxidant enzymes in rat retinal ischemia followed by reperfusion. *Metabolism*. 2006;55:892–898.
- Nycyk JA, Drury JA, Cooke RW. Breath pentane as a marker for lipid peroxidation and adverse outcome in preterm infants. *Arch Dis Child Fetal Neonatal Ed*. 1998;79:F67–F69.
- Dong A, Shen J, Krause M, et al. Superoxide dismutase 1 protects retinal cells from oxidative damage. *J Cell Physiol*. 2006;208:516–526.
- Salvemini D, Cuzzocrea S. Therapeutic potential of superoxide dismutase mimetics as therapeutic agents in critical care medicine. *Crit Care Med*. 2003;31(suppl 1):S29–S38.
- Usui S, Komeima K, Lee SY, et al. Increased expression of catalase and superoxide dismutase 2 reduces cone cell death in retinitis pigmentosa. *Mol Ther*. 2009;17:778–786.
- Cringle SJ, Yu DY. A multi-layer model of retinal oxygen supply and consumption helps explain the muted rise in inner retinal PO(2) during systemic hyperoxia. *Comp Biochem Physiol A Mol Integr Physiol*. 2002;132:61–66.
- Das A, McGuire PG. Retinal and choroidal angiogenesis: pathophysiology and strategies for inhibition. *Prog Retin Eye Res*. 2003;22:721–748.
- Pournaras CJ, Rungger-Brändle E, Riva CE, Hardarson SH, Stefansson E. Regulation of retinal blood flow in health and disease. *Prog Retin Eye Res*. 2008;27:284–330.
- Oshima Y, Oshima S, Nambu H, et al. Different effects of angiopoietin-2 in different vascular beds in the eye: new vessels are most sensitive. *FASEB J*. 2005;19:963–965.
- Cringle SJ, Yu DY. Oxygen supply and consumption in the retina: implications for studies of retinopathy of prematurity. *Doc Ophthalmol*. 2010;120:99–109.
- Keenaghan M, Cai CL, Kumar D, et al. Response of vascular endothelial growth factor and angiogenesis-related genes to stepwise increases in inspired oxygen in neonatal rat lungs. *Pediatr Res*. 2013;73:630–638.
- Dorfman AL, Joly S, Hardy P, Chemtob S, Lachapelle P. The effect of oxygen and light on the structure and function of the neonatal rat retina. *Doc Ophthalmol*. 2009;118:37–54.
- Binns KE, Salt TE. Post eye-opening maturation of visual receptive field diameters in superior colliculus of normal- and dark-reared rats. *Brain Res Dev Brain Res*. 1997;99:263–266.
- Zieske JD. Corneal development associated with eyelid opening. *Int J Dev Biol*. 2004;48:903–911.
- Robinson SR, Dreher B. The visual pathways of eutherian mammals and marsupials develop according to a common timetable. *Brain Behav Evol*. 1990;36:177–195.
- Dreher B, Robinson SR. Development of the retinofugal pathway in birds and mammals: evidence for a common “timetable.” *Brain Behav Evol*. 1988;31:369–390.
- Awad JA, Roberts LJ II, Burk RF, Morrow JD. Isoprostanes—prostaglandin-like compounds formed in vivo independently of cyclooxygenase: use as clinical indicators of oxidant damage. *Gastroenterol Clin North Am*. 1996;25:409–427.
- Roberts LJ II, Morrow JD. The generation and actions of isoprostanes. *Biochim Biophys Acta*. 1997;1345:121–135.
- Martin RJ, Wang K, Köroğlu O, Di Fiore J, Kc P. Intermittent hypoxic episodes in preterm infants: do they matter? *Neonatology*. 2011;100:303–310.

35. Martin RJ, Di Fiore JM, Macfarlane PM, Wilson CG. Physiologic basis for intermittent hypoxic episodes in preterm infants. *Adv Exp Med Biol*. 2012;758:351-358.
36. Peng YJ, Rennison J, Prabhakar NR. Intermittent hypoxia augments carotid body and ventilatory response to hypoxia in neonatal rat pups. *J Appl Physiol (1985)*. 2004;97:2020-2025.
37. Prabhakar NR. Oxygen sensing during intermittent hypoxia: cellular and molecular mechanisms. *J Appl Physiol (1985)*. 2001;90:1986-1994.
38. Kaur C, Sivakumar V, Foulds WS. Early response of neurons and glial cells to hypoxia in the retina. *Invest Ophthalmol Vis Sci*. 2006;47:1126-1141.
39. Tin W. Oxygen therapy: 50 years of uncertainty. *Pediatrics*. 2002;110:615-616.
40. Prabhakar NR. Sensing hypoxia: physiology, genetics and epigenetics. *J Physiol*. 2013;591:2245-2257.
41. Morrow JD, Awad JA, Boss HJ, Blair IA, Roberts LJ II. Noncyclooxygenase-derived prostanoids (F2-isoprostanes) are formed in situ on phospholipids. *Proc Natl Acad Sci U S A*. 1992;89:10721-10725.
42. Leitingner N, Huber J, Rizza C, et al. The isoprostane 8-iso-PGF(2 α) stimulates endothelial cells to bind monocytes: differences from thromboxane-mediated endothelial activation. *FASEB J*. 2001;15:1254-1256.
43. Fecondo JV, Augusteyn RC. Superoxide dismutase, catalase and glutathione peroxidase in the human cataractous lens. *Exp Eye Res*. 1983;36:15-23.
44. Usui S, Oveson BC, Iwase T, et al. Overexpression of SOD in retina: need for increase in H₂O₂-detoxifying enzyme in same cellular compartment. *Free Radic Biol Med*. 2011;51:1347-1354.
45. Saugstad OD. Hypoxanthine as an indicator of hypoxia: its role in health and disease through free radical production. *Pediatr Res*. 1988;23:143-150.
46. Cho M, Hunt TK, Hussain MZ. Hydrogen peroxide stimulates macrophage vascular endothelial growth factor release. *Am J Physiol Heart Circ Physiol*. 2001;280:H2357-H2363.
47. Sims NR, Anderson ME, Hobbs LM, et al. Impairment of brain mitochondrial function by hydrogen peroxide. *Brain Res Mol Brain Res*. 2000;77:176-184.
48. Sims NR. Energy metabolism and selective neuronal vulnerability following global cerebral ischemia. *Neurochem Res*. 1992;17:923-931.
49. Boveris A, Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem J*. 1973;134:707-716.
50. Maranzana ME, Barbero G, Falasca AI, Lenaz G, Genova ML. Mitochondrial respiratory supercomplex association limits production of reactive oxygen species from complex I. *Antioxid Redox Signal*. 2013;19:1469-1480.
51. Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnfsky EJ. Production of reactive oxygen species by mitochondria: central role of complex III. *J Biol Chem*. 2003;278:36027-36031.