The Range of $P_aO_2$ Variation Determines the Severity of Oxygen-Induced Retinopathy in Newborn Rats

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Purpose. This study was conducted to determine the potential influence of $P_aO_2$ fluctuation on the retinal neovascular response known to occur in newborn rats exposed to hyperoxic conditions. As an inherent corollary, the authors also defined the relationship between the fraction of inspired oxygen ($F_iO_2$) and the arterial blood oxygen tension ($P_aO_2$) in newborn rats.

Methods. Experiment 1 was composed of several oxygen-exposure protocols in which atmospheres of 10% oxygen concentration were alternated with different higher levels of ambient oxygen (50%, 40%, 30%, and room air). In experiment 2, two alternating oxygen concentrations were made to converge toward room air (20.9% oxygen) with each successive group of four treatment groups. These included another group exposed to alternating 50% and 10% oxygen, a group exposed to alternating 45% and 12.5% oxygen concentrations, one exposed to alternating concentrations of 40% and 15% oxygen, and a final group exposed to 35% and room air oxygen concentrations. In each case, oxygen was alternated between the two exposure concentrations every 24 hours. The term $\Delta F_iO_2$ is used to designate the difference in the two oxygen concentrations to which a treatment group was subjected, applying the units of fraction of inspired oxygen (i.e., $\Delta F_iO_2 = 0.5$ for the exposure to alternating 50% and 10% oxygen). At birth, litters of albino rats were placed in each of these environments for 15 or 14 days, after which $P_aO_2$ and retinal vascular development were assessed in some rats. The remainder were removed to room air for 4 days before the incidence and severity of abnormal neovascularization were measured.

Results. $P_aO_2$ and $F_iO_2$ were directly and linearly correlated ($r^2 = 0.998$). In experiment 1, the extent of retinal vascular development on removal from oxygen was a linear function of $\Delta F_iO_2$. Retinal neovascularization subsequently occurred in all rats exposed to alternating 50% and 10% or 40% and 10% oxygen concentrations, but only a third of the 30% and 10% exposure group, indicating a minimum threshold for proliferative disease at $\Delta F_iO_2 = 0.2$. In experiment 2, retinal avascularity also increased linearly with increasing $\Delta F_iO_2$. There was a threshold for neovascularization between the exposure to alternating 45% and 12.5% oxygen and the 40% and 15% oxygen exposure (100% versus 4.8% incidence of neovascularization), indicating a requirement of $\geq 12.5%$ oxygen episodes to stimulate a consistent proliferative response.

Conclusions. These results suggest that $P_aO_2$ fluctuation and degree of hypoxia may have more influence on proliferative retinal disease in newborn rats than the extended hyperoxia that has historically received greater attention. Experimental designs that address the inherent differences in pulmonary function between intrinsically healthy animals and compromised premature infants are of substantial value to our understanding of the pathogenesis of retinopathy of prematurity. Invest Ophthalmol Vis Sci. 1995;36:2063–2070.

Experimental models of retinopathy of prematurity (ROP) traditionally have involved exposure of newborn rats,1–3 cats,4–5 dogs,6–8 rabbits,9 or mice10 to constant levels of hyperoxia. Typically, the target level of oxygen is rigidly adhered to in these experiments. This is probably because in the clinical setting neonatologists attempt to provide consistent oxygen therapy—often a difficult task in the fragile, very low birth...
weight neonate. Yet, newborn rats have demonstrated a greater likelihood for preretinal neovascularization as the result of exposures to variable oxygen, particularly when the experimental protocols are designed to produce arterial oxygen partial pressures (PaO\textsubscript{2}) that are reflective of the sick premature infant's physiologic condition. The neonate's variable PaO\textsubscript{2} profile includes hypoxic episodes that are the consequence of apnea, bronchopulmonary dysplasia, and other respiratory or metabolic complications. Because the newborn rat is intrinsically healthy, ambient hypoxia is necessary to mimic the infant's episodes of systemic hypoxia.

We have established the relationship between the ambient oxygen concentration, expressed as the fraction of inspired oxygen (FiO\textsubscript{2}), and PaO\textsubscript{2} using newborn rats exposed to various oxygen environments. Our purpose in deriving this relationship was to determine the clinical relevance of our experimental oxygen treatments and to learn what exposure paradigm would most closely mimic the PaO\textsubscript{2} profile of premature infants with ROP. We also have attempted to define the degree of oxygen variability allowable in newborn rats before proliferative retinopathy results, thereby taking a substantial step toward understanding the importance of PaO\textsubscript{2} variation in the pathogenesis of the human disease.

**METHODS**

**Oxygen Exposures**

Two series of treatments were conducted. In the first series, newborn rats were subjected to oxygen concentrations alternating every 24 hours between 10% and some level of hyperoxia that differed between experimental groups (experiment 1). In the second series, both alternating oxygen concentrations—one hypoxic and the other hyperoxic—differed between treatment groups (experiment 2). For example, in experiment 1, one treatment group consisted of a litter of newborn albino rats placed at birth with its mother in a chamber containing 50% oxygen concentration. Twenty-four hours later, the oxygen concentration was quickly lowered to 10%, where it remained for 24 hours. The 10% oxygen atmosphere was produced by mixing appropriate fractions of pure nitrogen and room air. The oxygen level in the chamber continued to alternate between 50% and 10% every 24 hours for 14 days. This exposure is designated "50/10" in the subsequent text. Other litters were simultaneously exposed to atmospheres that alternated in the same manner between 40% and 10% oxygen concentration, between 30% and 10%, or between room air and 10% (designated 40/10, 30/10, and 21/10).

Both oxygen concentration (%) and fraction of inspired oxygen (FiO\textsubscript{2}) are used to describe the oxygen levels used in these experiments. In general, concentration is used when discussing the mechanics of oxygen delivery to the exposure chambers or when naming treatment groups (i.e., 50/10). FiO\textsubscript{2} is used in other contexts within the text and in the figures. The difference in the two oxygen concentrations to which a treatment group was exposed in experiment 1 was 40%, 30%, 20%, or 11%, respectively. The term ΔFiO\textsubscript{2} is used to define this parameter of the exposures, and its units are fractions: 0.4, 0.3, 0.2, and 0.11 for the four groups in experiment 1.

In experiment 2, the exposure to alternating oxygen concentrations of 50% and 10% was repeated in another litter. Three new treatment protocols were added in which the oxygen concentration alternated between 45% and 12.5%, between 40% and 15%, or between 35% and room air (designated 45/12.5, and so on). The ΔFiO\textsubscript{2} of these four exposures was 0.4, 0.325, 0.25, and 0.14, respectively. Length of exposure was, again, 14 days.

On removal from the exposure chamber, some rats were killed to determine the extent of retinal vascular development. The remainder were allowed 4 days after exposure in room air before they were killed to measure the incidence and severity of the retinal neovascular response. A second litter was subjected to each of the seven different exposure protocols. Oxygen was maintained within 0.3% of the intended level using an interactive controller designed and manufactured for this purpose (Reming Bioinstruments, Redfield, NY). Two hundred fourteen rats were treated with these exposures. All experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Rat Blood Gases**

In rats exposed to some of the conditions described above as well as a litter exposed to 80% constant oxygen, PaO\textsubscript{2} and PaCO\textsubscript{2} measurements were made on day 13 or 14 at least 6 hours after the time of oxygen change. The procedure involved deep anesthesia with 40 to 50 mg/kg sodium pentobarbital, followed by tracheotomy and ventilation of the lungs with a Harvard Rodent Ventilator (model 683; Harvard Apparatus, South Natick, MA). The source gas to the ventilator consisted of the ambient oxygen level for each exposure group (10%, 15%, 21%, 40%, 50%, or 80%), ventilation rates of 65 to 80 pulses per minute, and volumes of 0.25 to 0.4 ml. These parameters were chosen to match the rats' preanesthesia levels for each respective breathing environment. Measurements of blood gases were made using a Blood Gas Analyzer (model 1306; Instrumentation Laboratory, Lexington, MA), and 300 μl of blood taken from the left ventricle with a heparin-flushed 0.5 ml syringe and a 22-gauge needle.
The degree of retinal avascularity was assessed in 46 rats (n = 8 for the 40/15 and 30/10 exposure groups; n = 6 for the remaining groups) using an adaptation of the method of Smith et al. This entailed deep anesthesia with 40 mg/kg sodium pentobarbital and injection of 9.0 mg of fluorescein isothiocyanate-dextran (145,000 average MWt; Sigma, St. Louis, MO) in 40 μl of phosphate-buffered saline vehicle into the left ventricle. After 80 seconds, the rats were killed, the eyes were enucleated, and the retinas were dissected, flatmounted on microscope slides, and assessed using an inverted fluorescence microscope (Olympus IMT-2; Olympus Optical, Tokyo, Japan). Analysis of avascular regions was accomplished with the aid of a computer image analysis apparatus designed in this laboratory and described elsewhere. Briefly, images of the flattened, fluorescein-perfused retinas were captured with a digitizing camera. Avascular retinal regions were then outlined using an interactive digitizing stylus. The more central of the two major capillary-free zones (immediately surrounding the optic nerve head) was analyzed separately from the sum of the more peripheral avascular areas (adjacent to the ora) in the four quadrants of the flattened retinal tissue. Using Enhance software (MicroFrontier, Des Moines, IA), the fraction of the total retinal area contained in these two avascular regions was calculated. The two values obtained from the two retinas of each rat were averaged to constitute one data point.

Abnormal neovascularization was assessed on day 18 using dissected, flatmounted retinas stained for ADPase activity. This histochemical staining procedure is a previously described adaptation of a method developed by McLeod and Lutty. The method stains only retinal vascular endothelia and their stem cells in rats of this age. These retinas were flattened on microscope slides using four radial cuts marked for orientation of the superior quadrant and overslimped in 1:1 phosphate-buffered saline:glycerol. The presence of preretinal neovascularization was then determined by trained observers using adjustment of the plane of focus at high magnification. Counting the number of clock hours occupied by pathologic vessel growth is an established unit of clinical assessment of ROP. To grade severity of pathology, a clock face was superimposed on the retinal surface. Areas between the separated quadrants of the flattened retinas were omitted from the assessment. This approach yielded a semiquantifiable measure of severity. Retinas were scored independently by three masked investigators, and the three assessments were averaged for each retina.

Selected retinas were removed from the slides after staining and assessment and were processed for histologic sectioning to study the preretinal neovascularization identified in flatmounts. These retinas were fixed in 2.5% glutaraldehyde, dehydrated in an ethanol series, and infiltrated and embedded in Embed 812 (Polysciences, Warrington, PA). Sections were 0.5 μm in thickness and were stained with 1.0% toluidine blue.

The infant PaO2 range was derived from a retrospective case-control study of infants weighing 1500 g or less at birth. All infants were admitted to Arkansas Children’s Hospital neonatal intensive care unit within the first 3 days of life between June 1, 1991 and May 31, 1992. Infants surviving at least 6 weeks with their highest active stage of ROP documented were considered. Nine of the 106 surviving infants were discharged before examination. Both eyes of each remaining infant initially were examined 4 to 6 weeks after birth by an ophthalmologist specializing in pediatric or vitreoretinal diseases using indirect ophthalmoscopy. Depending on the severity of ROP, subsequent examinations occurred weekly or biweekly until retinal vasoformation was completed.

The most severe stage of ROP was identified using the International Classification of Retinopathy of Prematurity. Eyes were diagnosed as prethreshold (n = 13) or threshold ROP (n = 13) according to the standards established in the multicenter cryotherapy trial. A second examiner was required to confirm independently all diagnoses of threshold ROP.

Arterial blood gas values obtained during routine intermittent sampling throughout the first 30 days of life were extracted from hospital laboratory computer records. The majority of the samples were postductal and were obtained by indwelling umbilical arterial catheters. No capillary blood gas values were included. We recorded PaO2, FiO2, and the time of each determination. The 13 infants with threshold ROP had an average birth weight of 876 ± 245 g, were of 26.0 ± 2.0 weeks gestational age at birth, and had an average PaO2 during oxygen therapy of 71.7 ± 7.6 mm Hg.

For each of the 13 infants with threshold ROP, an average PaO2 was determined for each day on oxygen. Next, the highest of each infant’s daily average values and the lowest of each infant’s daily average values were used to determine a range of PaO2 for each infant. These 13 ranges are depicted as vertical lines on the right side of Figure 1. Then, the 13 high daily averages were averaged and the 13 low daily averages were averaged to create a range of average oxygen values for the entire group (the shaded rectangle in Fig. 1). Finally, because this range is conservative owing to the averaging process and, therefore, it may represent a relative underestimation of the true PaO2 variability, the extremes of the daily averages for the group are depicted as well (dotted horizontal lines).
FIGURE 1. The relationship between \( F_{\text{IO}_2} \) and \( P_{\text{O}_2} \) was found in newborn rats exposed to extended periods of various \( F_{\text{IO}_2} \). The relationship is linear \((r^2 = 0.998)\). The average range of \( P_{\text{O}_2} \) variation in a group of infants in whom threshold retinopathy of prematurity developed is superimposed upon the plot (shaded area). A less conservative estimation of \( P_{\text{O}_2} \) variation was calculated and also is depicted (dotted horizontal lines). The 13 vertical lines on the right side of the graph illustrate the individual \( P_{\text{O}_2} \) ranges of the 13 threshold infants. The \( x \)-axis has no bearing on the position of these lines. The \( F_{\text{IO}_2} \) at which the limits of the two estimated infant \( P_{\text{O}_2} \) ranges cross the relationship curve (arrows) may represent clinically relevant ranges of variable oxygen exposure for animal experiments.

This second range represents the difference between the highest daily average from any individual and the lowest daily average from any individual. The average number of blood gas determinations during days that contributed to the illustrated daily averages was 15.0. The average number of hours during which blood gases were measured on these days was 18.6. For all 13 infants, the highest and lowest daily averages occurred within the first 8 days of oxygen therapy.

RESULTS

The relationship between \( F_{\text{IO}_2} \) and \( P_{\text{O}_2} \) in the rats is shown in Figure 1. The rat data exhibit a linear relationship between the two parameters, with a correlation coefficient of 0.998. Superimposed on the data acquired from newborn rats is an illustration of the average range of \( P_{\text{O}_2} \) in the 13 infants with threshold ROP. The points at which this average range intersects the rat \( F_{\text{IO}_2} \) versus \( P_{\text{O}_2} \) relationship correspond to oxygen concentrations of 10.3% and 23.7%. Also depicted is a less conservative measure of \( P_{\text{O}_2} \) variation that used the highest of the 13 high daily average \( P_{\text{O}_2} \) values and the lowest of the 13 low daily averages. The intersections of this range with the rat data occur at 8.0% and 29.2% oxygen concentration. This means that exposing rats to oxygen varying within these ranges should result in \( P_{\text{O}_2} \) values representative of the fluctuating systemic oxygen in infants with threshold ROP. \( P_{\text{CO}_2} \) values from rats in each oxygen environment varied between 40.1 ± 5.2 and 52.6 ± 8.1 mm Hg (mean ± SD), with no significant differences between experimental groups.

Table 1 describes the degree of retinal avascularity that resulted from each exposure when measured at the time of removal from oxygen. These data are plotted in Figure 2. There was a linear relationship between the \( \Delta F_{\text{IO}_2} \) in the animal experiments and the degree of retinal nonperfusion that resulted from the exposure (Fig. 2, \( r^2 = 0.979 \)).

Figure 3 describes the incidence of proliferative retinopathy (i.e., neovascularization) in rats subjected to different degrees of oxygen variation followed by 4 days in room air. In experiment 1, rats exposed to oxygen levels varying by 0.3 \( \Delta F_{\text{IO}_2} \) or more sustained retinopathy 100% of the time; exposure groups that received an oxygen variation of 0.11 \( \Delta F_{\text{IO}_2} \) never developed retinopathy; the 30/10 group, representing \( 0.2 \Delta F_{\text{IO}_2} \), sustained an intermediate incidence of retinopathy—33%. Experiment 2 yielded similar results, with all animals subjected to oxygen variation of 0.325 \( \Delta F_{\text{IO}_2} \) or more developing retinopathy; animals that received a variation of 0.14 \( \Delta F_{\text{IO}_2} \) never developed retinopathy; and the 40/15 group, representing a variation of 0.25 \( \Delta F_{\text{IO}_2} \), sustained a 4.8% incidence of retinopathy.

The severity of retinopathy resulting from each exposure is described in Table 1. Severity is represented by the average number of clock hours of retinopathy considering only those retinas that displayed some degree of pathology. The findings typical of the more damaging exposures are illustrated in Figure 4. The formation of ridges (Fig. 4A), similar to those observed in human ROP, was seen only in the oxygen protocols that resulted in 100% incidence of retinopathy. Milder forms of neovascularization, such as hyperplastic tufts or spheres of endothelial cells that penetrated the inner limiting membrane (Fig. 4C), were found in the retinas of all exposure groups that displayed retinopathy. These tufts were located just central (posterior) to the leading edge of vessel advancement.

DISCUSSION

Clearly, rats that receive \( F_{\text{IO}_2} \) of 0.8 (the most common experimental level described in the literature) do not have \( P_{\text{O}_2} \) levels representative of a premature infant on oxygen therapy. According to our data, exposures
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**TABLE 1. Effect of Variable Oxygen Exposures**

<table>
<thead>
<tr>
<th>Treatment (sample size)</th>
<th>( \Delta FiO_2 )</th>
<th>Avascular Area* (% total retinal area) (mean ± SD)</th>
<th>Retinopathy†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Central</td>
<td>Peripheral</td>
</tr>
<tr>
<td>50/10 (42)</td>
<td>0.4</td>
<td>4.2 ± 2.1</td>
<td>25.2 ± 7.6</td>
</tr>
<tr>
<td>40/10 (29)</td>
<td>0.3</td>
<td>3.9 ± 1.8</td>
<td>16.9 ± 5.2</td>
</tr>
<tr>
<td>30/10 (41)</td>
<td>0.2</td>
<td>1.2 ± 0.6</td>
<td>8.9 ± 3.6</td>
</tr>
<tr>
<td>21/10 (29)</td>
<td>0.11</td>
<td>0</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td>45/12.5 (24)</td>
<td>0.325</td>
<td>2.6 ± 1.2</td>
<td>16.4 ± 5.8</td>
</tr>
<tr>
<td>40/15 (29)</td>
<td>0.25</td>
<td>1.0 ± 1.0</td>
<td>13.3 ± 3.9</td>
</tr>
<tr>
<td>35/21 (20)</td>
<td>0.14</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Assessed immediately on removal from oxygen exposure; † assessed after a 4-day postexposure period in room air; ‡ averages are calculated only from rats displaying some degree of retinopathy.

to oxygen concentrations varying between the range from approximately 10% to 25% or 30% would be appropriate for animal experiments because those are the values that produce \( Pao_2 \) in rats similar to those of infants in whom threshold ROP develops. This was determined by calculating the two locations in which the infant \( Pao_2 \) range intercepts the rat \( FiO_2 \) versus \( Pao_2 \) curve. In fact, similar exposures (30/10) caused a 33% incidence of severe proliferative retinopathy in newborn rats. In this case, our intent was not so much to create a model in which all animals sustain retinopathy (although several of our exposures produced this result) as it was to begin to develop a model with clinical relevance so rational experiments designed to illuminate the pathogenesis of ROP follow a natural progression. It should be emphasized that fewer than 10% of infants with early stages of the disease progress to "threshold" ROP. In fact, even if the analysis is limited to infants with "prethreshold" disease, threshold ROP develops in only 30% to 35%.

The degree of linearity shown by the \( FiO_2 \) versus \( Pao_2 \) relationship in rats is a fortunate result. That it exists despite long-term exposures to extreme hypoxia and hyperoxia is probably because of the resiliency of newborn rats' pulmonary function. The linearity allows for simple interpolation and calculation of \( Pao_2 \) values from \( FiO_2 \) values. This, in turn, facilitates dialogue between investigators conducting animal experiments and neonatologists caring for infants—an advantage for understanding and preventing the disease.

Our exposure protocols represent a first attempt to determine the degree of oxygen variability allowable before proliferative retinopathy results. Experiment 1 used the most extreme hypoxia consistent with 100% survival during the 24-hour episodes. Better management of oxygen delivery to premature infants is accomplished not only through limiting or eliminating hyperoxia, but through avoiding hypoxic episodes as well, although generally the latter is more difficult. Experiment 2 was designed to test the importance of the percentage of hypoxia while still providing \( \Delta FiO_2 \) values similar to those in experiment 1. The various exposure protocols reveal a minimum threshold for developing retinopathy at approximately 0.2 \( \Delta FiO_2 \), representing a range of \( Pao_2 \) variation of 90 mm Hg. Interestingly, this is exactly the range demonstrated...
FIGURE 3. $\Delta F_iO_2$ is related to the propensity for neovascularization as shown by the data from experiments 1 and 2. The shaded areas represent arbitrary thresholds of $\Delta F_iO_2$, below which little retinopathy occurs and above which all animals develop retinopathy. The extent of hypoxia during the episodes at low oxygen is probably responsible for the paradoxical outcome that the 30/10 exposure is more damaging than the 40/15 exposure.

by the less conservative means of calculating $P_aO_2$ variability in infants with threshold disease (dotted horizontal line, Fig. 1).

Previous experiments comparing exposure groups in which the level of absolute hyperoxia was different while $\Delta F_iO_2$ remained the same have proven that the extent of hyperoxia, per se, does not determine the propensity for neovascularization. These experiments compared a 24-hour alternating exposure using 80% and 40% oxygen to an identical exposure protocol using 50% and 10% oxygen ($\Delta F_iO_2 = 0.4$ in both cases). The 80/40 treatment group developed retinopathy 72% of the time, whereas the 50/10 group sustained 97% retinopathy. In those rats that suffered some degree of retinal pathology, the 80/40 group displayed only 4.2 clock hours of neovascularization on average, and the 50/10 group showed 8.0 clock hours. Clearly, any number of oxygen concentration combinations can result in the same $\Delta F_iO_2$, and we have tested the importance of $\Delta F_iO_2$ in a limited cross-section of those combinations. We chose the range of oxygen concentrations used in these experiments because of the $P_aO_2$ ranges that resulted from them. The middle ranges (i.e., 30/10 exposure) yielded clinically relevant $P_aO_2$ values, and the extreme ranges (50/10 and 21/10, for example) allowed us to observe the full spectrum of incidence and severity of retinopathy. In the current experiments, two exposures with identical levels of hyperoxia, 40/10 and 40/15, yielded incidences that were vastly different (100% versus 4.8%). If the differences in incidence of retinopathy cannot be explained entirely by the level of hyperoxia, other possibilities must be sought, and $\Delta F_iO_2$ is a reasonable candidate as evidenced by Figure 3. In the premature infant, it is extremely difficult to separate the parameter of systemic oxygen fluctuation from those respiratory and metabolic conditions that lead to it. Yet, the fact that the degree of $P_aO_2$ fluctuation is related to retinopathy in the newborn rat, where these conditions do not exist, supports the idea that the parameter is critical in the development of the human disease.

But $\Delta F_iO_2$ is not the only critical aspect of variable oxygen exposures. The 40/15 exposure of experiment 2, with a $\Delta F_iO_2$ of 0.25, yielded significantly less retinopathy than the 30/10 exposure of experiment 1, with a $\Delta F_iO_2$ of only 0.20. This implies that not only is the degree of oxygen variation important, the percentage of absolute episodic hypoxia is as well. The indication is that stimulation of a consistent proliferative response requires episodes of 12.5% oxygen or less, which corresponds to a $P_aO_2$ of 61.7 mm Hg. The results suggest that even infants whose oxygen therapy course is relatively well controlled are at risk if they sustain some extended periods of systemic hypoxia. Perhaps this explains how ROP develops in infants with cyanosis who never have hyperoxia.21 Another possible explanation is the presence of hypercarbia; recent clinical22–24 and experimental25–27 attention has been focused on this issue. However, because our rat blood gases were determined during mechanical ventilation, little can be concluded from the $P_aCO_2$ values other than the reliability of the concomitant $P_aO_2$ measurements.

If the $F_iO_2$ data are transformed to $P_aO_2$ using the relationship derived for newborn rats, one can begin to consider ranges of $P_aO_2$ variation allowable in humans. This practice naturally invites criticism of the validity of comparing pulmonary function in rats and infants, and we agree that caution is required. One of our exposures (30/10) does in fact closely mimic the clinical situation in terms of the range of $P_aO_2$ fluctuation it causes. But the frequency of oxygen variation in our experiments is dictated by the logistics of our oxygen control system, and it is not clinically relevant. Episodes of hypoxia are rigorously avoided in the neonatal intensive care unit, and although an infant’s $P_aO_2$ level can drop into the 50-mm Hg or even the
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FIGURE 4. The 50/10, 45/12.5, and 40/10 exposure protocols all caused the formation of ridges in some rats, similar to those seen in human retinopathy of prematurity. Panel A illustrates this form of pathology (black arrows) in the ADPase-stained retina of a rat from the 45/12.5 group killed 4 days after removal from the exposure chamber. Also included in this figure is a large-caliber shunt joining a major artery to an adjacent vein (white arrow). Panel B shows neovascular tissue that includes lu- menized vessels containing red blood cells (RBCs). The vessels and accompanying cells have penetrated the inner limiting membrane. Because this tissue section was not produced from an intact eyecup, the presence of extravascular RBCs in the vitreous (arrows) cannot be viewed as evidence of hemorrhage. Panel C demonstrates a milder form of neovascularization. All treatments that induced retinopathy caused the formation of hyperplastic spheres of endothelial cells (arrows) in some rats.

20-mm Hg range, the duration of these levels can be measured in minutes rather than hours. Further, the fluctuations in PaO$_2$ generally have a randomness that is difficult to reproduce in the experimental setting. We have conducted experiments designed to test the importance of the frequency of oxygen variation. However, the shortest period of alternating oxygen levels in these experiments was 6 hours—too long to be reflective of the infant's fluctuating systemic oxygen. These studies conclusively showed that the frequency of fluctuation, at least in the range between 6 and 48 hours, was critical when the oxygen was alternated between 80% and 40%. Prolonged exposures (48 hours) to relative hypoxia or hyperoxia were determined to be more detrimental than more frequent (every 6 hours) oxygen concentration changes. The 6-hour alternating exposure to 80% and 40% oxygen resulted in the least severe retinopathy as evaluated 4 days after removal from the chamber. Of course, this says nothing of the effect of fluctuation frequency in the range of oxygen concentrations used in the current set of experiments.

Finally, an aspect of proliferative retinopathies that has received considerable attention is the theory that neovascularization is driven by ischemia-induced hypoxia. Because no neovascularization was observed immediately on removal from the exposure, it is reasonable to assume that the postexposure environment promoted its stimulation. But all rats were given the same postexposure environment, so another difference between experimental groups must be sought to explain the differences observed in retinopathy, and the most obvious is the degree of avascularity with which they entered the postexposure period. If one assumes that the avascular retinal area becomes hypoxic on removal from oxygen, the relationship between hypoxia and neovascularization can be examined using the results obtained from these experiments. As Figure 2 illustrates, ΔFiO$_2$ and avascular area immediately on removal from the exposure are closely and linearly related ($r^2 = 0.979$). Within the boundaries of the various treatments studied here, one can predict with accuracy the avascular area that will result from a given exposure regimen. Based on this correlation, the theory that retinal nonperfusion and subsequent neovascularization are causally related appears well grounded. However, just as the relationship between ΔFiO$_2$ and neovascularization is not linear, a direct relationship between avascular area and subsequent neovascularization does not hold. This failure is again caused by the 30/10 and 40/15 exposures. There is a significant difference between the extent of avascular retina in these two exposure groups ($P < 0.01$; $n = 8$ for each exposure group). The protocol with the greater avascular area (40/15)
yields only a 4.8% incidence of retinopathy, whereas that with the smaller avascular area (30/10) yields a 33% incidence. Again, this is probably caused by the absolute percentage of episodic hypoxia, which is more extreme in the 30/10 exposure. Thus, the 30/10 group clearly suffered more retinal hypoxia during the exposure, but the 40/15 group probably suffered more upon removal to room air, which is when neovascularization commences. The resultant incidence of retinopathy in these two groups indicates that avascular area, although important, is not the single overriding factor in producing post-exposure neovascularization, and it calls into question the theory that ischemia-induced retinal hypoxia on removal from the exposure chamber is its sole driving force. It is likely that periods of absolute and relative retinal hypoxia during exposure (which are not caused solely by ischemia) are critical, even though they may not manifest themselves until after removal to room air.

Key Words
hypoxia, neovascularization, Pao2, rat, retinopathy of prematurity

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