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Exposure to Alternating Hypoxia and Hyperoxia Causes Severe Proliferative Retinopathy in the Newborn Rat

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ABSTRACT

Exposure to variable hyperoxia has recently been shown to be much more effective at producing proliferative retinopathy in the newborn rat than exposure to constant hyperoxia. To incorporate a more clinically relevant oxygen-exposure paradigm in our studies, we have now used a cycle between 50 and 10% oxygen and have compared its effects with those found using new exposures to the previously used 80/40% cycle. Starting at birth and continuing for 14 d, rats were exposed to environments that cycled between 50 and 10% oxygen or 80 and 40% oxygen every 24 h. After exposure, some rats were killed for assessment of retinal vascular development. Others were removed to room air for 4 d before killing and evaluation for the presence of abnormal neovascularization—a clinical consequence believed to be promoted by termination of oxygen therapy. The 50/10% cycle resulted in greater retardation of retinal blood vessel development during oxygen than that found in the 80/40% exposure group. After 4 d postexposure in room air, the incidence of preretinal neovascularization was 97% in the 50/10% rats and 72% in the 80/40% group. Clearly, the overall amount of oxygen the subject receives is less critical than other parameters of its administration in producing proliferative retinopathy. Also, the range of variation (40% in both cases) is not the controlling characteristic. Our results suggest that consistency of oxygen level and avoidance of hypoxic levels should be important concerns in neonatal oxygen therapy. (***Pediatr Res* 36: 724–731, 1994**)

Abbreviations

ROP, retinopathy of prematurity
FiO_2, fractional inspired oxygen
Pao_2, arterial blood oxygen partial pressure
$Paco_2$, arterial blood carbon dioxide partial pressure
ADPase, adenosine diphosphatase
ICU, intensive care unit

ROP is a complex disease involving multiple factors. Oxygen was first recognized as a critical factor in the early 1950s (1–3), but the potential for prolonged hyperoxia alone to cause or exacerbate ROP is still not clearly defined. The disease persists despite the attention now placed on careful monitoring and limited oxygen delivery. Additional doubt about the role of hyperoxia in the pathogenesis of ROP has been raised with the finding that cyanotic premature infants can develop ROP in the absence of oxygen therapy (4). The theory (5) that the developing retina is highly sensitive to any disruption of its oxygen supply, whether hyperoxemic or hypoxemic, warrants consideration. Recent studies in animals (6, 7) have addressed this issue.

Exposure to variable hyperoxia has been shown to be a much more effective stimulus of proliferative retinopathy in the newborn rat than exposure to constant hyperoxia (6). This previous study compared the effect of a cyclic variation of oxygen between 80 and 40% with that of a constant 80% exposure. An exposure paradigm that incorporates fluctuations in oxygen level is clearly more representative of the neonatal setting than the constant exposures typical of animal studies. Still, variations in inspired oxygen (FiO_2) between 80 and 40% do not accurately reflect the therapeutic levels received by premature infants. Neonates suffering apnea, patent ductus arteriosis, bronchopulmonary dysplasia, or one of a number of other respiratory complications often fluctuate between hyperoxic and hypoxic arterial partial pressures during therapy. This subpopulation of neonates is at high risk for ROP (8, 9). To more closely model the clinical setting, we have now used an exposure cycle that varies

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between 50 and 10% FiO_2. Ambient hypoxia is necessary to produce episodes of low Pao_2 in intrinsically healthy newborn rats.

METHODS

Upon birth, litters of Sprague-Dawley rats and their mothers were placed in a variable oxygen environment that consisted of 24 h of 50% oxygen followed by 24 h of 10% oxygen. The 10% oxygen atmosphere was produced by mixing appropriate fractions of pure nitrogen and room air. The oxygen level continued to alternate between 50 and 10% every 24 h for 14 d, the time required for a room-air raised rat to complete retinal vascular formation. Simultaneously, rats were exposed to a similarly alternating exposure composed of 80 and 40% oxygen levels (Fig. 1). Other litters were raised in room-air conditions as a control. Some rats from each exposure group were killed on d 14 immediately after exposure to assess retinas for degree of avascularity. The remaining rats were removed to room air on d 14 and were killed on d 18 to assess retinas for abnormal neovascularization. A total of 16 litters (169 rats) were used for the experiments. There was no mortality caused by the oxygen treatments, although preliminary trials showed that extending exposure to 10% oxygen environments beyond 24 h could be lethal. Treated rats were weighed upon removal from oxygen, as were room-air–raised rats at the same age. All animal experimentation was performed with the highest standards of humane care and conformed to the *NIH Guide for the Care and Use of Laboratory Animals.*

Retinal avascularity. The degree of retinal avascularity was calculated for 21 rats (n = 15 and n = 6 for 50/10% and 80/40% treatments, respectively) using an adaptation of the method of D'Amato and Smith (10). This entailed deeply anesthetizing the ratling with 40 mg/kg sodium pentobarbital and injecting 9.0 mg of fluorescein isothiocyanate-dextran (145 000 molecular weight, Sigma Chemical Co., St. Louis, MO) in a 40-μL PBS vehicle into the left ventricle. After 80 s, the ratlings were killed, the eyes enucleated, and the retinas dissected and flat-mounted on microscope slides and assessed using an inverted fluorescence microscope (Olympus IMT-2, Olympus Optical Co., Tokyo, Japan). Analysis of avascular regions required the aid of a computer-image analysis apparatus described elsewhere (6). The diameter of major arteries and veins was measured 1.0 mm from the optic nerve head at ×1000 magnification with the aid of an ocular micrometer.

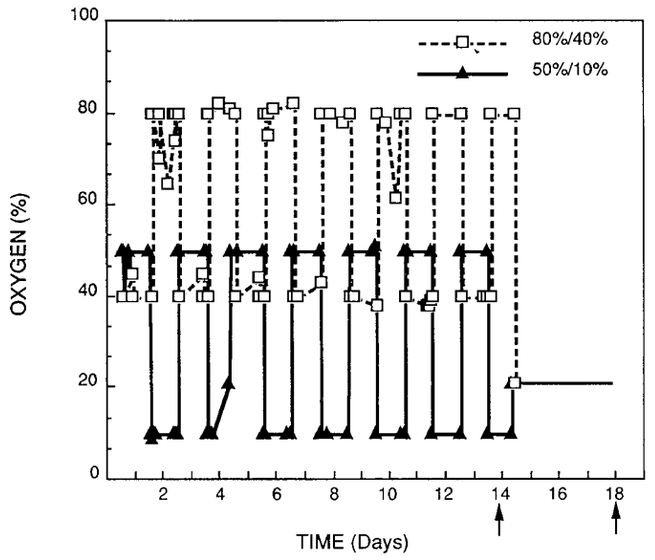

Figure 1. Oxygen exposure paradigm. This schematic is a real-time plot of the course of two typical oxygen exposures. The litters are placed in incubators within 4 h after birth and the oxygen level is adjusted every 24 h thereafter. The *arrows* denote times of killing.

Retinopathy. Abnormal neovascularization was assessed in 73 rats on d 18 using fresh retinal dissections to produce flat-mounted retinas that were stained for ADPase activity. This histochemical staining procedure is a previously described adaptation (6) of a method developed by Flower *et al.* (11). This method stains only retinal vascular endothelia and their stem cells in rats of this age (12). The presence of preretinal neovascularization was then determined by trained observers using adjustment of the plane of focus at high magnification. 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. Counting the number of clock hours occupied by pathologic vessel growth, the established unit of clinical assessment of ROP, yielded a semiquantifiable measure of severity. Retinas were scored independently by three masked investigators and the three assessments averaged for each retina. Selected retinas were removed from the slides after staining and assessment and were processed for histologic sectioning to confirm the presence of preretinal neovascularization identified in flat mounts. Tissue was 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.

A few rats were allowed to remain in room air for 14 d after 50/10% (n = 5) or 80/40% exposures (n = 5) before killing. These rats were enucleated, and the whole eyes were processed for histology by the same means as those used for flat-mounted retinas.

Systemic oxygen. To establish the clinical relevance of the 50/10 variable oxygen exposure, arterial partial pressures of oxygen were measured in five rats at the end of the last 50% oxygen-exposure period (d 13) and in five rats at the end of the last 10% oxygen-exposure period (d 14). Age-matched room-air rats (n = 4) were also assessed. Measurements were made from 300 μL of blood taken from the left ventricle of deeply anesthetized animals using a heparin-flushed 0.5-mL syringe with a 22-gauge needle. During the procedure, rats were ventilated using a Harvard Rodent Ventilator (Model 683, Harvard Apparatus, South Natick, MA) at a rate and volume matched to that observed before anesthesia for each

Table 1. *Effect of variable oxygen exposures**

Parameter	50/10%	80/40%
Peripheral avascularity† (% retinal area)	25.2 ± 7.6 (15)	8.1 ± 3.4‡ (6)
Central avascularity† (% retinal area)	4.2 ± 2.1 (15)	14.1 ± 3.2‡ (6)
Neovascularization incidence§ (%)	97.3 (36/37)	72.2 (26/36)
Severity§ (average clock hours/retina)	8.0 ± 2.2 (36)	4.2 ± 2.1‡ (26)

* Values are mean ± SD. Sample sizes are in parentheses.
† Assessment was made immediately after 14-d oxygen exposure.
‡ Significantly different from corresponding 50/10% value ($p \leq 0.005$).
§ Assessment of severity and incidence was made after 14-d oxygen exposure and 4 d postexposure in room air.

treatment group (60 to 75 pulses per minute and 0.25 to 0.4 cc) and using the ambient oxygen level for each group (10, 50, or 21%). Blood gases (Pao_2 and $Paco_2$) were analyzed using a Blood Gas Analyzer (Model 1306, Instrumentation Laboratory, Lexington, MA).

RESULTS

Body weights at 14 d were 24.3 ± 2.8 g for room-air–raised rats, 26.8 ± 3.4 g for the 80/40% exposure group, and 19.8 ± 2.3 g for the 50/10% group.

Retinal avascularity. Table 1 describes several parameters related to the pathology induced by the two variable oxygen exposure paradigms. The extent of avascular retinal area immediately upon removal from oxygen was 29.4% of the total retinal area in rats exposed to the 50/10% paradigm and 22.2% for rats in the 80/40% paradigm ($p \geq 0.05$). The pattern of avascularity was different in the two groups: 50/10% rats displayed 3 times more avascular area in the peripheral retina than 80/40% rats (25.2 *versus* 8.1%, respectively), but 80/40% rats had 3 times more avascular area in the central region (14.1 *versus* 4.2%). Figure 2 illustrates the appearance of fluorescein-infused retinas from rats raised in room air and the two variable oxygen exposures. In addition to differences in the degree of retinal avascularity of the two exposure groups, there was also a substantial difference in vessel diameter as assessed with fluorescein infusion (Table 2). In 50/10% rats, major retinal arteries and veins had an average diameter of 45.1 and 45.7 μm, respectively. In 80/40% rats, measurements averaged 27.8 and 35.3 μm, respectively ($p \leq 0.001$, for both artery and vein caliber).

Retinopathy. Four days after exposure, the incidence of abnormal retinal neovascularization in the two exposure groups was 97.3% for 50/10% rats, and 72.2% for 80/40% rats. The severity of neovascularization when it occurred was also greater in the 50/10%-exposure group. Abnormal vessel growth was found in 8.0 average clock h of

Figure 2. Oxygen-induced retinal nonperfusion. These three panels illustrate the degree of retinal nonperfusion at the end of the 14-d exposure period and at the same time in room-air–raised controls. Vessels have been perfused with fluorescein-labeled dextran. *Top panel*, room air; *middle panel*, 80/40% oxygen exposure; *bottom panel*, 50/10% oxygen exposure. The increased caliber of major vessels and capillaries in the retinas of the 50/10%-exposure group can be appreciated even at this low magnification.

Table 2. *Effect of variable oxygen exposure on vessel diameter*

	Vessel diameter		
	50/10%	80/40%†	Room air†
Major arteries	45.1 ± 6.5 (13)	27.8 ± 5.3‡ (12)	28.7 ± 3.6‡ (11)
Major veins	45.7 ± 8.0 (13)	35.3 ± 6.1‡ (13)	38.6 ± 4.8‡ (11)

* Values are mean ± SD. Sample sizes are in parentheses.

† Significant difference between arteries and veins within exposure group ($p \leq 0.005$).

‡ Significantly different from 50/10% values ($p \leq 0.001$).

retina in this group compared with 4.2 average clock h in the 80/40% exposure group. Figure 3 illustrates ADPase-stained retinas from the two oxygen exposure groups and from a room-air–raised rat of the same age.

Observation of retinas from the 50/10% exposure group at higher magnification revealed preretinal vascular tufts that arose primarily at the peripheral-most extent of the major veins (*arrow*, Fig. 4*A*). Also evident were a high degree of tortuosity of the major arteries and frequent abnormal capillary buds at the peripheral extent of major arteries (*arrows*, Fig. 4*B*). In contrast, rats exposed to the 80/40% paradigm generally displayed narrow bands or ridges of preretinal vascular growth along the vascular/avascular interface. These bands spanned the region between major arteries and veins.

Vitreal hemorrhages were seldom observed in the eyes of rats from the 80/40% exposure group (five of 42, 12%), but severe vitreal hemorrhages were found in nearly half of rats (22 of 52, 42%) exposed to the 50/10% paradigm. Figure 5*A* depicts one such case in which the hemorrhage apparently emanated from preretinal vessels (*thin black arrows*, Fig. 5*B*, *inset*). The inner limiting membrane appeared relatively intact in this retinal location (*thick black arrows*, Fig. 5*B*, *inset*). There existed membranous extensions from the retinal surface that encompassed most, but not all, of the preretinal growth. Preretinal tufts like those often found in 80/40% rats were seen in these retinas (*white on black arrow*, Fig. 5*B*), but retinas from the 80/40% exposure group seldom displayed the membranous extensions. The retina in Figure 5*B* was detached in the area immediately beneath the vitreal hemorrhage. This may have been due to the large dysplastic retinal fold underlying the hemorrhage or to tractional force provided by the membranous extensions and preretinal vessels. Because similar detachments were never seen in room-air rats, they are not believed to be artifactual, or if artifactual, the variable oxygen-exposed rats were much more susceptible to such artifact. A very limited sample of

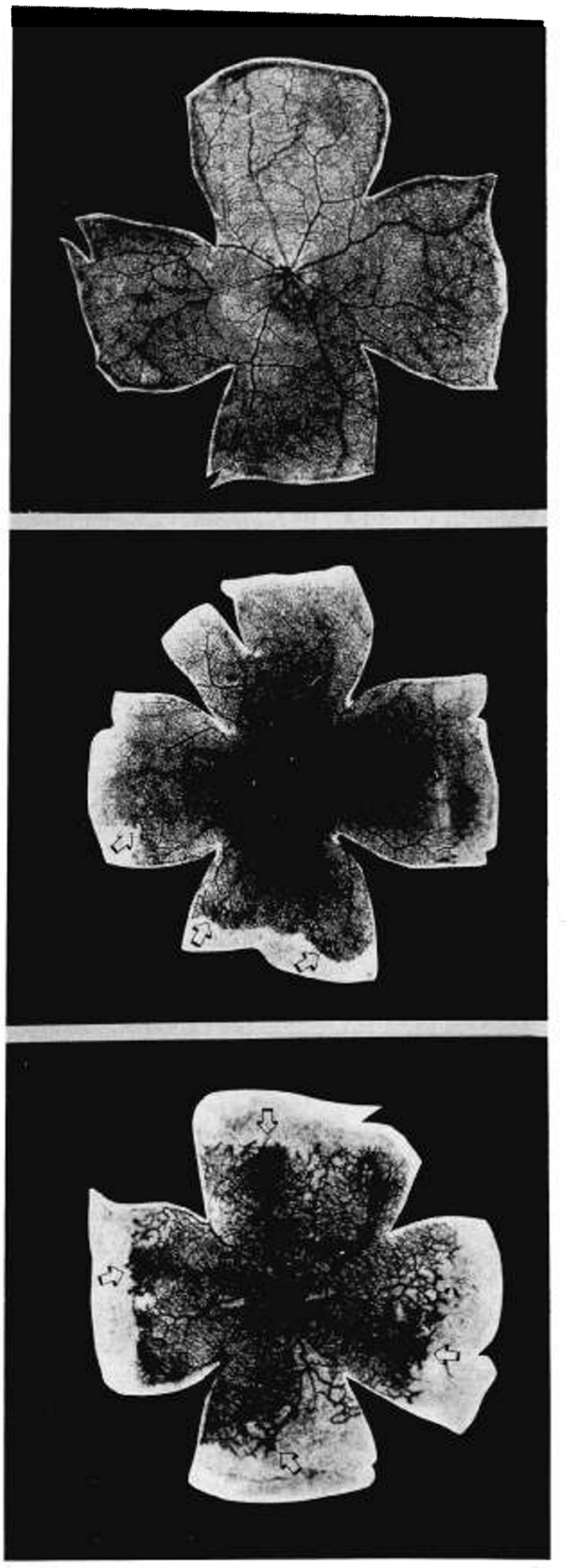

Figure 3. Retinal neovascularization. These three panels illustrate the degree of abnormal vessel growth after a 4-d postexposure period in room air. Vessels are marked by ADPase activity. *Top panel*, room-air–raised rat shows no retinal pathology or avascular area at 18 d of age; *middle panel*, the 80/40% exposure group still has a small avascular zone in the retinal periphery which is bordered by abnormal capillary tufts (*arrows*); *bottom panel*, the 50/10%-exposure group displays substantial peripheral avascularity and severe abnormal vessel proliferation, particularly at the peripheral extent of major veins (*arrows*).

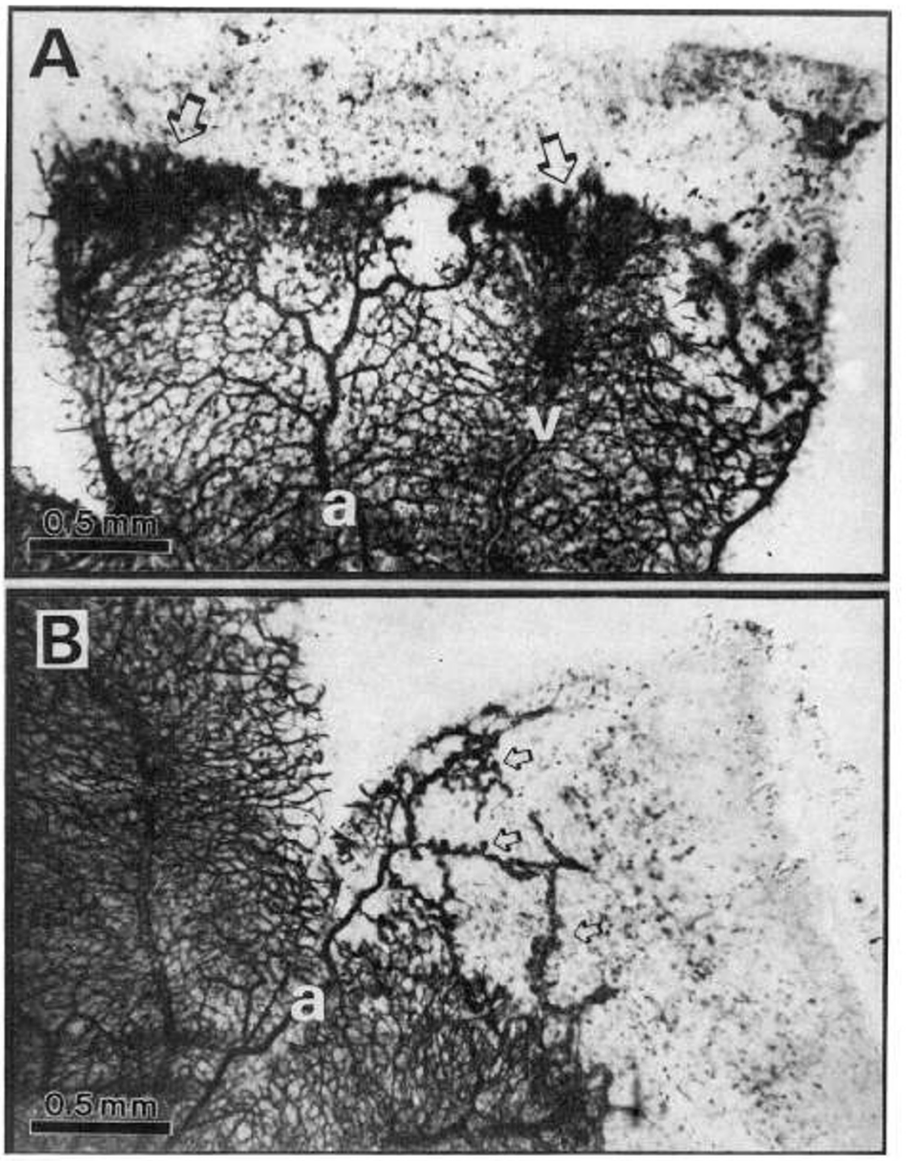

Figure 4. Vascular pathology in retinal flat-mount. At higher magnification, the abnormal vascular development of 50/10% retinas is seen to manifest itself in at least two ways: *Panel A*, dense sheets of endothelial cells (*arrows*) overlay the peripheral extent of major veins, in many cases penetrating into the vitreous (*a*, artery; *v*, vein); *panel B*, abnormal buds (*arrows*) extend from the peripheral extent of major arteries (*a*, artery). New capillaries in the superficial retina normally arise by differentiation of stem cells, rather than by endothelial budding.

50/10% rats (n = 5) were maintained in room air for 14 d postexposure before killing. Two exhibited peripheral retinal detachment without vitreal hemorrhage. The other three appeared normal with the exception of sporadic dysplastic retinal folds like that illustrated in Figure 5*B*. The 80/40% exposure group yielded unremarkable retinas at this time.

Systemic oxygen. Results of the Pao_2 assessment are shown in Table 3. Rats assessed at the end of the last period in 50% FiO_2 had an average Pao_2 over 4 times higher than that of rats at the end of the last period in 10% FiO_2 (kPa = 29.0 *versus* 6.1, respectively; 217.8 *versus* 45.8 mm Hg). Room-air–raised rats had an average Pao_2 of 12.6 kPa (94.8 mm Hg), which agrees well with previously published values (*e.g.* 13) and suggests that our apparatus functioned well for all other measurements. The Pao_2 and $Paco_2$ values precisely agree with gas-exchange theory for the 10 and 21% determinations, indicating that respiratory volume and frequency during blood collection were appropriately controlled. The 50% determination yielded low Pao_2 values, which may be explained by compromised pulmonary function caused by the extended exposure.

DISCUSSION

With respect to oxygen-induced retinopathy, the overall amount of oxygen that a subject receives is far less critical than other parameters of its administration. Raising rats in an environment cycling between 50 and 10%

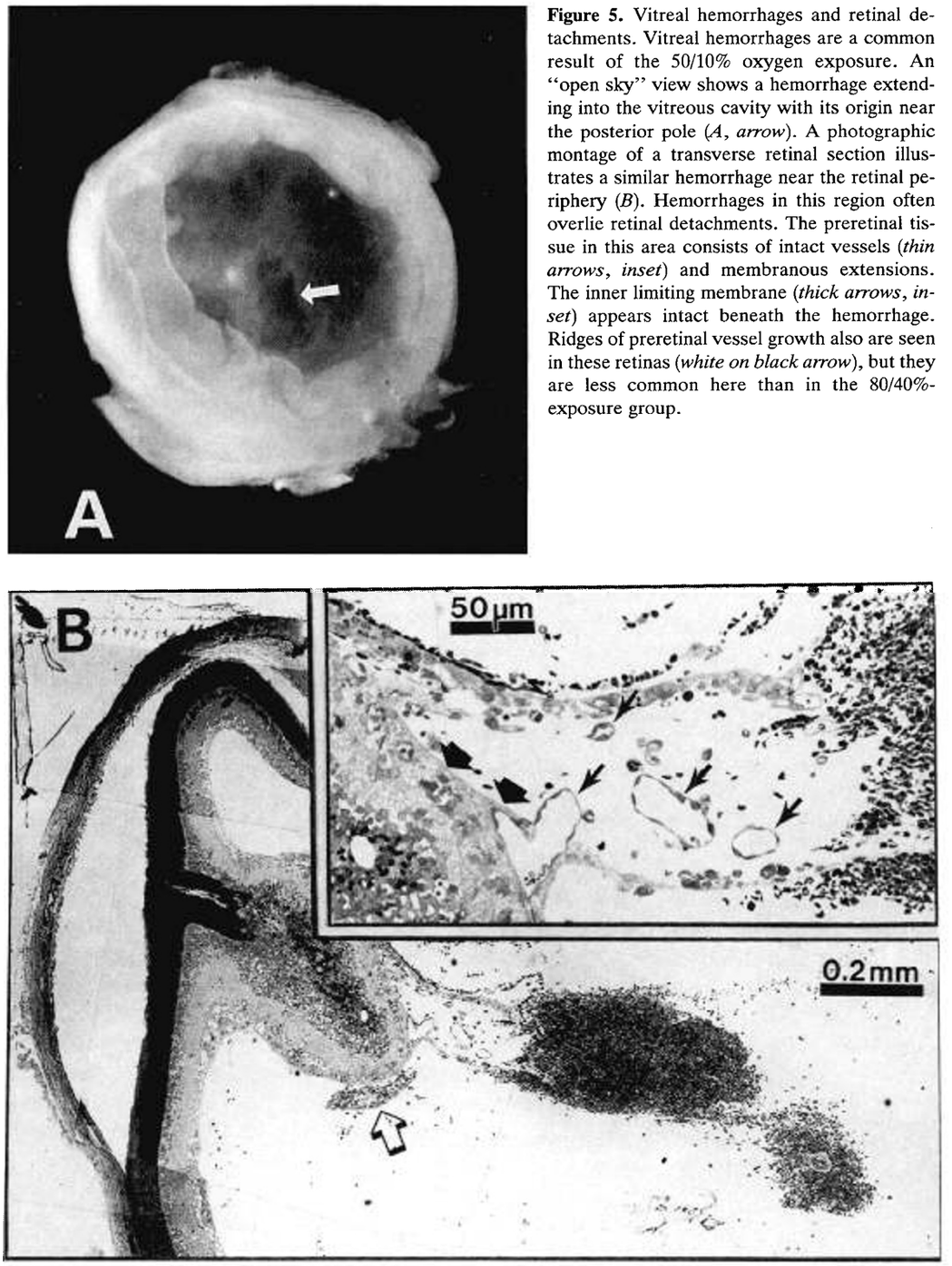

Figure 5. Vitreal hemorrhages and retinal detachments. Vitreal hemorrhages are a common result of the 50/10% oxygen exposure. An "open sky" view shows a hemorrhage extending into the vitreous cavity with its origin near the posterior pole (*A*, *arrow*). A photographic montage of a transverse retinal section illustrates a similar hemorrhage near the retinal periphery (*B*). Hemorrhages in this region often overlie retinal detachments. The preretinal tissue in this area consists of intact vessels (*thin arrows*, *inset*) and membranous extensions. The inner limiting membrane (*thick arrows*, *inset*) appears intact beneath the hemorrhage. Ridges of preretinal vessel growth also are seen in these retinas (*white on black arrow*), but they are less common here than in the 80/40%-exposure group.

oxygen resulted in a much higher incidence and severity of retinopathy than did raising them in an 80/40% cycle, even though the latter received much more oxygen during the exposure period. This provides evidence that excessive oxygen alone is not the single overriding factor in retinopathy of prematurity and that episodal hypoxia may play a critical role in the pathogenesis.

Many studies have addressed the effect of local hypoxia on retinal structure and function (14–17) including experiments describing a stimulation of vascular endothelial mitosis in the rat retina (18). A recent study (19) suggests that vascular endothelial growth factor functions as a hypoxia-inducible angiogenic agent in some cell cultures and in tumors *in situ*. Furthermore, although inherently different by design, experiments conducted by Phelps and Rosenbaum (20) examined the effect of recovery in hypoxia after hyperoxic exposure of kittens. The authors found strikingly similar incidence of vitreal hemorrhage (46%) as that found in the 50/10%-exposure group in this study (42%). Kittens that recovered in room air developed vitreal hemorrhages only 9% of the time. It should also be noted kittens exposed to hypoxia in this

Table 3. *Exposure effects on blood partial pressures**

	Pao_2		$Paco_2$	
FiO_2 (%)	mm Hg	kPa	mm Hg	kPa
50	217.8 ± 48.7 (n = 5)	29.0 ± 6.5	39.0 ± 5.2 (n = 5)	5.2 ± 0.9
10	45.8 ± 4.7 (n = 5)	6.1 ± 0.6	52.6 ± 12.6 (n = 5)	7.0 ± 1.7
21	94.8 ± 14.5 (n = 4)	12.6 ± 1.9	42.2 ± 4.1 (n = 4)	5.6 ± 0.5

* Values are means ± SD.

study showed poor weight gain, just as the 50/10% exposure caused in these experiments. Lastly, direct evidence of the clinical significance of hypoxia was presented by Katzman *et al.* (21), who found that more severe stages of ROP were closely associated with hours spent by infants with Pao_2 below 50 mm Hg and capillary Po_2 below 35 mm Hg.

Just as important are several studies suggesting a relationship between fluctuation in retinal Po_2 and ROP (22–25). The most rigorous of these is a study by Saito *et al.* (25) who showed a significant relationship between "hour-to-hour fluctuation" in arterial oxygen as measured by transcutaneous monitoring and progression of ROP. Of course, fluctuations in Po_2 may simply be reflective of fragile infants who, because of other factors related to their condition, are at increased risk for ROP. The authors addressed this inherent complication by stringently limiting their study population and carefully assessing the infants' clinical conditions.

In comparing rats raised in the 80/40% cycle with those raised in the 50/10% cycle, another issue can be considered—namely, the relationship between the degree of retinal avascularity upon removal from the exposure chamber and the propensity for subsequent abnormal neovascularization. Several retinal conditions, including sickle cell retinopathy (26), diabetic retinopathy (27), and branch vein occlusion retinopathy (28), have in common a degree of retinal nonperfusion before abnormal vascular growth. This has led to the suggestion that the retinal vascular proliferation is the result of ischemia-induced hypoxia. It is not proven, however, that retinal nonperfusion leads to tissue hypoxia, and therefore a causal relationship between nonperfusion and vasoproliferation remains hypothetical for these pathologies.

Results of the present experiments support this relationship between the degree of retinal avascularity and subsequent abnormal proliferation. The 50/10% cycle caused abnormal vessel growth in 97% of the rats, whereas the 80/40% exposure caused this pathology in 72% of the rats exposed in that manner. Also, the degree of pathology was 8.0 average clock h of retina in the former group, but only 4.2 in the latter. The 50/10% rats had significantly ($p \leq 0.05$) more avascular retinal area upon removal from the exposure chamber than did the 80/40% experimental group (29.4 *versus* 22.2% of total retinal area). If the stimulus for pathologic vascular proliferation is ischemia-induced hypoxia, the extent of which may well correlate to retinal avascularity, one would expect this relationship. Perhaps the extreme dilation of major vessels and capillaries in 50/10% rats reflects an attempt to lessen the degree of retinal ischemia.

Interestingly, the pattern of retinal avascularity was different in the two exposure groups (Fig. 2). The vasoattenuation that characterizes oxygen-induced retinopathy in the rat is partly composed of two major capillary-free zones—one in the central retina surrounding the origin of vessels and one encompassing the retinal periphery (6). It has been proposed that the central avascular area is the result of obliteration of preexisting capillaries (29), and the peripheral avascular area is due to a slowing of the development of the retina's vascular network (30). Although we have no experimental evidence for either of these mechanisms, it is reasonable to assume that higher ambient oxygen would lead to higher tissue oxygen levels in the central retina where major retinal arteries are in close proximity. Diffusion of oxygen from these arteries may lead to death of existing vascular cells (vasoobliteration) by free-radical–mediated damage. Several studies have demonstrated biochemical evidence of such damage in retinas of oxygen-exposed animals (31–33). Conversely, a central retinal capillary-free zone might also be caused under these conditions by a cessation of capillary formation due to elevated tissue Po_2 in combination with continued growth and peripheral extension of the retina. In either case, the 80/40% exposure would be expected to exert a greater effect on the central retina than the 50/10% exposure, and it does, creating a 3-fold–larger central avascular area.

The peripheral avascular zone, on the other hand, is three times larger in the 50/10% exposure group. We must assume that diffusion of oxygen from the choroid vasculature and existing retinal vessels creates a higher Po_2 in peripheral retinal tissue of 80/40% rats than 50/10% rats, so hyperoxia alone cannot explain the retardation of centrifugal vessel growth in the latter exposure group. It is possible that, although the relatively high Po_2 in the peripheral retina of 80/40% rats is an inhibitor of vessel growth in that region, the hypoxic insult of 10% FiO_2 to the newly forming vessels and/or their precursors in the 50/10% paradigm is more damaging to the vasoformation process. This would explain the larger avascular area found in this region of the 50/10% exposure group.

The site of abnormal neovascular growth is the midperipheral vascular/avascular boundary in rats as it is in humans. This is expected if one assumes that local retinal ischemia is the stimulus for this growth. The existing retinal vasculature must provide the origin of the new abnormal vessels, but the adjacent avascular retina would be the likely source of the growth stimulus. It is, therefore, logical that 50/10% rats suffer the more severe effects of the two groups, because the degree of peripheral retinal nonperfusion is much greater in 50/10% rats than in their 80/40% counterparts.

Our blood-gas analysis of 50/10% rats demonstrated Pao_2 levels like those commonly found in sick infants monitored in neonatal ICU (25). Partial pressures as low as 5 kPa (~35 mm Hg) are frequently seen as the result of apnea or other respiratory complications. Pressures as high as 25 to 30 kPa (~185 to 225 mm Hg) are periodically seen after therapeutic oxygen levels are increased to counter respiratory illness. It appears that the more we fashion our animal exposures to model the conditions experienced by sick neonates at risk for ROP, the more severe and consistent our pathologic results become.

Another possible explanation for the higher incidence and severity of retinopathy in the 50/10%-treatment group is the ratio of the two oxygen levels. In the 50/10% group, the ratio of highest to lowest ambient oxygen level is 5, and the systemic result is a Pao_2 that varies by nearly 5 times. The 80/40% exposure constitutes an ambient oxygen ratio of only 2. Because the range of FiO_2 variation remains at 40%, the absolute range of Pao_2 that results from this treatment is probably similar to that caused by the 50/10% exposure. However, one would expect a 2-fold or less variation in Pao_2 in the 80/40% treatment, which may constitute a less severe insult.

The high incidence (97%) of proliferative retinopathy found in 50/10% rats is significant, not only because it implies a role of hypoxia in the pathogenesis, but because it constitutes a model in which potential therapies designed to inhibit this process can be reliably tested. It is reasonable to suggest that hypoxic episodes and fluctuations of Pao_2 are critical factors in the etiology of ROP. Our findings suggest that avoidance of these two perturbations should be emphasized by neonatalogists.

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