The Regional Distribution of Vitamins E and C in Mature and Premature Human Retinas

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Vitamin E is used to ameliorate retinopathy of prematurity, but little is known about baseline vitamin E levels in retinas of premature infants or the effect of vitamin E supplementation on these levels. Vitamin E and C levels were measured in mature retinas (1 month to 73 years) and in retinas of premature infants (22 to 33 weeks of gestation). The infants fell into two groups: (1) those who survived <12 hr and received no vitamin E, and (2) those who survived >4 days and received vitamin E supplementation. Premature infants are born with 5 to 12 percent the vitamin E levels found in mature retinas. Vitamin E levels in vascular and avascular retina of premature infants increased with gestation. Infants born >27 weeks gestation and surviving at least 4 days with vitamin E supplementation demonstrated markedly elevated vitamin E levels in vascular and avascular retina when compared to supplemented infants < 27 weeks gestation. Premature infants possessed 35-50% higher levels of retinal vitamin C than those found in mature retinas. These data demonstrate that premature infants are born with relatively low levels of retinal vitamin E, particularly in the avascular region, but contain an abundance of retinal vitamin C. These data further suggest that vitamin E supplementation results in a rapid increase in retinal vitamin E levels, particularly in infants > 27 weeks gestational age. Invest Ophthalmol Vis Sci 29:22-26, 1987

In 1956 oxygen was identified as the primary etiological factor in retinopathy of prematurity (ROP). Oxygen toxicity may be mediated by highly reactive metabolites of molecular oxygen. Since some of these reactive oxygen species are products of normal oxidation-reduction processes of living cells, it is not surprising that cells have several defense mechanisms against oxidative attack, among these, vitamins E and C. Premature human infants are deficient in plasma vitamin E, and in 1949 it was suggested that E-deficiency might play a role in the etiology of ROP. Support for this hypothesis comes from studies showing that animals deficient in vitamin E are more susceptible to the toxic effect of elevated oxygen. Also, an increase in gap junctions between adjacent spindle cells, the earliest morphological manifestation of the development of human infant ROP, is suppressed by early vitamin E supplementation.

There is controversy regarding the results of the numerous clinical trials performed to appraise the efficacy of vitamin E supplementation in suppressing the development of severe ROP. Some of this controversy may involve differences in experimental design. The four most recent double-masked clinical trials, with the exception of route of vitamin E administration, employed similar experimental criteria: infants < 1500 g birth weight, large population size, randomization, early initiation and continuous vitamin E supplementation, control population vitamin E-deficient, and frequent ophthalmologic examinations. When these studies were compared, initial oral vitamin E, initial intramuscular vitamin E acetate, and initial intravenous vitamin E showed efficacy. In another study, initial intravenous vitamin E was non-efficacious. Reconciliation of disparate results of clinical trials may rely on a further understanding of the content and uptake of vitamin E in premature infant retinas as a function of gestational age. We have compared the regional distribution of vitamin E in retinas of premature infants to that in mature retinas. Also, we have compared retinal vitamin E levels in vitamin E-supplemented and -unsupplemented infants. Vitamin C levels were also determined in some of these retinas.

Materials and Methods

Whole eye donations were obtained from nine premature infants (22–33 weeks of gestation) who survived <12 hr and received no vitamin E supplemen-
tation and from 6 premature infants (23-31 weeks of gestation) who survived from 4 to 42 days and received the standard protocol of vitamin E supplementation at the Baylor College of Medicine affiliated level III neonatal intensive care units. Supplemented infants received IM injections of aqueous tocopherol (15, 10 and 10 mg/kg on days 1, 2 and 4, respectively, in alternate thighs) concomitantly with oral tocopheryl acetate in medium chain triglycerides (100 mg/kg/day).13 All eyes were removed within 3 hr of death and transported to the lab on ice and dissected immediately. Thirteen mature retinas (ages 1 month to 73 years) were obtained from eyes donated to the Lions Eyes of Texas Eye Bank. These eyes were obtained within the same time frame and treated the same way as the premature eyes. The birth weights, gestational ages, and survival times of the infants included in this study are given in Table 1.

Table 1. Birth weight and duration of life as a function of gestational age

<table>
<thead>
<tr>
<th>Gestational age (weeks)</th>
<th>Birth weight (g)</th>
<th>Life (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>360</td>
<td>&lt;1</td>
</tr>
<tr>
<td>22</td>
<td>420</td>
<td>&lt;1</td>
</tr>
<tr>
<td>22</td>
<td>470</td>
<td>&lt;1</td>
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<tr>
<td>23</td>
<td>440</td>
<td>&lt;1</td>
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<tr>
<td>23</td>
<td>530</td>
<td>10</td>
</tr>
<tr>
<td>23</td>
<td>530</td>
<td>&lt;1</td>
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<tr>
<td>23</td>
<td>540</td>
<td>11</td>
</tr>
<tr>
<td>25</td>
<td>660</td>
<td>20</td>
</tr>
<tr>
<td>26</td>
<td>700</td>
<td>&lt;1</td>
</tr>
<tr>
<td>27</td>
<td>800</td>
<td>&lt;1</td>
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<tr>
<td>28</td>
<td>*580</td>
<td>&lt;1</td>
</tr>
<tr>
<td>28</td>
<td>950</td>
<td>42</td>
</tr>
<tr>
<td>28</td>
<td>950</td>
<td>14</td>
</tr>
<tr>
<td>31</td>
<td>1200</td>
<td>4</td>
</tr>
<tr>
<td>33</td>
<td>1320</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* Low birth weight because this infant was a triplet.

Retinas were dissected from whole eye donations under a stream of argon in the following solution: 140 mM NaCl, 2.7 mM KCl, 11 mM dextrose, 0.05 mM diethylenetriamine pentaacetic acid (DTPA), and 10 mM HEPES, pH 7.4. In order to minimize oxidation, all solutions and solvents were purged with argon and the study was done under an atmosphere of argon whenever possible.

Three millimeter trephined punches were taken from within the temporal arcades (vascular region) and the temporal periphery (avascular region) of premature infant retinas. For purposes of comparison, 3 mm punches were taken from mature retinas from within the temporal vascular arcades, avoiding the fovea (central region), and just anterior to the temporal equator (peripheral region). Care was taken to ensure that no major blood vessels were present in the trephined punches. However, the material removed from the vascularized retina also contained some blood. No attempt was made to correct for vitamin E or C that might have been present in this blood.

An internal standard of \( \alpha \)-tocopheryl acetate, along with 250 \( \mu \)g t-butyl hydroxytoluene (BHT), was added to each sample. Each trephined punch was homogenized in chloroform-methanol (2:1 by volume) and centrifuged at 4000 \( g \) for 15 min. The supernatant was carefully transferred to a 12 ml screw cap tube and washed once with a 20% volume of water. The pellet was saved for DNA analysis. After vortexing, the samples were centrifuged briefly at 2000 \( g \) to produce a biphasic. The lower organic phase was transferred, evaporated under a stream of argon, re-dissolved in 10 \( \mu l \) methanol, and analyzed for \( \alpha \)-tocopherol by high pressure liquid chromatography (HPLC).

A Rainin (Woburn, MA) Microsorb C-18 reverse phase column, coupled to a Kratos (Ramsey, NJ) Spectronow \( / \beta \) variable wavelength detector, and a Spectra-Physics (Houston, TX) 4270 integrator were used. The mobile phase was methanol-water (98:2 by volume) run at 2 ml/min. The eluent was monitored at 288 nm. The details of this procedure are published elsewhere.14,15

DNA was analyzed by a fluorometric method16 which was slightly modified by increasing the sample concentration of the fluorochrome, bisbenzimide, to 2 \( \mu \)g/ml.

When vitamin C was assayed, two trephined punches were homogenized in \( O_2 \)-free water and aliquots were then taken for vitamin C, vitamin E, and DNA analysis. In some cases, an aliquot was also taken for protein analysis. The vitamin C aliquot was placed immediately into a known volume of perchloric acid, to which an internal standard of fumaric acid had been added to give a final perchloric acid concentration of 4%. These samples were vortexed and centrifuged at 3000 \( g \) for 15 min. The supernatant was analyzed by HPLC, using the same column and system as for the vitamin E analysis, except the mobile phase was 70 mM \( Na_2HPO_4 \) buffer (pH 2.8) run at 1 ml/min. The eluent was monitored at 245 nm. The vitamin E aliquot was placed immediately into a known volume of chloroform-methanol (2:1 by volume) containing 250 \( \mu \)g BHT and the internal standard, \( \alpha \)-tocopheryl acetate. The final ratio of chloroform-methanol-water was identical to that achieved with the 20% volume water wash described above, and the rest of the analysis was carried out as described above.

The protein concentration was determined by the method of Lowry17 using bovine serum albumin as a standard.
Table 2. Distribution of vitamins E and C and DNA in mature and unsupplemented premature human retina

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Retinal region</th>
<th>Vitamin E</th>
<th>Vitamin C</th>
<th>DNA</th>
<th>Protein/DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nmole/mg DNA</td>
<td>nmole/cm² retina</td>
<td>nmole/mg DNA</td>
<td>nmole/cm² retina</td>
</tr>
<tr>
<td>Premature retina</td>
<td>Vascular</td>
<td>*1.4 ± 1.1 (0.51-3.8)</td>
<td>0.47 ± 0.32 (0.21-1.1)</td>
<td>244 ± 38 n = 4</td>
<td>71.3 ± 15.2 n = 4</td>
</tr>
<tr>
<td></td>
<td>Avascular</td>
<td>0.73 ± 0.56 (0.22-1.8)</td>
<td>0.28 ± 0.18 (0.12-0.58)</td>
<td>236 ± 66 n = 4</td>
<td>95.2 ± 22.1 n = 3</td>
</tr>
<tr>
<td>Mature retina</td>
<td>Central</td>
<td>10.9 ± 6.0 (6.2-24)</td>
<td>3.04 ± 1.34 (1.6-5.2)</td>
<td>162 ± 33 n = 5</td>
<td>38.3 ± 8.8 n = 5</td>
</tr>
<tr>
<td></td>
<td>Peripheral</td>
<td>14.1 ± 8.3 (5.1-37)</td>
<td>2.41 ± 1.43 (0.74-5.2)</td>
<td>174 ± 44 n = 5</td>
<td>25.5 ± 7.1 n = 5</td>
</tr>
</tbody>
</table>

*Mean values ± standard deviation. Ranges are given in parentheses.

For statistical analysis, whenever regions within a retina were compared, the paired t-test was used; when differences between retinas were compared, the student two-tailed t-test was used. Linear regression analysis was used to compare age versus α-tocopherol concentration in premature retinas. The data are reported as mean ± standard deviation (SD).

**Results**

Since there are significant morphological differences between the avascular and the vascular regions of premature retina, an ideal denominator for vitamin E and C concentrations is not readily available. We therefore elected to use two separate denominators, DNA weight and retinal area. The DNA content per cm² retinal area significantly decreases from central to peripheral mature retina (P < 0.001) (Table 2). The vascular region of premature retina has 20% more DNA/cm² retinal area than the central region of mature retina (P < 0.001) (Table 2). The avascular region of premature retina has 36% more DNA/cm² retinal area than the vascular region in premature retina. Thus, the regional difference we report within the same group of retinas may be a reflection of cell density and/or volume. The protein-to-DNA ratio given in Table 2 allows comparison of our data with that from other labs that may express their data per unit of protein.

In comparing the vitamin E-supplemented with the unsupplemented infants, it should be noted that the unsupplemented infants did not survive a significant period of time and therefore are not a true control group for the supplemented infants, but rather demonstrate the retinal vitamin E in an infant at birth. All but one infant had similar birth weights relative to gestational age and all infants were anomalously free.

Relative to DNA, the central region of mature retinas has eight times (P < 0.001) the α-tocopherol found in the vascular region of premature infant retinas (Table 2). Relative to retinal area, the difference is about six-fold (P < 0.001). The avascular region of premature infant retinas has 52% (P < 0.001) the α-tocopherol found in the vascular region when related to DNA. This pattern is similar when the α-tocopherol is related to retinal area. Conversely, in mature retinas, the α-tocopherol per DNA increases significantly from central to peripheral retina. Like the premature infant retina, the vitamin E level in mature retinas decreases from central to peripheral retina when related to retinal area.

The data presented in Table 2 for the levels of vitamin E in premature infant retinas are the average of values for all gestational ages. If these results are plotted against gestational age, it can be seen that the retinal vitamin E increases gradually with gestation in unsupplemented infants (Fig. 1). From linear regression analysis, the regression coefficients, correlation coefficients and probability values were 0.26, 0.95, and <0.001 for avascular retina (Fig. 1A) and 0.13, 0.86, and <0.005 for vascular retina (Fig. 1B), respectively. Vitamin E levels for infants supplemented with vitamin E are also presented in Figure 1. A theoretical dotted line connects the predicted value at the age of birth with the actual value at the age (gestational age plus weeks of life) of death. With vitamin E supplementation, infants > 27 weeks gestational age appear to achieve much higher retinal levels of vitamin E than less mature infants.

The vitamin C level in the vascular region of premature retinas as compared with the central region of mature retinas is significantly greater (P < 0.02) when related to either DNA or retinal area (Table 2).
vascular region of premature retinas is also significantly different from the peripheral region of mature retinas. The avascular and vascular regions of premature retinas have similar vitamin C levels. In mature retinas, vitamin C decreases from the central to the peripheral region \((P < 0.05)\) when related to retinal area. However, when related to DNA, there is no significant difference between these regions. Unlike vitamin E, there does not appear to be significantly less vitamin C in the avascular region of premature retina as compared with the vascular region when related to either denominator.

**Discussion**

Retinas of premature human infants are clearly deficient in vitamin E when compared to mature human retinas. These differences are too great to be explained by variations in retinal thickness or DNA content between premature and mature retinas. As a result of the 4:1 placental vitamin E permeability barrier, premature infants have low plasma levels of vitamin E \((0.4 \text{ mg}%)^2\) as compared with adults \((2 \text{ mg}%).\) This, in part, would account for the low retinal vitamin E concentration found in premature infants. However, it is of interest that the difference in the vitamin E levels between mature and unsupplemented premature retinas is greater than the relative plasma difference, particularly in the younger infants. The retinas of the premature infants appear to have difficulty sequestering vitamin E from the blood.

In the three vitamin E-supplemented infants \(< 27\) weeks gestational age, supplementation produced minimal elevation of retinal vitamin E in the avascular retina. Minimal retinal increase in vitamin E with supplementation in this age group might explain why supplementation does not suppress the development of severe ROP in infants < 27 weeks gestational age.\(^{10,18,19}\) In contrast, in the three supplemented infants > 27 weeks gestational age, supplementation appeared to result in an elevation of vitamin E in the avascular retina. This increase with supplementation is consistent with the finding that vitamin E supplementation in infants > 27 weeks gestational age suppresses severe forms of ROP in infants in this age group.\(^{10,18}\) In the vascular region, the gradual increase in vitamin E relative to gestational age is probably in part a result of photoreceptor outer segment lengthening.\(^{20}\) In addition, it has been suggested that interstitial retinol binding protein (IRBP) may play a role in the transport of vitamin E into premature infant retina.\(^{21}\) Indeed, the radial distribution of IRBP in the subretinal space increases as a function of gestational age.\(^{22}\) It might be that the slowly rising vitamin E levels in the peripheral avascular retina reflect a state of retinal development in which photoreceptor maturation in avascular retina leads to the secretion of a threshold level of IRBP into the subretinal space to sequester greater amounts of plasma vitamin E.\(^{20-22}\)

The endogenous retinal vitamin E levels we report for unsupplemented premature infants are similar to those previously reported\(^{23}\) when we convert our data to \(\mu\text{g vitamin E/mg protein}.\) In this previous study, the four infants surviving less than 2 days appeared to demonstrate an increase in total retinal vitamin E with gestation; however, it should be noted that two of these infants did receive one IM injection of vitamin E. Four of the infants in this study were >33 weeks gestational age. Only one infant survived >4 days after receiving vitamin E supplementation. This infant was 23½ weeks gestation and survived almost 6 weeks. This infant had an elevated total retinal vitamin E level that is consistent with our finding of a slow rise in total (vascular and avascular) retinal vit-
min E level in vitamin E-supplemented infants of this gestational age. The rest of the infants in this study were either older than our infants or did not receive uniform vitamin E supplementation, making further comparisons difficult.

In contrast to vitamin E, the vitamin C levels in premature retinas are greater than in mature retinas. This increase in retinal vitamin C level in premature infants is consistent with the finding that fetal plasma vitamin C levels are two to four times higher than maternal plasma levels.

Our results show that premature human infant retinas, while having an abundance of vitamin C, are "deficient" in vitamin E when compared to mature retinas. The difference is of such magnitude that it cannot be explained on the basis of relative DNA content or retinal thickness. Since both vascular and avascular areas are affected, the presence of a blood supply cannot explain the difference, either.

**Key words:** vitamin E, vitamin C, premature infants, retinopathy of prematurity, human retina

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**References**