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The Impact of Hyperoxia on the Neonatal and Adult Developing Dendritic Cell

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ABSTRACT: Oxygen is essential therapy for neonates with acute respiratory failure, including those with infections. However, high oxygen levels may be counterproductive for overcoming infections because hyperoxia may kill cells, including dendritic cells that are essential to the emergence of the pulmonary immune system and pivotal in mounting immune responses to infections. We studied the impact of hyperoxia on developing dendritic cells from neonatal cord blood and adult blood monocytes, comparing viability, development of maturation, and endocytic function. Our data suggest that cord blood–derived dendritic cells may be more resistant to hyperoxic-induced cell death than adult blood–derived cells. Moreover, the surviving cells in either group are those that maintain an immature phenotype. This may impair their ability to perform optimal immune function. **(*Pediatr Res* 62: 78–82, 2007)**

Infection is one of the most important causes of mortality and morbidity among newborns with acute respiratory failure (1). Inhaled oxygen is a vital therapy for these infants, despite the potential risks of hyperoxia to the lung, including affecting the DNA integrity of type II pneumocytes (2), and normal immune responses in the lungs (3,4). Hyperoxia alters the pulmonary tissue immune response by up-regulating proinflammatory cytokines and inducing neutrophil infiltration in the alveolar spaces (5). It also increases alveolar macrophage apoptosis (4), further weakening the immune response (3). We investigated whether hyperoxia might likewise interfere with the development of DC, immune cells that are more important for presenting antigen to T cells than are macrophages.

While the majority of term infants exposed to hyperoxia recover, occasionally with some residual morbidity, adults under similar conditions do not survive beyond a few days (6,7). To investigate whether this differential response to oxygen is reflected in DC function and viability, we compared the hyperoxic effect on developing DC from neonates *versus* adults.

MATERIALS AND METHODS

Cell culture and hyperoxia. Pulmonary dendritic cells, which are exposed to the highest levels of oxygen, are predominantly of myeloid origin (8) and resemble monocyte-derived DC from cord or adult peripheral blood (9). After institutional board review and informed consent, cord blood from normal term pregnancies was collected into sterile collection bags and processed within 24 h. Some cord blood was supplied by the St. Louis Cord Blood Bank and solely used for determining the optimal duration of hyperoxia exposure. Although the blood from preterm cord blood would be more reflective of the clinical setting underlying this study, limited volume and, therefore, smaller cell numbers made it impractical. Adult blood was collected by venipuncture from healthy volunteers. The dendritic progenitor cells were extracted from mononuclear cells as described previously (10). Briefly, the mononuclear cells were separated from red cells by sedimentation with a solution of hydroxyethyl starch followed by density centrifugation over Histopaque-1077 (Sigma Chemical Co., St. Louis, MO). The cells adhering after 1-h incubation (monocyte lineage cells) were cultured at 37°C in RPMI 1640 and 10% FCS enriched with 800 U/mL rhGM-CSF and 80 ng/mL rhIL-4. (R & D Systems, Minneapolis, MN). To induce maturation of the DC, 20-ng/mL lipopolysaccharide (LPS, Sigma Chemical Co.) was added to the culture at d 5.

Hyperoxic conditions were created by incubating half of the cell cultures in an oxygen-saturated chamber (Billups-Rothenberg, Del Mar, CA), as previously described (11), with a gas mixture of 95% O_2 and 5% CO_2 for 2, 5, or 7 consecutive days. This oxygen level is chosen to produce the maximal exposure and is comparable to that exposed to the sickest patients. Two, five, and seven days of exposure to hyperoxia had been predetermined experimentally, *via* a doses-response study, as optimal for detecting differences in viability of the cells from both cord and adult blood (data not shown). Control cells were incubated in 21% O_2 and 5% CO_2. To account for any confounding effects of incubation before hyperoxia, all cells were incubated for 1 h at 37°C, 21% O_2 and 5% CO_2, before being exposed to 95% or 21% oxygen The oxygen was thereafter reintroduced every 2 d to maintain the stated level (11).

At the end of 2, 5, and 7 d, the cells were enumerated and viability was determined by negative staining with trypan blue. The ratio of remaining cells after exposure to 95% oxygen to remaining cells following 21% O_2 was expressed as the "hyperoxia index."

Phenotypic marker analysis. At the end of a 7-d incubation period, the cells were incubated with mouse anti-human fluorescent MAb against CD1a (immatured-DC marker), CD83 (matured-DC marker), HLA-DP/Q/R (HLA-II), or with isotype-matched antibodies (all from BD PharMingen, San Diego, CA) for 60 min at 4°C. Flow cytometry data were acquired using an Epics-XL-MCL flow cytometer (Beckman Coulter, Inc., Fullerton, CA). A subpopulation was defined for its high concentration of cells that shared similar forward as well as side scatter and tendency to express DC markers. All data were analyzed for marker quantity (percentage of cells that expressed DC markers within the subpopulation) and for marker intensity (MFI, mean fluorescence intensity: the number of markers expressed per cell) using Cytomics RXP software (Beckman Coulter).

Measurement of endocytic activity. The ability of cells to take up and catalyze the self-quenched green bodipy dye conjugate of BSA (DQ-BSA) (Molecular Probes, Eugene, OR) was used to measure endocytosis, a function of immature DC (12). Cells were incubated with DQ-BSA (10 μg/mL in RPMI 1640 containing 10% fetal bovine serum) for 1 h at 37°C (controls were incubated on ice), washed twice with PBS containing 0.1% NaN_3 and 0.1% BSA (Sigma Chemical Co.), and endocytic activity was measured by flow cytometry.

Cell viability analysis. The changes in cell viability were quantified by determining the amount of bound FITC-conjugated Annexin-V (AV-FITC) (BD PharMingen) and propidium iodide (PI). Per manufacturer's recommendations, 5 μL AV-FITC was incubated with 10^5 cells for 15 min at room temperature in the dark followed by 5 μL of 50 μg/mL PI for another 15 min, then the whole promptly analyzed by flow cytometry. Cells in apoptosis (annexin-V positive, PI negative), apoptosis-necrosis (annexin-V positive and

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Abbreviations: AB, adult blood; **CB,** cord blood; **DC,** dendritic cells; **ROS,** reactive oxygen species

PI positive), and necrotic cells (annexin-V negative, PI positive) were then quantified using flow cytometry.

Quantification of ROS. Cells cultured for 7 d were exposed to either 21% or 95% O_2 for 1, 4, or 24 h. The ROS reagent (Molecular Probes) reacts specifically with hydrogen peroxide (H_2O_2), which converts 2′, 7′-dichlorodihydrofluorescein diacetate into 2′, 7′-dichlorofluorescein (13). It was freshly reconstituted with anhydrous DMSO and added to the cells at a concentration of 1 μg/mL of media during the last 15 min of O_2 exposure (14). The cells were washed twice with PBS and ROS were immediately quantified by determining the mean fluorescence intensity from each treatment group by flow cytometry.

Statistical methods. All experiments in this study were conducted three or more times. The marker patterns within each experiment for Figure 1 were reproducible but the magnitude of responses between experiments was variable, prompting us to illustrate the results of one individual experiment accompanied by a table summarizing the first phenotypic marker experiments (Fig. 1). To minimize individual variation, some of the data were represented as the ratio of the treated group to the control group for marker quantity and intensity of positive cells within the DC subpopulation (Table 1, Figs. 2 and 3). Mean values of the ratios and their SD were calculated for each variable. Data were analyzed using *t* test with Bonferroni correction for multiple comparisons where appropriate.

RESULTS

Hyperoxia reduced the number of mature DC. We first determined the effect of hyperoxia on the expression of CD1a and CD83 (Fig. 1). Double-negative ($CD1a^-/CD83^-$) cells derived from both CB and AB increased after 5 d of hyperoxia, as noted by the increased density in the lower left portion of the dot plots. However, the proportion of CB-derived cells that expressed CD1a and the intensity of that expression, were not affected by hyperoxic exposure, indicating a resistance to change of the number of CD1a CB-derived cells. In AB-derived cells, 5 d of hyperoxia significantly decreased both the number and the intensity of CD1a expression ($p \geq 0.001$ and 0.004, respectively), indicating that early in maturation, AB-derived DC are susceptible to hyperoxic damage. The direction and magnitude of change in the percentage of CD1a CB-derived DC relative to that of CD1a AB-derived DC due to hyperoxia was remarkable: under hyperoxic conditions, the percentage of CB-derived CD1a DC remained the same as was found under normoxic conditions (23% *versus* 26%), but the percentage of AB-derived CD1a DC decreased (from 59% to 16%), indicating a resistance of young CB-derived DC to hyperoxia compared with young AB-derived DC.

Concomitantly, both the CB-derived and AB-derived DC expressing the later maturity marker, CD83, decreased to <5%. Altogether, these data imply that, although the less mature CB-derived DC are more resistant than AB-derived DC to hyperoxic injury, both CB-derived mature DC, as well as AB-derived mature DC, are vulnerable to hyperoxic damage.

Two days of hyperoxia diminished marker intensity of mature CB-derived DC more than AB-derived DC. To study the effects of hyperoxia on the maturation process, we compared the CB- and AB-derived DC ratios of the hyperoxic to

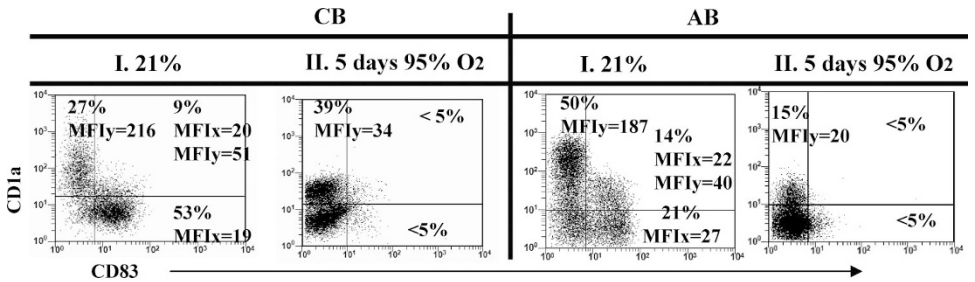

Figure 1. Hyperoxia (5 d 95% oxygen exposure) reduced the number of mature (CD83) DC in both CB and AB cells but immature (CD1a) CB-DC less than in AB-DC. [a]Significant reduction in the number of CD83-positive cells incubated on 21% *vs* 95% oxygen with $p < 0.0253$ (Bonferroni correction for two comparisons). $n = 3$ or more individuals per group treatment.

Surface cell markers		CB with Oxygen			AB with Oxygen		
		I. 21%	II. 95%	p^a	I. 21%	II. 95%	p^a
1.CD1a	a. %	23 ± 8	26 ± 8	0.16	59 ± 18	16 ± 6	0.001
	b. MFI	105 ± 63	39 ± 6	0.04	154 ± 73	27 ± 13	0.004
2. CD83	a. %	34 ± 22	4 ± 1.2	0.01	37 ± 6	4.5 ± 1	0.001
	b. MFI	57 ± 5	35 ± 11	0.11	27 ± 0.4	13 ± 4	0.002

Table 1. *Comparison of CB-DC vs AB-DC after 2 (I) and 5 (II) days of continuous hyperoxia*

Ratio of measured-values on 95% *vs* 21% O_2	I. 2-d exposure			II. 5-d exposure		
	CB	AB	*p*	CB	AB	*p*
1. CD83						
a. %	1.0 ± 0.2	1.0 ± 0.01	0.49	0.2 ± 0.1	0.1 ± 0.01	0.45
b. MFI	0.7 ± 0.1	1.0 ± 0.1	0.04	0.6 ± 0.3	0.5 ± 0.1	0.25
2. HLA-II						
a. %	1.0 ± 0.02	1.0 ± 0.02	0.37	0.8 ± 0.03	1.0 ± 0.01	0.0001*
b. MFI	0.6 ± 0.1	1.1 ± 0.1	0.002*	0.3 ± 0.1	0.2 ± 0.03	0.27
3. Endocytosis						
a. %	0.9 ± 0.1	1.0 ± 0.01	0.19	0.5 ± 0.4	0.4 ± 0.2	0.17
b. MFI	0.7 ± 0.1	0.8 ± 0.2	0.33	0.5 ± 0.1	0.3 ± 0.1	0.02

* $p < 0.017$ for CB-DC *vs* AB-DC after Bonferroni correction. $n = 3$–5 individuals per group treatment.

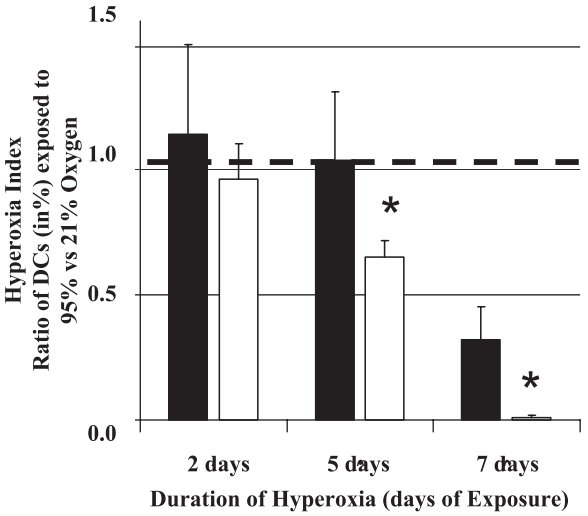

Figure 2. Prolonged hyperoxia decreased the number of cells less significantly in CB (■) compared with AB (□). The "Hyperoxia Index" is the ratio of remaining cells after exposure to 95% O_2over remaining cells following 21% O_2. The horizontal broken line represents the hyperoxia index of 21% O_2 treated cells from each individual, which is equal to 1. $n = 5$–9 in each group; * $p < 0.05$. *t* test was used to analyze the difference between 95% *vs* 21% O_2 exposures from the same group of cells; $p = 0.04$ for 5 d and $p = 0.02$ for 7 d hyperoxia group, respectively.

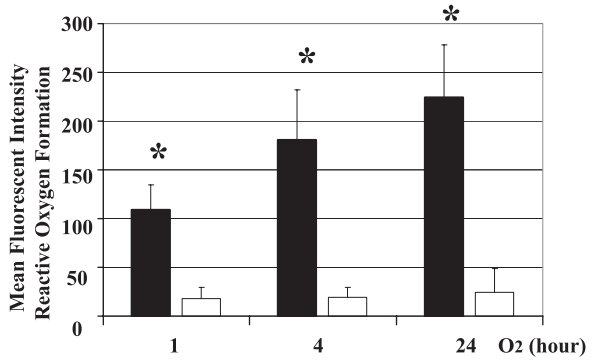

Figure 4. Hyperoxia caused higher ROS formation in CB-DC (■) than AB-DC (□). The formation of intracellular ROS was measured by FACS quantification of 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA). Data represents mean ± SD. *$p <$ 0.05 for CB-DC *vs* AB-DC.

normoxic marker quantity and intensity (Table 1). After 2 d of hyperoxia (column I), there was no significant difference in the relative number of DC expressing CD83 or HLA-II in both CB- and AB-derived DC in the hyperoxic groups compared with the normoxic group (rows: 1a and 2a). The ratio of the intensity of expression of both markers was lowered in CB-derived DC compared with AB-derived DC, although the effect is modest, and after Bonferroni correction, only those of AB-derived DC were significantly lowered (row 1b: $p = 0.04$ for CD83 and 2b: $p = 0.002$ for HLA-II).

After 5 d of exposure, the quantity and intensity of CD83-mature DC from either CB or AB were both reduced. The difference in the HLA-II positive proportion of cells at d 5 between CB-derived and AB-derived DC, while statistically highly significant, is of quite modest degree (Table 1). We postulate this to be due to the effect on the corresponding ration of the much more dramatic reduction in intensity of the AB-derived than CB-derived cells going from 2 to 5 d of hyperoxia (Table 1). Moreover, these findings may indicate that longer exposure indiscriminately depressed the quantity and intensity of DC expressing maturation marker CD83 and HLA-II in both CB- and AB-DC.

Hyperoxia affected endocytic activity. The ability of DC to capture antigen (endocytosis) is an important characteristic of immature DC, as this function declines with DC maturation (15). Neither a 2- nor 5-d exposure to O_2 alters the endocytic activity of either CB or AB-DC (Table 1, column I, rows 3a and 3b), suggesting that the hyperoxia did not alter the function of DC.

Hyperoxia decreased the number of viable AB cells more rapidly than CB cells. To assess the impact of hyperoxia on cells' viability, we compared the ratio of cells at the end of a 7-d culture between the hyperoxic (2, 5, or 7 d-exposure) *versus* normoxic groups (Fig. 2). Continuous hyperoxia for 5 d or more significantly decreased the number of AB cells (CB cells: $p = 0.25$; AB cells: $p = 0.02$), while CB cells were affected only after 7 d (CB cells: $p = 0.004$; AB cells: $p = 0.003$).

Hyperoxia caused necrosis and apoptosis in AB cells earlier than CB cells. We further compared the course of hyperoxic injury that leads to cell death by analyzing the percentage of necrotic, necrotic-apoptotic, or apoptotic cells after continuous hyperoxic exposure for 2 and 5 d (Fig. 3). After as little as 2 d of hyperoxia, the percentage of necrosed AB cells increased more than 3-fold, approximately double that of the CB cells ($p = 0.03$; Fig. 3, left panel first column). This difference disappeared when the cells were exposed for 5 d (Fig. 3, *left panel*; second column: both necrotic CB and AB cells increased 10-fold over the normoxic groups). The proportion also holds true for the necrotic-apoptotic cells (Fig. 3, *middle panel*). There was no difference in the degree of apoptotic cells from CB or AB cells, exposed either for 2 or 5 d (Fig. 3, *right panel*).

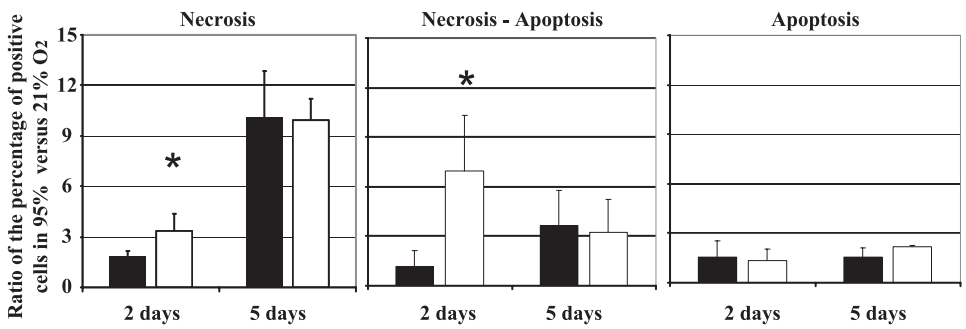

Figure 3. Hyperoxia induced cell death more gradually in CB- than AB-DC. The cells derived from CB (■) or AB (□) exposed to either 2 d and 5 d of 21% or 95% O_2 were assessed at the end of the 7 d culture for necrosis and apoptosis. In 2 d, AB-DC showed significant necrosis ($p = 0.03$) and necrosis-apoptosis ($p = 0.02$), where $p <$ 0.05. *t* test (with Bonferroni correction) was used to assess the significance of the difference between CB *vs* AB. Data represents mean ± SD of six individual treatment groups.

Hyperoxia produced higher ROS levels in CB than in AB cells. To investigate the dichotomy of the response to hyperoxic injury in CB *versus* AB cells, we measured the level of ROS at varying durations of oxygen exposure (Fig. 4). To provide more accurate interpretation of the impact of hyperoxia on the production of ROS and rapid decline of viable cells, only the first 24-h measurement was analyzed. In CB cells, ROS increased directly with increasing exposure to hyperoxia and the amount of intracellular ROS was significantly higher in CB than AB-derived cells.

DISCUSSION

Many cell types demonstrate an increased susceptibility to hyperoxic injury in more mature cells (7,16). Our study extends this to dendritic cells. There is a definite predilection to hyperoxic injury by more mature dendritic cells either within the cell lineage (Fig. 1: more CD83 positive cells were reduced than CD1a-positive cells in both CB and AB cells) or within different chronological ages (Fig. 1: more CD1a- and CD83-positive cells significantly reduced in AB- than CB-DC; Fig. 2: higher reduction in cell number in AB than CB cells). The consistent pattern of this effect supports the conclusion that immaturity confers some protection against hyperoxic injury. Specifically, our study also showed that the length of hyperoxia determined the gravity of the negative effect on both matured CB- and AB-DC (Table 1), and the induction of apoptosis-necrosis in the developing CB and AB cells (Fig. 3), similar to the reduction in cell viability observed when other forms of oxidative stress, such as hydrogen peroxide, have been used (17,18).

Our results support that of others that showed hyperoxia induced cell death through apoptotic (19,20), nonapoptotic (21), or both pathways (22). This is in contrast to our previous study where dexamethasone preferentially induced apoptosis and much less necrosis, in the immature dendritic cells (23). CB cells were more resistant to hyperoxia than AB cells (Fig. 2 and 3) in the present study, despite a higher level of reactive oxygen species in CB cells (Fig. 4). Additionally, the CB-derived cells showed a small but significant effect of hyperoxia on HLA-II expression (Table 1).

This paradox of increased oxidative stress from hyperoxia but less effect on immature DC or cells from cord-blood-derived cells may be explained by several previous observations. Adult cells have been shown to have lesser amounts or activity of antioxidative enzymes (24,25). Adults also produce higher amounts of monocyte chemotactic protein-1 after hyperoxic exposure, which can amplify the production of inflammatory cytokines (26) that may result in cell death. In addition, there is a relative maturational difference in lung nuclear factor (NF)-κB activation (27), a gradual switching of mitochondrial phosphorylation from glycolytic to oxidative metabolism (28,29) and higher level of oxidative phosphorylation (30), resulting in higher amplification of oxygen toxicity in adult cells. Individually or in combination, these findings could explain why the adult or more mature cells appeared more susceptible to the damaging effects of hyperoxia in this study. The relative predominance of anti-inflammatory cytokines such as IL-10 in the newborn compared with adult (10,31,32) may also contribute to a protective mechanism toward neonate cells against hyperoxia. We acknowledge that further work needs to clarify any effect of DMSO, the diluent used in stabilizing the dye in quantifying the oxygen reactive species, on the membrane permeability (33) of CB- *versus* AB-DC.

Despite their resistance toward hyperoxia, neonatal DC were more readily altered by hyperoxic exposure. For example, our study showed that within 2 d of hyperoxia, the number of necrotic cells was significantly higher in AB- than CB-DC, but that the CB-DC showed a significant lowering of maturation markers' expression intensity per cell (Table 1), suggesting that, in the remaining viable CB-DC, maturation was impeded. Five-day hyperoxia exposure caused the number of remaining viable cells capable of endocytic capacity to be reduced by half in both CB- and AB-DC, most probably *via* actin-dysfunction (34). However, the CB-DC capable of endocytic activity showed significantly higher ability than AB-DC to endocytose, supporting the cell marker findings that the remaining viable CB-DC were immature. There are potentially interesting implications of these findings. Immature DC activate regulatory T cells, promoting tolerance (35–37). Tolerance is vital in preventing premature rejection of the fetus by facilitating the suppressive effect on the maternal immune system (38,39). On the other hand, tolerance increases the risk that pathogens might evade the weakened protective immunity (36,37,40). It may be worth investigating whether immature neonatal DC, which are more resistant to hyperoxia, are then less able to help the immune system mount a response against invading pathogens. Baleeiro *et al.* (3) described the impairment of innate immunity upon exposure to sublethal hyperoxia in the adult animal. Given our current findings, investigating less mature hosts could yield significant results.

While results from our study are consistent and intriguing, several limitations and questions need to be addressed. Hyperoxia would most affect pulmonary DC and, although monocyte-derived DC strongly resemble pulmonary DC (8,9), there is some evidence that the local microenvironment can affect terminal differentiation (41). Only one high concentration of oxygen was used so it is not clear whether, for example, there is a different threshold for oxygen effects in the CB- *versus* AB-derived cells. We did not further characterize the cells that became CD1a and CD83 negative after 5 d in hyperoxia.

In summary, our findings demonstrate that although CB-DC are more resistant to hyperoxic-induced death than AB-DC, the surviving CB-DC are immature and dysfunctional. The survival of immature and dysfunctional DC may interfere with development of adaptive immunity in those infants with acute respiratory failure who are exposed to prolonged hyperoxia. This finding may provoke a need to reexamine the development of induced adaptive immunity through vaccination of infants exposed to prolonged hyperoxia.

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