The Effect of Oxygen on Corneal Neovascularization

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Since tissue oxygen levels are believed to play a pivotal role in new vessel growth in several situations, we studied the effect of several oxygen concentrations (0, 10, 21, 50, 75, or 100%) on corneal vascularization induced in the rat by chemical cautery. We achieved this by perfusing known concentrations of oxygen through goggles fitted over both eyes of the rat after corneal cautery. Neovascularization was measured in flat corneal preparations with India ink-filled vessels 4 days postcautery using computerized image analysis. The angiogenic response of rats whose eyes were continuously exposed to 0–75% oxygen were not significantly different from each other. The mean response in corneas exposed to 100% oxygen was 10–21% lower than all of the other groups, and this difference was statistically significant when compared to oxygen concentrations of 0, 21 and 75%. The reason for the inhibitory effect of 100% oxygen remains to be determined, but it may represent a toxic effect of oxygen free radicals on the vascular endothelium. Invest Ophthalmol Vis Sci 31:1277–1281, 1990

Tissue hypoxia is considered to play an essential role in the induction of neovascularization in certain disorders, such as the retinopathies of prematurity and diabetes mellitus1,2 and in wound-healing.3 Hypoxia also leads to an increased capillary density in skeletal and cardiac muscle.4–8 Conversely, direct vasodilator and constrictive effects of oxygen have been observed in the mammalian retina,9 and increased oxygen tension is believed to underlie the relative absence of capillaries around retinal arterioles as compared to venules.10,11 In a clinical study on a few patients using a subjective rating scale based on slit-lamp biomicroscopic examination and minimal follow-up, subconjunctival oxygen injections have been reported to have a vasodilator and vasoinhibitory effect on small superficial corneal vessels in humans.12,13

Several investigators have studied the effect of hypoxia and hyperbaric oxygen on neovascularization induced by corneal injuries in rabbits and guinea pigs.9,14–17 All showed no effect of oxygen on the corneal neovascular response, but all of these studies lacked objective quantitative methods for evaluating the angiogenic response. We investigated the effect of various oxygen concentrations on corneal neovascularization induced in the rat by chemical cautery. For these studies we constructed special goggles that could be perfused with known concentrations of oxygen without influencing the breathing environment of the animal. In this way, extreme hyperoxia could be studied for longer periods of exposure without the limiting constraint of animal morbidity and mortality due to pulmonary oxygen toxicity. Likewise, the effect of extreme local hypoxia could be evaluated. The degree of corneal neovascularization in the different experimental groups was compared using computerized image analysis of corneal flat preparations with India ink-filled vessels.

Materials and Methods

Corneal Cauterization

Both eyes of Sprague Dawley male rats weighing 250–375 g (Harlan Sprague Dawley, Indianapolis, IN) were cauterized by applying the tip of a silver/potassium nitrate applicator (75% silver nitrate / 25% potassium nitrate, no. 2867-1590; Graham-Field Surgical, New Hyde Park, NY) to the center of the cornea of ether anesthetized rats for 4.5 sec. A fresh applicator stick was used for each cornea. Immediately after cauterization, the cautery site was gently blotted with tissue paper. The care and maintenance of the rats used in these experiments conformed to the ARVO Resolution on the Use of Animals in Research.

Goggles

To permit the maintenance of local nonatmospheric oxygen concentrations to the eyes, goggles

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were constructed from soft rubber microscope eye pieces (Carl Zeiss, Oberkochen, West Germany), which were contoured to fit a rat's head. Plexiglass® (Rohm and Haas, Philadelphia, PA) covers were molded and cut to the curvature of the eyepieces and attached circumferentially with epoxy resin. Holes were then drilled in the right and left sides of the Plexiglass® plates for the insertion of stainless steel inlet and outlet ports to which flexible plastic tubing was attached. The soft rubber rim of the goggles was secured to the head of anesthetized animals with tape (Durapore; 3M, St. Paul, MN), a thin circumferential strip of foam underpadding (Depend underpads; Kimberly-Clark Health Care Products Group, Roswell, GA), and cyanoacrylate glue (Duro Quick-Gel, No-Run Superglue; Loctite, Cleveland, OH) (Fig. 1). The tape and foam padding were necessary to ensure an airtight seal. When in place, the eyes of each animal were enclosed, with a margin left between the eyes and the edge of the mask to allow free movement of the eyelids.

Animal Preparation and Maintenance

To prevent the animals from removing their goggles, each rat was restrained in a harness (Harvard Biosciences, South Natick, MA) that allowed free movement of all four limbs, the tail, head, and neck. Prior to placement in the harness, the upper halves of the forelegs and shoulders of each rat were wrapped with surgical tape (Durapore; 3M). Each animal, within its harness/frame apparatus, was provided constant access to food and water.

Administration of Oxygen

Different mixtures of oxygen and nitrogen (High Purity Nitrogen/Extra Dry Grade Oxygen; National Welders, Raleigh, NC) were used to achieve oxygen concentrations of 0, 10, 21 (simulating room air), 50, 75, and 100. The eyes of 20 rats were continuously exposed to each of the different oxygen concentrations for 4 days via the goggles. The gas was distributed through plastic tubing and circulated through a humidifier before reaching the inlet port of the goggles. The regulation of gas flow from the gas tanks was accomplished through the use of low pressure, two-stage regulators (Mathison Instruments, Newark, NJ). When 100% oxygen or nitrogen was administered, the gas was channeled directly from the tanks to the humidifier. The gas finally reached the goggles through plastic tubing attached directly to the metallic inlet port positioned over the left eye. A section of the tubing was attached to the outlet port over the right eye and was vented over the edge of the rodent cage (Fig. 1). For mixing of oxygen and nitrogen, a gas proportioner (Mathison Instruments), was used. The tubing was connected to each gas proportioner so that the gas was channeled through the same system of humidification and Y-tubing. The flow rate was approximately 0.5 liters/min.

Gas Sampling

On days 1 and 3 of each experiment, the oxygen concentration reaching the corneas was determined by slowly withdrawing 20 ml of the gas from the outlet tubing of the goggles into a plastic syringe, which was quickly sealed with an airtight rubber cap. Confirmation of the oxygen concentration in the specimens with 0 and 100% concentrations was performed using a pH/blood gas analyzer (Instrument Laboratory Systems; Lexington, MA) that gave oxygen readings in mmHg. Determination of the oxygen concentration in the oxygen/nitrogen mixtures was performed with a pH/blood gas analyzer (Instrument Laboratory Systems) that provided readings as percentage oxygen concentration and was accurate to within 0.1%.

Visualization of Corneal Blood Vessels

Four days after corneal cauterization, an interval that consistently provides prominent corneal vascularization in this experimental model,18 each rat was deeply anesthetized with intraperitoneal 1.0% sodium pentobarbital (50 mg/kg) and the upper torso was perfused with a mixture of 11% gelatin–10% India ink–lactated Ringer's solution. The degree of vascularization of each cornea was quantitated in
corneal flat preparations by computerized image analysis as described by Proia et al.\textsuperscript{19}

**Results**

The corneas in all of the experimental groups manifested prominent corneal vascularization (Fig. 2). Animals whose eyes were exposed to 0, 10, 21, 50, and 75% oxygen did not differ from each other, but corneas exposed to 100% oxygen had 10–21% less neovascularization than all of these groups after chemical cautery (Fig. 3). The differences were statistically significant when compared to animals exposed to 0, 21, and 75% oxygen. The size of the cauterized sites and the distance between the periphery of the corneal injury and the corneoscleral limbus did not differ among any of the groups.

**Discussion**

This investigation differs from previous related studies\textsuperscript{9,14–17} in two important ways. First, by using goggles to control the gaseous environment of the corneas, we were able to evaluate the effect of a wider range of oxygen concentrations over several days on corneal neovascularization. Second, unlike past studies, which relied on less precise indicators of corneal vascularization, the current one used computerized image analysis to quantitate corneal neovascularization objectively.\textsuperscript{19}

The current study provides evidence that hyperoxia exerts a small but significant inhibitory effect on corneal vascularization in the experimental model studied. In a variety of other situations also, oxygen appears to suppress vascular growth. As pointed out by Michaelson\textsuperscript{15} over four decades ago, capillaries arise almost solely from venules in the normal retina, and a capillary-free zone surrounds the arterioles in this tissue. The increased oxygen tension around the arterioles is believed to cause this periarteriolar capillary-free zone, since the width of it is diminished experimentally in newborn rats after exposure to a lowered partial pressure of oxygen.\textsuperscript{10} Also, oxygen causes a vasoobliterative effect on retinal vessels in the immature kitten and rat.\textsuperscript{9,20,21}

Capillary endothelial cells are the primary site of
this oxygen-induced damage, which apparently begins on the arterial side of the capillary bed before extending to other vessels. The observations that the endothelium in capillaries and larger vessels are affected similarly and that the pericytes are spared until much later argues in favor of the vascular endothelium being particularly susceptible to a toxic action of oxygen.20,21 Exposure of chicken eggs to hyperoxia also inhibits capillary development in the chorioallantoic membrane.22 The delivery of increased oxygen levels to the center of healing wounds within rabbit ear chambers leads to a cessation of capillary growth. In addition, high oxygen concentrations cause a relative decrease in the lung capillary density of newborn rats.23 This susceptibility to hyperoxia also has been demonstrated also in cultured cells, in which hyperoxia changes the shape, increases the surface area, and inhibits the proliferation of microvascular endothelial cells, but does not affect pericytes.24 The reason why hyperoxia diminishes microvascular proliferation in the corneas of chemically cauterized rats as well as in other situations remains to be determined, but it may be a sequel to oxygen free-radical damage to the vascular endothelium.

Because hypoxia did not enhance neovascularization under the conditions studied in our experiment, it cannot be implicated as an accentuator of corneal neovascularization induced by chemical cauterization. Aside from the association of tissue hypoxia with retinal neovascularization,25-27 a variety of experimental studies support the view that hypoxia stimulates new vessel formation. The mechanism by which hypoxia induces angiogenesis remains to be determined, but may involve the release of angiogenic substances. The observation that corneal neovascularization can be induced in rats by depriving them of dietary riboflavin,25-27 a respiratory coenzyme, together with reports that riboflavin is beneficial in the treatment of rosacea keratitis, a condition associated with riboflavin deficiency and most cases do not respond to riboflavin.30

Several experimental studies are relevant to the role of hypoxia in corneal neovascularization. An intracameral injection of alloxan, a stimulus for corneal neovascularization, is followed by stromal edema and a transient decrease in the corneal lactic acid concentration (expressed in milligrams percent wet weight).31 Langham31 interpreted this initial drop in lactic acid concentration as due to an inhibition of glycolysis or to an increased diffusion of lactic acid from the cornea. Alloxan administration also reversibly inhibited oxygen uptake by the cornea, supporting the view that corneal glycolysis was inhibited reversibly prior to angiogenesis in the alloxan model of angiogenesis. The possibility that an increase in corneal anaerobic glycolysis may trigger vessel growth was raised also by the observation that an elevated lactic acid concentration in the peripheral, but not the central, cornea preceded neovascularization induced in rabbits,32 and that injections of lactic acid into rabbit corneas induce corneal vascularization.33

Also, since corneal hypoxia is the major consequence of prolonged contact lens wear, it has been suspected of causing the superficial corneal neovascularization that sometimes complicates contact lens wear.34-38 Indeed, in rabbits, gas-permeable hard lenses are associated with less vascularization than gas-impermeable hard lenses. The above findings suggest that when the cornea is faced with increased metabolic needs, but insufficient oxygen to meet these needs, the cornea undergoes anaerobic glycolysis, leading to an increased production of lactic acid. Lactic acid has been implicated as an angiogenic factor,33 and it is noteworthy that lactic acid and hypoxia stimulates macrophages to release angiogenic activity.39,40

Nevertheless, despite evidence implicating hypoxia in corneal vascularization, a larger body of information points to an intimate relationship between the inflammatory response and the growth of blood vessels into the cornea.41 This association includes the angiogenesis of riboflavin deficiency, in which inflammation precedes corneal neovascularization.27 Even the corneal neovascularization induced by prolonged wear of hard contact lenses in rabbits is associated with inflammation, since the vascularization is inhibited by flurbiprofen, an inhibitor of prostaglandin synthesis.37

There are three possibilities for our inability to detect a hypoxic accentuation of the angiogenic response to silver/potassium nitrate cauterization, as follows. 1) The stimulus for corneal neovascularization in this model may not involve hypoxia. 2) The hypoxic environment created at the corneal surface may have been insufficient to induce hypoxia within the corneal tissue. Although oxygen reaches the cornea by diffusion from the pericorneal vessels, aqueous, and atmosphere, the pericorneal vasculature and aqueous do not provide sufficient oxygen to maintain corneal metabolism in the absence of atmospheric oxygen.42 However, because the conjunctiva provides 55-60 mmHg oxygen at the surface of the cornea when the eyelids are closed35,43 the conjunctiva may have negated any potential angiogenic effect provided by the anoxic environment within the gogles. 3) Corneal cauterization may provide a maximal angiogenic stimulus, such that additional hypoxia is not able to accentuate the neovascularization.
In summary, we have shown that perfusing the corneal surface with 100% oxygen causes a small but significant reduction in corneal neovascularization after silver nitrate/potassium nitrate cauterization. Hypoxia had no affect on the neovascular response in this model. These results suggest that tissue oxygen levels are not a major influence on corneal neovascularization in our experimental model.

**Key words:** hypoxia, hyperoxia, angiogenesis, rat, cornea

**References**