Oxygen-Induced Retinopathy in the Rat

Vitamins C and E as Potential Therapies

John S. Penn,* Lisa A. Thum,† and Muna I. Naash†

Oxygen-induced retinopathy (OIR) was produced by subjecting newborn albino rats to a 60% oxygen atmosphere for 14 days before they were killed and retinal analysis was done. The extent of OIR was measured by estimating the severity of retinal vasoobliteration in ink-perfused flat-mounted retinas. This was done with the aid of a digitizing camera and an image-analysis system designed to create binary images of the retinal blood vessels. Retinal levels of several antioxidant molecules also were measured. Alpha-tocopherol and ascorbic acid were reduced in oxygen-exposed rats by 34% and 20%, respectively, compared with room air-raised control animals. Retinal glutathione reductase, S-transferase, and peroxidase showed no differences between oxygen-treated and -untreated rats. Attempts to increase the newborn rats' retinal ascorbic acid by administering daily subcutaneous injections (5 g/kg body weight) to the mother rats were unsuccessful. However, the level of retinal alpha-tocopherol of newborn rats could be altered by dietary manipulation of the mothers. The mothers were fed diets containing either 1 g alpha-tocopherol acetate/kg food or none, starting 21–25 days before the birth of their litters and lasting throughout the exposure period. This treatment resulted in three- to fourfold differences in the retinal alpha-tocopherol levels of the pups. The combination of dietary and oxygen treatments also resulted in significant differences in retinal glutathione peroxidase activity, with the vitamin E-deprived, oxygen-exposed group having highest levels. Newborn rats both supplemented with and deprived of alpha-tocopherol had less vasoobliteration than did those nursed by mothers fed rat chow.

It is now more than 40 years since the first report1 was published indicating that vitamin E therapy may reduce the severity of retinopathy of prematurity (ROP). However, since then, approximately 20 clinical studies have yielded conflicting results. In 1986, a committee of the Institute of Medicine reviewed these studies and reported that "the evidence from existing studies is not convincing that the incidence or severity of ROP is modified by prophylactic administration of vitamin E given orally, intramuscularly, or intravenously from birth."2 This conclusion was reached irrespective of a 2:1 ratio in these studies that statistically favored the efficacy of vitamin E therapy. The result of this controversy is nonuniform treatment in intensive care units nationwide. Some treat all premature infants receiving oxygen therapy with vitamin E, some treat only a select population, and others do not treat such infants with vitamin E. Clearly these infants are treated with many things—not just vitamin E.

The retina is particularly susceptible to oxygen-mediated damage for several reasons: (1) its cells have a rapid rate of oxygen consumption, even under normal oxygen conditions;3 (2) it has the highest levels of polyunsaturated fatty acids of any known tissue, and these molecules are preferred substrates for damaging peroxidation reactions;4 and (3) it processes light, a known initiator of oxygen radical formation.5 Under elevated levels of oxygen, the first aspect, that of oxygen flux, is likely to be enhanced.

The importance of oxygen-mediated damage to the retina also is suggested by its elaborate defense system. The retina can combat peroxidation by enzymatic detoxification of reactive oxygen molecules or termination of radical chain propagation by vitamin E. The rationale for using vitamin E as treatment for ROP stems from the idea that peroxidative damage to cells is a causal factor and that, by acting as a scavenger of the reactive molecules that lead to peroxidation, vitamin E may prevent this damage. Oxidative
damage to cells often is attributed specifically to peroxidation of the polyunsaturated fatty acids in the lipid of membranes; this causes loss of fluidity and breakdown of membrane transport functions that control transmembrane ionic gradients. Because vitamin E is an integral part of the lipid bilayer, it can react with alkyl and alkoxy radicals at this site by donating a hydrogen atom to them. Vitamin E then becomes a radical itself, but because of its relative stability, it is unable to continue the chain propagation of lipid peroxidation.

Enzymatic detoxification of reactive oxygen molecules is done by various endogenous agents, including superoxide dismutase (SOD), catalase, and peroxidases. The enzyme SOD converts the superoxide radical to hydrogen peroxide and oxygen. The hydrogen peroxide produced is metabolized by catalase and glutathione peroxidase. Vitamin E, SOD, and glutathione peroxidase are all present in the retina. The retina's antioxidant system also may extend to vitamin C and glutathione through a coupled exchange of unpaired electrons. In part, the scheme depicted in Figure 1 was proposed originally by Tappel and has been considered by others in the context of the retina.

One obvious suggestion might be that, in the case of the premature infant, the antioxidant system has not had adequate time to develop all its weapons. It is true that premature infants are born with only 10% of adult levels of retinal vitamin E. However, glutathione peroxidase and glutathione S-transferase activities are substantially greater in premature infant retinas than in mature retinas, and vitamin C levels also are greater in premature retinas than in adult ones. In each instance, these values reflect both vascular and avascular regions of the premature retina compared with central and peripheral portions of the mature retina.

The lack of agreement in past clinical studies over the efficacy of vitamin E may stem, in part, from many contributing factors. These include the direct effect of duration of oxygen therapy and retinal immaturity, as defined by birth weight and gestational age, and the indirect effects of other complicating health factors such as the presence or absence of intraventricular hemorrhage, sepsis, and bronchopulmonary dysplasia. The retinal cells of the premature infant who has these clinical complications may have their antioxidant defense system severely compromised, and they may be unable to withstand levels of oxygen that would otherwise be benign. In the experimental animal, where only inspired oxygen is altered, there are fewer complicating factors.

Direct evidence from animal models that free radicals are involved in retinal oxygen toxicity has come from several studies. A reduction of retinal SOD activity was found in newborn kittens exposed to 72 hr of 80% oxygen, suggesting a reduced ability of the kitten retina to defend itself against attack by oxygen radicals. Earlier supplementation with vitamin E protected against loss of retinal SOD. Increased levels of lipid peroxides were reported in the retina and blood of kittens exposed to 48 hr of 70% oxygen from day 3 of life. A free radical reaction with the lipids of cell membranes was suggested as the source of these peroxides. If a subcutaneous injection of tocopherol acetate was administered during and after exposure, the peroxide levels were reduced. Elevated lipidoperoxide levels were found in both the blood and retinas of chick embryos after exposure to 95% oxygen for 12 or 24 hr. Others measured increases in liperoxides in rabbit retinas after 12 hr at 90-95% oxygen. Finally, in a study using kittens exposed to 2-3 days of 79% oxygen from day 3 of life, treatment from day 1 with tocopherol acetate resulted in less severe retinopathy.
One drawback of this earlier work addressing the effect of vitamin E on oxygen-induced retinopathy in animals is the lack of regard for the interrelationship between vitamin E and other retinal antioxidants. We wished to (1) document the effects of manipulating retinal vitamin C and vitamin E on oxygen-induced vasoobliteration in the rat model of ROP and (2) monitor the effect of these manipulations on the effectiveness or activity of other retinal antioxidants. We chose the newborn rat for this set of experiments because its retina can be damaged by oxygen, its retinal vasculature develops in a manner similar to that of the human, but postnatally, facilitating its study; and the rat is born with a vitamin E-deficient retina, like the human infant.

**Materials and Methods**

**Oxygen Exposure**

Sprague Dawley albino rats were allowed to give birth to litters in room air. Experimental litters then were transferred immediately, mother included, to 60% oxygen, where they remained for 14 days. The oxygen level was maintained within a range of ±2% for the entire length of the experiment. Control litters were raised simultaneously in room air, with all conditions other than atmospheric oxygen being identical. All rats were fed rat chow (Purina, St. Louis, MO) ad libitum. At the end of the 14-day experiment, some rats from each group were designated for morphologic scrutiny of retinal vasculature and the remainder for determination of the levels or activities of certain retinal antioxidants.

**Vitamin C Supplementation**

In a separate experiment, similar groups of hyperoxic or normoxic newborn rats were supplemented or not with vitamin C. This was accomplished by giving mother rats daily subcutaneous injections of sodium ascorbate 0.5 g/kg body weight (Sigma, St. Louis, MO) or of the water vehicle, beginning day 1 of exposure to oxygen and continuing through day 14. Again, the young rats were separated into two groups at the time they were killed: one for morphologic assessment and the other for biochemical determinations. This protocol produced four separate treatment groups of 14-day old rats: (1) vitamin C supplemented normoxic (C+/RA), (2) normal vitamin C normoxic (C-/RA), (3) vitamin C supplemented hyperoxic (C+/O2), and (4) normal vitamin C hyperoxic (C-/O2). Lastly, serum levels of vitamin C were measured in the mothers and pups.

**Vitamin E Manipulation**

A final set of treatments was conducted in which breeding pairs were placed together and fed one of two diets manufactured by Dyets, Inc. (Bethlehem, PA). These contained either alpha-tocopherol acetate 1.0 g/kg of food or no Vitamin E. Dietary supplementation or deprivation of vitamin E continued through gestation. Again, the rats were allowed to give birth to litters in room air, at which time some litters were placed in 60% oxygen, mother included, where they remained for 14 days. Control litters simultaneously were raised in room air. Dietary vitamin E manipulation continued throughout the exposure period (a total of 35–39 days). This experimental scheme also produced four treatment groups: E+/RA, E-/RA, E+/O2, and E-/O2. The animal experiments conformed to the ARVO Resolution on the Use of Animals in Research.

**Retinal Whole Mounts**

Deeply anesthetized ratlings were perfused through the left ventricle with 5–7 ml of sodium nitroprusside 0.2% in India ink. Their eyecups were removed, and their retinas were dissected, marked for orientation, and dried on microscope slides. The resulting whole mounts then were cover slipped in glycerin and photographed with a Nikon Multiphot Macrocamera (Tokyo, Japan). We used an image-analysis system consisting of the following components: an Olympus SZ40 dissection stereomicroscope with advanced transmitted light base (Delta, Little Rock, AR), a DAGE-MTI CCD-72 series camera (Michigan City, IN), Neotech Image Grabber frame-capture hardware (Hampshire, UK), and Enhance image enhancement and analysis software (Micro-Frontier, Des Moines, IA). Two measurements of vascular extent were made: (1) the percent of total retinal area containing blood vessels (vascular area divided by vascular plus avascular area) and (2) the density of retinal vessels as calculated from a computer-derived binary image (number of black pixels in the retinal image divided by the total number of pixels in the image). The former measurement appears in Tables 1, 2, and 3. Examples of the binary images from which the latter were derived can be found in Figure 3.

**Glutathione Enzymes**

The retinas were homogenized in 300 μl of 50 mmol/l sodium phosphate buffer (pH 7.0) with 1.0 mmol/l ethylenediaminetetraacetic acid (EDTA). A 70-μl aliquot of the homogenate was transferred immediately into 5.0 mmol/l potassium phosphate buffer.
buffer (containing 140 mmol/l KCl, 1.0 mmol/l EDTA, and 1.0 mmol/l dithiothreitol, pH 7.0) for later determination of glutathione reductase. Each homogenate was centrifuged at 100,000 × g for 1 hr, and enzymatic activities were measured in the supernatant. These activities also were measured in the resulting pellet to prove the efficiency of the homogenization process in releasing all the enzyme. The activity of each enzyme was determined spectrophotometrically by following the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) or the reduction of NADP at 340 nm. This level of NADPH was at least tenfold the enzyme’s Michaelis constant for each assay.

**Glutathione peroxidase:** Glutathione peroxidase activity was measured in an appropriate amount of the supernatant by modifying an earlier procedure. Oxidation of glutathione (5.0 mmol/l) was coupled to NADPH (0.25 mmol/l) oxidation by glutathione reductase (1 unit/ml), in the presence of a 0.6 mmol/l cumene hydroperoxidase substrate, to measure total peroxidase activity. The selenium-dependent peroxidase was measured using 0.2 mmol/l hydrogen peroxide as substrate.

**Glutathione S-transferase:** Glutathione S-transferase was determined by measuring the conjugation of glutathione (1.0 mmol/l) with 1-chloro-2,4-dinitrobenzene (1.0 mmol/l). The supernatant was assayed in the presence of 0.1 mol/l sodium phosphate buffer (pH 6.5) and 1.0 mmol/l EDTA.

**Glutathione reductase:** This assay was done by measuring the reduction of oxidized glutathione (0.25 mmol/l) by NADPH (0.13 mmol/l). Supernatant levels were measured in 0.1 mol/l potassium phosphate buffer (pH 6.6) and 1.0 mmol/l EDTA by an adaptation of an earlier method.

**Antioxidant Molecules**

The retinas were homogenized in 1.0 ml of argon-purged distilled water. A 700-μl aliquot of the homogenate was removed for vitamin E and lipid phosphorus measurements; 100 μl of the remaining homogenate was used for ascorbic acid measurements. The rest of the homogenate underwent cytoplasmic protein determinations.

**Vitamin E:** A known amount of vitamin E acetate internal standard and 250 μg of butylated hydroxytoluene were added to the aliquot taken for vitamin E analysis. After extraction of lipid components in chloroform and methanol (2:1), one fourth of the total was removed for lipid phosphorus determination. The remaining solvent was evaporated under argon, and the sample was dissolved in methanol and injected into a

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**Table 1. The effect of oxygen exposure**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Room air</th>
<th>60% Oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinal vit. C (nm/mg cyt pro)</td>
<td>69.3 ± 7.9 (17)</td>
<td>58.6 ± 11.0 (17)</td>
</tr>
<tr>
<td>Retinal vit. E (mmole/mole PL)</td>
<td>0.96 ± 0.29 (14)</td>
<td>(0.005)*</td>
</tr>
<tr>
<td>GSH-red (sp. act.)</td>
<td>41.7 ± 8.6 (16)</td>
<td>39.0 ± 12.6 (18)</td>
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<tr>
<td>GSH-tase (sp. act.)</td>
<td>55.3 ± 9.6 (16)</td>
<td>61.2 ± 15.1 (16)</td>
</tr>
<tr>
<td>GSH-px (sp. act.)</td>
<td>89.6 ± 16.8 (15)</td>
<td>88.2 ± 14.4 (16)</td>
</tr>
<tr>
<td>% Retinal area vascularized</td>
<td>94.4 (25)</td>
<td>58.0 (23)</td>
</tr>
</tbody>
</table>

Value ± standard deviation (sample size).
No statistical analyses were performed on the % retinal area vascularized.
* Significant difference between atmospheric treatments.
† Significant difference between room air and oxygen treatments irrespec- tive of vitamin C supplementation (confidence level in parentheses).
‡ Significant difference between vitamin C treatments within room air or oxygen treatments (confidence level in parentheses).

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**Table 2. The effect of vitamin C supplementation**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Room air</th>
<th>60% Oxygen</th>
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</thead>
<tbody>
<tr>
<td>Mother’s serum vit. C (mg/dl)</td>
<td>0.62 ± 0.03 (3)</td>
<td>0.55 ± 0.04 (3)</td>
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<tr>
<td>Pups’ serum vit. C (mg/dl)</td>
<td>0.75 ± 0.20 (5)</td>
<td>0.44 ± 0.07 (5)</td>
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<td>Retinal vit. C (nm/mg cyt pro)</td>
<td>75.1 ± 7.9 (5)</td>
<td>62.0 ± 7.1 (5)</td>
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<td>Retinal vit. E (mmole/mole PL)</td>
<td>1.03 ± 0.20 (5)</td>
<td>0.67 ± 0.02 (5)</td>
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<td>GSH-red (sp. act.)</td>
<td>48.2 ± 1.5 (5)</td>
<td>47.5 ± 5.7 (7)</td>
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<tr>
<td>GSH-tase (sp. act.)</td>
<td>58.3 ± 1.3 (5)</td>
<td>66.5 ± 9.7 (6)</td>
</tr>
<tr>
<td>GSH-px (sp. act.)</td>
<td>96.3 ± 24.8 (4)</td>
<td>83.5 ± 13.8 (5)</td>
</tr>
<tr>
<td>% Retinal area vascularized</td>
<td>94.9 (4)</td>
<td>56.0 (4)</td>
</tr>
</tbody>
</table>

Value ± standard deviation (sample size).
No statistical analyses were performed on the % retinal area vascularized.
* Significant difference between room air and oxygen treatments irrespec- tive of vitamin C supplementation (confidence level in parentheses).
† Significant difference between vitamin C treatments within room air or oxygen treatments (confidence level in parentheses).
Table 3. The effect of vitamin E manipulation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Room air</th>
<th>60% Oxygen</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>E+</td>
<td>E-</td>
</tr>
<tr>
<td>Retinal vit. C (nm/mg cyt pro)</td>
<td>72.6 ± 14.5 (12)</td>
<td>79.6 ± 15.0 (12)</td>
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<tr>
<td>Retinal vit. E (nmol/mg cyt pro)</td>
<td>1.55 ± 0.56 (12)</td>
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<td>GSH-red (sp. act.)</td>
<td>36.9 ± 9.0 (9)</td>
<td>37.5 ± 8.5 (8)</td>
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<tr>
<td>GSH-tase (sp. act.)</td>
<td>57.8 ± 13.4 (12)</td>
<td>62.6 ± 9.8 (12)</td>
</tr>
<tr>
<td>GSH-px (sp. act.)</td>
<td>88.0 ± 18.0 (9)</td>
<td>87.2 ± 13.1 (8)</td>
</tr>
<tr>
<td>% Retinal area vascularized</td>
<td>94.5 (9)</td>
<td>90.1 (10)</td>
</tr>
</tbody>
</table>

Value ± standard deviation (sample size).

No statistical analyses were performed on the % retinal area vascularized.

* Significant difference between room air and oxygen treatments, irrespective of vitamin E manipulation (confidence level in parentheses).

† Significant difference between dietary treatments within room air or oxygen treatments (confidence level in parentheses).

C18 reverse-phase liquid-chromatography column. Isocratic elution was done with a mobile phase of methanol 98% in water at a flow rate of 2.0 ml/min.27 Absorbance was monitored at 288 nm.

Ascorbic acid: We placed 100 μl of the homogenate immediately in perchloric acid to which an internal standard of fumaric acid had been added to give a final perchloric acid concentration of 4%. The samples were vortexed and centrifuged at 3000 x g for 10 min. Ascorbic acid was measured in the supernatant by liquid chromatography, again using a C18 reverse-phase column. We used a mobile phase of 37 mmol/l sodium phosphate buffer (pH 2.8). Absorbance was monitored at 245 nm.

Statistical differences between treatment groups were determined using analysis of variance and Dunnett’s post hoc analysis. In all instances, the differences were considered statistically significant if they satisfied a 95% confidence limit.

Results

Effect of Oxygen Exposure on Vascular Morphology and Retinal Antioxidants

Figure 2 shows retinal vasoobliteration caused by 14 days of exposure to 60% oxygen. The difference in vascular architecture between the two treatments consists of three primary components: (1) retardation of the progression of vessels toward the retinal periphery, (2) obliteration or lack of development of the central and periarterial capillaries, and (3) complete loss of the “deep” vascular network throughout. Digital analysis revealed that the retinal area containing blood vessels was reduced by 35% in the oxygen-exposed group. Vessel density measurements agreed ex-

Fig. 2. Ink-perfused, flat-mounted retinas from a room air-raised 14-day-old rat (left) and from an age-matched animal raised in 60% oxygen (right). Vaso-obliteration of previously formed capillaries has led to a central avascular region in the latter case. In addition, retardation of new vessel development has created an avascular zone in the retinal periphery.
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Fig. 3. Binary images of the two ink-perfused retinas in Figure 2. It is from these images that estimates of vaso-obliteration are made by two methods. One method reflects the portion of retinal area that contains vessels; the other addresses the density of vessels within that vascular area.

Fig. 3. Binary images of the two ink-perfused retinas in Figure 2. It is from these images that estimates of vaso-obliteration are made by two methods. One method reflects the portion of retinal area that contains vessels; the other addresses the density of vessels within that vascular area.

acctly; there was a 35% reduction in the retinas of oxygen-exposed rats (Fig. 3).

Table 1 shows the antioxidant profiles for the two groups of rats. There were significant differences in the levels of retinal vitamin E and vitamin C, with normoxic rats having 35% and 20% higher levels, respectively, than hyperoxic rats. No differences were found in the activity levels of glutathione enzymes between the two groups.

Effect of Vitamin C Supplementation

Table 2 shows the antioxidant profiles for the four groups in this experiment. Significant differences ($P < 0.0002$) were found between oxygen- and room air-exposed mother rats with respect to serum vitamin C levels. Vitamin C supplementation also affected this parameter significantly. The pup's serum vitamin C levels also were affected by oxygen exposure. Oxygen-exposed rat pups' had an average serum vitamin C level of 0.33 ± 0.19 mg/dl; the room air-raised pups' level was 0.77 ± 0.16 ($n = 10, P < 0.0005$). Supplementation did not cause a difference in serum vitamin C levels between the two room-air groups. However, supplemented rats raised in oxygen ($C+/O_2$) had significantly higher serum levels than the $C-/O_2$ group ($n = 5, P < 0.05$).

This experiment was designed to produce a difference in retinal vitamin C between $C+/O_2$ rats and $C-/O_2$ rats. However, retinal vitamin C was not altered by vitamin C supplementation for either hyperoxic or normoxic rats. Retinal vitamin C levels reflected an expected decrease in all oxygen-exposed rats that was not compensated for by supplementation in the $C+/O_2$ group. All room air-reared rats combined had an average level of 74.0 ± 7.3 nm/mg cytoplasmic protein, and all oxygen-reared rats had an average level of 61.3 ± 9.8 nm/mg cytoplasmic protein ($n = 10, P < 0.0025$).

Retinal vitamin E also was significantly different between exposure treatments, as it was in the rat chow-fed pups. All room air-reared rats combined had an average retinal level of 0.89 ± 0.21 mmol/mol phospholipid; all oxygen-reared rats had an average of 0.66 ± 0.03 mmol/mol phospholipid ($n = 10, P < 0.002$). Furthermore, there was also a significant difference in vitamin E levels between $C+$ and $C-$ rats in the room-air groups ($P < 0.025$). This suggested that retinal vitamin C may have been affected transiently in these rats and may have, in turn, initiated a longer-term alteration of vitamin E. Glutathione enzyme activities reflected no differences between any of the four groups.

Digital analysis of vascular profiles showed no effect of vitamin C treatment in the room air- or oxygen-treated groups. The morphologic difference between exposure treatments was the expected reduction in retinal vessel density of about 35% in all oxygen-reared pups.

Effect of Vitamin E Manipulation

Table 3 lists the antioxidant profiles for the four groups of rats with vitamin E manipulation. Three- to
fourfold increases in retinal vitamin E were achieved by this manipulation in both oxygen-exposed and normoxic rats. Also, the typical oxygen-induced reduction in retinal vitamin E was reversed completely in the E+/O₂ rats.

Retinal vitamin C reflected the lower levels characteristic of oxygen-exposed rats. All room air-reared rats had an average retinal vitamin C level of 77.4 ± 13.5 nm/mg cytoplasmic protein; oxygen-reared rats had an average retinal level of 56.1 ± 16.5 nm/mg cytoplasmic protein (n = 24, P < 0.0005). Retinal vitamin C was not affected by dietary manipulation of vitamin E. That is, no difference in retinal vitamin C was found between E+/RA and E−/RA nor between E+/O₂ and E−/O₂.

Neither glutathione reductase activity nor glutathione S-transferase activity showed a significant difference across exposure or diet treatment groups. Glutathione peroxidase was significantly higher in oxygen-exposed rats than in their room-air counterparts (118.5 ± 20.2 nm NADPH oxidized/min/mg protein versus 87.6 ± 15.4, respectively). Also, in the oxygen group, vitamin E-deprived rats had significantly higher levels than those that were supplemented with vitamin E.

There was a difference in oxygen-induced vasooblitera-tion between vitamin E-supplemented and -deprived rats of 11% in terms of vascular area. In terms of vessel density, the difference was 14%. Qualitatively, this difference appeared to be caused primarily by a markedly reduced capillary-free zone in the center of the retinas of the supplemented group (Fig. 4). However, both E-supplemented and -deprived rats raised in oxygen had less vasoobliteration than the oxygen-exposed rats whose mothers were fed rat chow (Table 1). The avascular area was reduced by 23% in E+/O₂ rats and by 12% in E−/O₂ rats compared with rat-chow fed/O₂ rats. This difference was found in the retinal periphery, where both E+/O₂ and E−/O₂ rats contained a smaller avascular area than did rat-chow fed/O₂ rats. This difference was found in the retinal periphery, where both E+/O₂ and E−/O₂ rats contained a smaller avascular area than did rat-chow fed/O₂ rats and E−/O₂ rats had a vessel density 11% greater. This close agreement of the two methods of vascular assessment suggests that differences in overall retinal vessel density between treatments were caused primarily by differences in the relative size of vascular and avascular areas of retina rather than differences in the density of vessels in the vascular areas.

Discussion

Exposure of newborn rats to 60% atmospheric oxygen for 14 days causes a significant reduction of retinal vitamin E and vitamin C. Having discovered this, an attempt was made to ameliorate oxygen-induced retinal vasoobliteration by increasing the levels of these two substances in the retinas of newborn rats. An effort aimed at altering retinal vitamin C levels met with limited success. Our attempt to supplement vitamin C did not result in a difference in its retinal
levels between treated and nontreated pups at 14 days. However, a significant difference in retinal vitamin E levels suggested that vitamin C may have been altered transiently in room air-raised rats. The treatment did not alter oxygen-induced vasooobliteration. Difficulties in manipulating vitamin C in this experiment may stem from the fact that, in rats, this vitamin is not a true essential vitamin; they can manufacture it. Therefore, rats are difficult to make scorbuitic. Even in cases where serum levels are low, such as in the oxygen-reared and nonvitamin C-supplemented group (C−/O2), retinal levels reflect a preferential sequestering of vitamin C.

Much has been written of the possible interaction between vitamins C and E in their role as antioxidants. It has been known since 1941 that vitamin C enhances the antioxidant activity of vitamin E. Many later studies, testing various in vitro systems, have established this relationship by spectrophotometric analysis of pulse radiolysis11 and by electron-spin resonance spectroscopy.29 The relationship also can be deduced from in vivo studies.30,31 The clearest experimental evidence comes from studies using water- and lipid-soluble radical initiators that generate an initiating free radical at a controlled rate and site.32,33

Using soybean phosphatidylcholine liposomes, when a water-soluble initiator was added to the system, free radicals were generated in the aqueous phase.32 If vitamin C was added to the aqueous phase, oxidation was suppressed greatly. If vitamin E was incorporated into the liposomal membranes, oxidation also was reduced. In the presence of both vitamins C and E, the suppression was equal to the sum of that of the two individual agents. Synergism was not observed.

When a lipid-soluble initiator was incorporated into the membrane, radicals were generated within the bilayer.32 Vitamin E was effective at suppressing oxidation. Addition of vitamin C alone had little effect, suggesting that vitamin C, located in the aqueous phase could not scavenge the radicals in the membrane. When vitamins E and C were added to the liposomal bilayer and the aqueous, respectively, the suppression of oxidation was greater than the sum of that for the two alone. That is, vitamin C was effective in suppressing the oxidation of liposomal membranes, even when initiating radicals were generated in the membranes where vitamin C could not scavenge them. Vitamin C was believed to accomplish this feat by regenerating vitamin E from the tocopheroxyl radical, its product of reaction with a free radical. This was confirmed by the rates of consumption of the two agents in the presence of lipid-soluble initiators.33 In the absence of vitamin C, vitamin E was consumed linearly with time, and when it was exhausted, a rapid increase in oxidation occurred. When vitamin C was added to the aqueous phase, the rate of consumption of vitamin E was reduced greatly, and vitamin C disappeared more rapidly. When vitamin C was exhausted, vitamin E was consumed more rapidly, at a rate similar to that in the absence of vitamin C.33

We hoped to observe this effect of vitamin C supplementation on oxygen-exposed rats, and it is tempting to speculate that a limited protective effect of vitamin C on vitamin E was observed in nonexposed rats supplemented with vitamin C (C+/RA). These rats had 36% more retinal vitamin E than their nonvitamin C-supplemented counterparts in room air (P<0.025), even though vitamin E was not manipulated in their diets. In the presence of oxygen, where no effect of vitamin C supplementation was seen on retinal vitamin E levels, the system probably was overwhelmed. Vitamin C was consumed rapidly, and after its disappearance, vitamin E also was exhausted. The result was that both antioxidants were found at minimal levels in the retinal tissue of oxygen-reared animals (approximately 60 nm/mg cytoplasmic protein for vitamin C and 0.65 mm/mol phospholipid for vitamin E).

Our attempt to alter retinal vitamin E was more successful than that for vitamin C. Three- to fourfold differences were caused by the dietary changes. Also, the typical oxygen-induced reduction of retinal vitamin E was suppressed completely in the supplemented group (E+/O2). The reduction in oxygen-induced vasooobliteration caused by vitamin E supplementation was expected. However, we were surprised that deprived rats sustained less loss of vessels than oxygen-exposed pups whose mothers were fed rat chow. The cause of this effect requires additional study. Perhaps a good starting point is the relationship between vitamin E and glutathione peroxidase.

Glutathione peroxidase exists in two forms in most tissues, including the retina.10 One is dependent on selenium as a co-factor; the other is not. In the retinas of all groups of 14-day-old rats in this study, virtually all of the glutathione peroxidase activity could be attributed to the selenium-dependent variety, with one exception. The additional activity seen in the E−/O2 group was contributed by the selenium-independent form of the enzyme. This intriguing result asks more questions than it answers. If the 14-day-old rat retina was too immature for the selenium-independent enzyme to be present (by 28 days of age in our control rats, this form constitutes 25% of the retinal total), what induced this early activity in E−/O2 rats? It was not vitamin E deprivation alone, nor was it oxygen exposure alone. Each would have caused the same activity increase in other groups of animals. It has
been suggested for human infant retinas that "since premature retinas are deficient in vitamin E in both the vascular and avascular regions, the early antioxidant protection is provided in part by GSH-px ... whose activity is substantially greater than [that] present in mature retinas."\(^{10}\) Perhaps the reason that \(E+/O_2\) and \(E-/O_2\) rats sustained less vasoobliteration than rat chow-fed oxygen-exposed groups was that glutathione peroxidase activities were abnormally high, particularly the relative activity of the \(E-/O_2\) condition. These activities were 123 nmol NADPH oxidized/min/mg cytoplasmic protein versus 88 nmol for \(E-/O_2\) and rat chow/O_2 rats, respectively. The extent of vasculature was 70% and 58%, respectively, for these two groups. Was glutathione peroxidase capable of compensating for retinal vitamin E deficiency in preventing oxygen-induced vasoobliteration? We plan to pursue the potential efficacy of glutathione peroxidase in future experiments.

This study presents evidence supporting a role of oxygen toxicity in the pathogenesis of ROP. It also reemphasized that vitamin E may not be the panacea for which we have searched in vain to ameliorate this condition. However, vitamin E affords consistent, albeit limited, protection in the oxygen-exposed rat, and its role as an antioxidant in this protection must be considered seriously. A difficult problem, even in well-controlled animal studies, is that there are many complicating factors. The retinal antioxidant system is an extremely intricate one in which subtle interactions occur between many agents. Our results should, if nothing else, serve to remind us of this caveat as follows. In assessing the effects of any one antioxidant on oxygen-induced retinopathy, the relative contributions of other antioxidants also must be considered. The actual contribution that glutathione peroxidase makes in defending the immature retina from oxygen-induced retinopathy is unknown for animals or humans. However, vitamin E is not the only antioxidant in the retina, and we should appreciate the value of other agents as potential therapies.

**Key words:** oxygen-induced retinopathy, vitamin E, vitamin C, retinal blood vessel, vasoobliteration

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