Fluorescein Angiography of the Newborn Rot
Implications in Oxygen-Induced Retinopathy

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The current technique was developed to characterize the morphologic changes in the retinas of oxygen-reared rats, as an animal model of retinopathy of prematurity. Past studies have used ink perfusion to observe the retinal vasculature, but this method is static and requires the sacrifice of the subject. Fluorescein angiography, however, is dynamic and relatively noninvasive, and allows the survival of the animal for further study. The fundus camera cannot be used because the source of light that is focused in an annulus is too large for the pupil size of a young (~14-day-old) rat. To overcome this, a Nikon inverted microscope (Diaphot-TMD) was used. Using the proper exciting and barrier filters for fluorescein, a photographic sequence was made by rapidly focusing to the plane of the retinal vessels. To our knowledge, similar photographs have not been previously published. This technique was used in newborn pigmented rats that were 1) exposed to 80% oxygen for the first 14 days of life; 2) exposed to 80% oxygen for the first 21 days of life; or 3) exposed for the first 14 days followed by 7 days in room air. Age-matched controls were raised simultaneously in room air and evaluated with the same technique. Differences were observed between treatments in the amount of retinal capillary loss, and in the tortuosity and diameter of the major retinal vessels. The hyaloid system also varied between treatment groups. Oxygen-exposed rats showed a persistence of the hyaloid vessels that was particularly prominent in the group returned to room air before analysis.

Comparisons are made to past results obtained with other histologic techniques. Invest Ophthalmol Vis Sci 31:810-818, 1990

The recent increase in the survival of premature infants due to improving neonatal care has caused an increased incidence of retinopathy of prematurity (ROP), but also a reduction in eye donations for basic research. This has led to renewed interest in the development of a suitable animal model for ROP. For a number of reasons, much attention has been focused on the rat in this role. First, the rat is an inexpensive and easily maintained model in which the retinal vasculature develops postnatally, facilitating its study. Over decades of research, newborn rats have shown consistent susceptibility to oxygen-induced retinopathy. Further, the ontogeny of the rat retina parallels that of the human infant, particularly with respect to vascular development. Finally, the techniques previously perfected to test retinal integrity in the rat were believed to be appropriate and adequate.

The preferred method of studying retinal vasculature morphology has been ink perfusion, a technique that requires the sacrifice of the animal. For decades, this method has been used extensively to describe alterations of the retinal blood vessels in response to hypoxic exposure of newborn animals. However, results obtained with this technique have been inconsistent. This is particularly true with respect to the possible occurrence of neovascularization. The argument over the presence or absence of neovascularization in oxygen-exposed rats began with the work of Patz and co-workers, who, using standard histologic techniques, found evidence of “capillary proliferation from the retina into the vitreous” and “intraocular hemorrhage” in newborn rats exposed to 80% oxygen. These findings were challenged by Ashton and Blach, who, using ink perfused and histologically sectioned retinas, reported no evidence of abnormal neovascularization or hemorrhages in similarly treated rats. Patz was later unable to reproduce his original results.

Recent work by Ricci and Calogero has rekindled this controversy. These authors described both retinal neovascularization and hemorrhagic lesions in ink-perfused retinas from rats exposed to 80% oxygen for 10 days followed by room air for 15 days. In some cases the neovascularization was described as “extraretinal.”
Over the past 2 yr our laboratory has processed approximately 200 ink-perfused retinas from newborn rats exposed for various durations to elevated oxygen and room air. It has been our experience that the presence of "hemorrhagic lesions," as Ricci and Calogero describe them, is difficult to distinguish from pressure-induced leaks of ink. With careful administration of ink, we have eliminated the occurrence of leaks, leading us to believe that they are most often, if not always, attributable to procedural artifact. Further, we see no evidence of neovascularization and little evidence of other forms of abnormal capillary growth or capillary "tuft" formation with ink. The true presence of hemorrhages or abnormal neovascularization probably can be determined with extensive histologic scrutiny of retinal tissue. However, we have chosen an alternative path for addressing past contradictions resulting from ink perfusion.

Retinal fluorescein angiography has been used largely in clinical diagnosis. Its advantage is that true vessel patency under physiologic conditions can be evaluated. For studies using animals, sacrifice of the subject is not necessary, and it is possible to follow progression of, or recovery from, vascular deficit within the same retina. Also, vessel integrity can be determined in vivo, without the complication of pressure artifacts, and thus, some understanding of the true integrity of the vessels can be gained from the presence or absence of fluorescein leaks.

Fluorescein angiography has been achieved successfully in the adult rat with the clinical fundus camera, but in younger animals its use has been impossible because of problems with illumination. The source of light in this instrument is focused in an annulus, and spreads out behind the pupil to illuminate the retina, allowing the image forming rays to return to the camera through the center of the annulus. This design eliminates light reflection by the cornea and lens. Unfortunately, the size of the newborn rat's entire eye is smaller than the center of the annulus, making it impossible to illuminate the retina with the fundus camera.

Fluorescence is made possible by a process of absorption and emittance of light in different wavelengths. Using the proper exciting and barrier filters, fluorescein angiography can be achieved in very small eyes with a fluorescence microscope. The problem of illumination is solved because, even though no annulus is used, the barrier filter renders the camera blind to the exiting wavelength, and scattered and reflected light is virtually eliminated.

This report describes an attempt to apply fluorescein angiography to the study of oxygen-induced retinopathy in newborn rats. To our knowledge, this is the first such attempt made. Further, we believe that these experiments mark the first use of the fluorescence microscope for angiography of the retina in eyes of this size. Comparisons are made to past results obtained using ink perfusion, with particular emphasis on those facets of retinal vasculature that may be explained by pressure artifact.

**Materials and Methods**

Eight litters of Long Evans pigmented rats were used. Immediately after birth, some litters were placed with their mothers in 80% atmospheric oxygen. Other litters of control animals were simultaneously placed in room air. Some rats received a combination of oxygen and room air maintenance. Surrogate mothers were alternated from room air to oxygen at regular intervals (3–4 days) to avoid respiratory distress. Mothers were fed rat chow ad libitum, and experiments were conducted under 12-hr light lighting of 500 lux illuminance. All animals were maintained in accordance with the ARVO Resolution on the Use of Animals in Research.

The rats were divided into five groups: 1) room air for 14 days; 2) 80% oxygen for 14 days; 3) room air for 21 days; 4) 80% oxygen for 21 days; and 5) 80% oxygen for 14 days, followed by 7 days in room air (called the "recovery group").

Fluorescein angiography was performed at the end of each experimental period with a Nikon inverted fluorescence microscope (Diaphot-TMD; Nikon, Tokyo, Japan) equipped with a 100-W mercury lamp and a 3.2× objective lens. Details of this procedure...
Table 1. Qualitative assessment* of fluorescein angiograms of oxygen- and room air-reared newborn rats

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<thead>
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<th>Treatment</th>
<th>Hyaloid</th>
<th>Retina</th>
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<td>Extent</td>
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<tr>
<td>14 days room air (n = 7)</td>
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<td>14 days O₂ 80% (n = 6)</td>
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<td>21 days room air (n = 6)</td>
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<td>21 days O₂ 80% (n = 6)</td>
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<tr>
<td>14 days O₂ 80% + 7 days room air (n = 9)</td>
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* Range of observation from - (no occurrence) to ++++ (extensive occurrence); +/- (inconsistent presence).

are described elsewhere.¹⁰ The camera was a Nikon F-3, set for automatic exposure time at ASA 3200. We used Kodak T-Max 3200 Professional Film, developed according to manufacturer instructions at standard film speed.

Typically, a ratling was anesthetized with sodium pentobarbital (50 mg/kg) injected intraperitoneally. In the 14-day-old animals, a canthotomy was performed occasionally in order to retract the eyelids fully. The pupil was dilated with 1% phenylephrine and 1% tropicamide, and the animal was placed over a heating pad to avoid hypothermia. At least 10 min were allowed for complete dilation. The ratlings then were injected intracardially with 10 mg/kg of 1% sodium fluorescein. This method of administration was necessary because of the difficulty of canalizing veins in animals of this age. A coverslip with a drop of 2.5% methylcellulose was placed on the warmed stage of the inverted microscope. Immediately after injection, the ratling was placed on the stage with its eye contacting the coverslip (Fig. 1). This created a negative lens and allowed for focusing in the plane of the retinal and hyaloid vessels. A photographic sequence was made, with the exposure times ranging between 0.5 and 1.5 sec. The eye was reoriented manually by moving the head of the animal, in order to photograph the different areas of the retina and hyaloid system.

Fig. 2. Retinal fluorescein angiograms. (a) 14-day-old rat reared in room air, showing major arteries and veins radiating from the optic disc and a fully arborized capillary network. (b) 14-day-old rat reared in 80% oxygen, showing narrowed major vessels and missing capillary network.
Fig. 3. Retinal fluorescein angiograms. (a) 21-day-old rat reared in room air, showing major arteries and veins radiating from the optic disc and a visible fully arborized capillary network. Details are clearer than in the 14-day-old room air-reared rat due to increased size of the eye and less persistent hyaloid system. (b) 21-day-old rat reared in 80% oxygen, showing narrowing of some major vessels and missing capillary network in the central retina and portions of the periphery. (c) 21-day-old rat reared in 80% oxygen for 14 days and then returned to room air for 7 days, showing striking tortuosity and slight narrowing of the major vessels, and persistence of missing capillary network.

Results

Our qualitative interpretation of the fluorescein angiograms is summarized in Table 1. Examples of angiograms are found in composite Figures 2–7.

Figure 2a depicts the retina of a 14-day-old control rat. The loss of retinal capillaries and the narrowing of the major vessels of the retina, both results of the 14-day oxygen exposure, are evident in the exposed counterpart (2b).

Figure 3a clearly shows the presence of capillaries under room-air conditions in the 21-day-old. Exposure to oxygen for this period (3b) resulted in the prolonged loss of most capillaries, with the exception of a limited number in the periphery of one or two retinal quadrants. Also, some tortuosity of the major vessels occurred during this extended exposure. This latter phenomenon was not observed in rats exposed for 14 days. The recovery animals (3c) showed no substantial increase in physiologically patent capillaries during the room-air period, and also showed an impressive degree of tortuosity of all major vessels.

Figures 4 and 5 depict the hyaloid systems of 14-day- and 21-day-old rats, respectively. The 14-day-old reared in 80% oxygen (Fig. 4b) maintains a slightly more extensive hyaloid network than the room-air counterpart (Fig. 4a), and the vessels of the former are slightly more dilated. In the 21-day-old control (5a), only a small number of very narrow hyaloid vessels remains. The oxygen-exposed 21-day-old (5b) has retained a more extensive system with greater arborization and vessel diameter. Rats raised for the first 14 days of life in 80% oxygen, followed by 7 days in room air (5c), display an extensive hyaloid network. Vessels are dilated and extremely tortuous, often displaying loops along their length from the optic nerve head to the rear of the lens.

Figure 6 reveals the possible presence of abnormal vessel growth and capillary formation in recovery animals. First, the formation of tuftlike capillary beds is depicted in Figure 6a. This single recovery animal is our only evidence of "capillary tufts" obtained with fluorescein for any experimental treatment. We have...
Fig. 4. Hyaloid system fluorescein angiograms. (a) 14-day-old rat reared in room air, showing the normal extent found at this age. (b) 14-day-old rat reared in 80% oxygen, showing a more fully arborized system than the room-air control.

never witnessed unequivocal evidence of tuft formation using ink perfusion. Second, vessel shunts can be documented with a battery of photographs taken at slightly different angles. Figure 6b is one of such a series. The fluorescein is routed directly from a major artery to a major vein with little capillary deviation.

Figure 7 compares one of our ink-perfused retinas with a fluorescein angiogram from a rat that received an identical treatment. Each animal was in the recovery group. This comparison emphasizes two major differences in the results of the two techniques: 1) the fluorescein angiograms show no significant capillary recovery during the postexposure week in room air, indicating that those capillaries seen with ink in this group are not physiologically patent; and 2) the tortuosity of major vessels in the recovery group, which is made obvious with fluorescein angiography, is not evident in ink-perfused retinas, except in rare cases in the far periphery.

Discussion

This work adds a new tool to the study of retinal vascular degeneration in small animals. It is particularly well suited for the study of oxygen-induced retinopathy in rats. The conditions under which the data is recorded allow for a truer physiologic evaluation of vascular integrity. This is especially important during development of the eye when the newest vessels are very fragile or are only patent under nonphysiologic pressures.

It was necessary to solve several technical problems in order to visualize ocular blood vessels with this technique, and some problems still remain. We were unable to adapt an electronic flash to our photographic equipment, so the amount of light returning from the retina after filtering and reflection was small, and highly sensitive film was required. The disadvantage of this film is increased granularity, resulting in a loss of photographic quality, both aesthetically and in the resolution of small objects.

Because the lens of the rat is almost flat at birth and rapidly becomes more spherical, occupying most of the ocular space at the 16th day of life, the hyaloid system is very active during this time. It is highly fluorescent during the angiography, and it is located between the retina and the camera. For these reasons, its scattered fluorescence partially obscures optical resolution of the retinal plane. If the hyaloid system is more developed or persistent, it becomes more difficult to visualize retinal vessels, because of the increased scattered fluorescence in the optical pathway.

We were unable to use our preferred model, the albino rat, because its retinal epithelium is not pigmented. The lack of pigment in the albino would have allowed the fluorescence from the choroidal vessels to mask that of the retinal vessels. Also, because of the immaturity of the ratlings, they had to be
closely monitored during anesthesia, with special attention paid to the dose of anesthetic and the body temperature. Several animals were lost during recovery from anesthesia, after the angiography.

The sum of the above difficulties was manifested in poor capillary resolution in comparison with that of ink perfusion. However, we were able to record data with fluorescein angiography which reflects the true physiology of the circulatory system of the eye. This is impossible in ink-perfused retinas for a number of reasons, the most notable of which is the likelihood of pressure artifact.

In an attempt to determine if the ink leaks observed by Ricci and Calogero in perfused retinal flat mounts and reported as “hemorrhagic lesions” could have been the result of pressure artifact, we scrutinized our fluorescein angiograms of similarly treated animals for fluorescein leaks. We were unable to demonstrate any unequivocal leakiness of retinal vessels in angiograms. Also, in our ink-perfused retinas, we were unable to observe any leakiness of retinal vessels that could not be the possible result of abnormal pressure. Moreover, we never observed capillary tufts with the ink procedure; however, the presence of tufts, albeit rare, was confirmed by the angiography (Fig. 6a).

Based on ink perfusions, this laboratory has suggested previously that complete recovery of the capillary net occurs within a week after return to room air, after a 14-day exposure to 80% oxygen. This result was not supported by the angiograms of animals with identical experimental treatment (Fig. 7b). The striking tortuosity of major vessels and the presence of large capillary-free zones were not evidenced in ink-perfused retinas. This vast discrepancy of vascular morphology serves to emphasize the advantage of observation under physiologic conditions, such as is achieved with fluorescein angiography. It should be noted that this tortuosity of the major vessels greatly resembles the so-called “plus disease” used in the classification of ROP. This phenomenon, as Flynn describes it, “is predictable and, in fact, inevitable” in cases where large capillary-free zones and vascular shunting are evident in the infant retina.
There has been some question in the past about the timing of the normal disappearance of the hyaloid system. Cairns,\(^2\) using ink perfusion, reported that the hyaloid system was vestigial or absent by the 11th day of life. In other, more recent work employing scanning electron microscopy, Hollenberg and Dick-

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**Fig. 6.** Retinal fluorescein angiogram of a 21-day-old rat reared in 80% oxygen for 14 days and then returned to room air for 7 days. (a) Tuftlike formation in the periphery. (b) Shuntlike formation in the periphery.

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**Fig. 7.** Comparison between ink perfusion (a) and retinal fluorescein angiography (b) of 21-day-old rats reared in 80% oxygen for 14 days and then returned to room air for 7 days. Note two clear differences: the extent of tortuosity with narrowing and dilation of different major vessels, and the degree of capillary presence.
son demonstrated the patency of the hyaloid system on the 14th day after birth. Its extent at this age was similar to that which we see in our angiograms (Fig. 4a).

There also has been controversy over the persistence of the hyaloid system in oxygen-treated animals. Ashton and Blach found no evidence of abnormal hyaloid persistence in oxygen-exposed rats with air survival after hyperoxia, but it should be noted that in their experiments the rats were not exposed immediately after birth in most cases. On the other hand, Patz et al. found histologic evidence of persistence of the hyaloid system in oxygen-treated animals, occasionally in 14-day-olds and consistently in 21-day-olds. Bischoff et al. found the same results in mice reared in oxygen, using scanning electron microscopy. The fluorescein angiograms in the current study corroborate the latter two studies. The hyaloid appears markedly more persistent in oxygen-treated rats than in controls, at both 14 and 21 days of age (Figs. 4, 5). According to Bischoff et al., since the retinal and hyaloid arteries share a common blood supply, retinal vasoconstriction resulting from oxygen exposure causes an elevation of pressure in the hyaloid system which inhibits the normal process of hyaloid vascular atrophy.

The persistence of the hyaloid in recovery animals was even more striking, with dilation and extreme tortuosity of the vessels. When similarly treated recovery rats (14 days, 60 or 80% oxygen; 7 days room air) are perfused with ink, the retina appears completely rearborized. Since, in this technique, both retinal and hyaloid vessels can be studied under physiologic conditions, and the possibility of sustained application of an abnormally high pressure, the marker prefers the hyaloid. Yet the two systems are hydrostatically coupled at their origin within the optic nerve head. The apparent persistence of the hyaloid evidenced by angiography in recovery rats is not easily explained. Bischoff et al. theorized that regression of the hyaloid vasculature is inhibited by the combination of sustained application of an abnormally high pressure gradient through it and the presence of an abnormally high intravascular oxygen concentration within it. When that oxygen concentration suddenly drops to a normal level, the hyaloid vessel walls may suddenly undergo rapid atrophic changes which lead to even greater distention while the pressure gradient remains high.

This condition, the above authors argue, would remain until the retinal vessels reopen after further recovery in normoxia. The current study does not address this question and sheds no new light on the persistence of the hyaloid, other than to document it in yet another manner.

Originally, retrolental fibroplasia was believed to be due to "persistence and thickening of the posterior fibrovascular sheath of the lens." Later experimentation pointed toward the retinal vessels as the sole mediator, and our attention has been focused on them since. Clearly, in both rats and humans, the hyaloid and retinal vessels, sharing a common source, are intimately connected. The effect of hyperoxia on the hyaloid system appears at least as drastic as its effect on the retina. The suggestions of Bischoff's group warrant further study, and fluorescein angiography is an effective tool with which to do so. With this technique, both retinal and hyaloid vessels can be studied under physiologic conditions, and the possible role of the hyaloid system in the development of retinopathy of prematurity can be reexamined.

Summary

We make the following conclusions with respect to fluorescein angiography and its role in the study of oxygen-induced retinopathy:

1. Satisfactory fluorescein angiography can be accomplished using the fluorescence microscope on eyes too small for the fundus camera.
2. Fluorescein angiography is a powerful tool for studying oxygen-induced alterations of retinal vasculature, eliminating many of the possible artifacts inherent in other methods.
3. Fluorescein angiography yields a new view of the effect of oxygen on the newborn rat retina with respect to each of the following:
   A. capillary loss and recovery
   B. major vessel diameter
   C. tortuosity of major vessels
4. Fluorescein angiography also can be useful in the study of the hyaloid. Inasmuch as the atrophy of hyaloid vessels seems directly coupled to the development of retinal vessels, the role of this system in the development of ROP warrants further investigation.

Key words: fluorescein angiography, retinal vascular degeneration, hyaloid system, newborn rat, oxygen-induced retinopathy

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