PURPOSE. Previous studies have shown that newborn rats exposed to hyperoxia within the first 2 weeks of life develop vasculopathy in addition to permanent changes in retinal structure and function. It has also been suggested that free radicals may be the source of these pathologic effects. Trolox C, a water-soluble analogue of vitamin E, was previously shown to limit the vascular consequences of exposure to postnatal hyperoxia. The aim of this study was to investigate whether trolox C could also help prevent the functional (electroretinography) and structural (retinal histology) consequences associated with oxygen-induced retinopathy (OIR).

METHODS. Newborn albino Sprague-Dawley rats exposed or not exposed to hyperoxia received daily injections of trolox C in doses of 300, 600, and 900 μg/kg (total volume, 50 μL). The effect of treatment was evaluated through electroretinography and retinal histology.

RESULTS. Although trolox C tended to have a retinoactive effect on the normal retina, normalization of the hyperoxia-treated group to hyperoxic control and of the normoxia-treated group to normoxic control revealed that the a-wave remained relatively unaffected by hyperoxia exposure and by treatment with trolox C, the efficacy of trolox C at doses of 600 and 900 μg/kg largely outweighed the retinoactive effect, and the oscillatory potentials (OPs) benefited to the greatest extent from trolox C treatment. Furthermore, trolox C was able to limit the reduction in outer plexiform layer thickness but not the concomitant reduction of the horizontal cell count, each of which is associated with OIR.

CONCLUSIONS. These results show that, as had been previously demonstrated with retinal vasculature, trolox C limited the retinal functional and structural damages inherent in the rat model of OIR. However, despite treatment, there were still signs (albeit less severe) indicative of OIR. This suggests, as previously advanced, that the pathophysiology of OIR is not solely caused by the action of free radicals or that trolox C is inadequate in treating all aspects of OIR. (Invest Ophthalmol Vis Sci. 2006;47:1101–1108) DOI:10.1167/iovs.05-0727
ducted to determine whether trolox C could also protect the structure and function of the retina after postnatal hyperoxia.

METHODS

The experimental protocol was approved by the McGill University/Montreal Children’s Hospital Research Institute Animal Care committee according to the guidelines of the Canadian Council on Animal Care. Experiments were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. newborn litters of Sprague-Dawley (SD) rats (Charles River Laboratories, St. Constant, Quebec, Canada) were exposed to 80% oxygen (mixture of medical grade 100% O2 and room air measured with a MaxO2 ceramic atmospheric oxygen meter, model OM25-ME, Medicana Inc., Montreal, Canada) from postnatal day 0 through postnatal day 14 for 22.5 hours daily, interrupted three times for a 30-minute period of dark adaptation. The rats were then placed in a light-proof recording chamber preamplifiers (P511; Grass Instruments, Quincy, MA). A fiber light source (50,000 lux, dim red light illumination) with intramuscular injections of ketamine (100 mg/kg) and xylazine (6 mg/kg). Pupils were dilated to a diameter preamplifiers (P511; Grass Instruments, Quincy, MA). A fiber light source (50,000 lux, dim red light illumination) with intramuscular injections of ketamine (100 mg/kg) and xylazine (6 mg/kg). Pupils were dilated to a diameter of 10 measurements from each was obtained. Pictures were obtained using a photograph microscope (Acti; Zeiss, Oberkochen, Germany).

Data Analysis

Amplitudes of ERG components were measured according to a method previously described. Briefly, the amplitude of the a-wave was measured from baseline to trough, and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. Oscillatory potentials were measured in a similar fashion from the preceding trough to the peak of the OP evaluated and were reported individually or as the sum of OPs (OP = OP + OP + OP + OP).

For each animal, scotopic luminance-response function curves were derived by plotting b-wave amplitudes against flash intensities. A sigmoidal-intensity response regression curve was then used to fit the data (Prism 3.00 software; GraphPad, San Diego, CA), from which the rod Vmax, maximal rod response was calculated. In addition, the ERG response evoked to the brightest flash delivered in scotopic conditions (the mixed rod-cone response) was also included in the analysis. Two-way repeated measures ANOVA (P < 0.05) with Bonferroni post-tests were used to determine the effect of hyperoxia and trolox C treatment on the different parameters of the ERG and on retinal histologic structures, with maturation (30 and 60 days) as the repeated factor and treatment group as the independent factor. Data are presented as the mean ± 1 SD.

Results

Amplitudes of ERG components were measured according to a method previously described. Briefly, the amplitude of the a-wave was measured from baseline to trough, and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. Oscillatory potentials were measured in a similar fashion from the preceding trough to the peak of the OP evaluated and were reported individually or as the sum of OPs (OP = OP + OP + OP + OP).

For each animal, scotopic luminance-response function curves were derived by plotting b-wave amplitudes against flash intensities. A sigmoidal-intensity response regression curve was then used to fit the data (Prism 3.00 software; GraphPad, San Diego, CA), from which the rod Vmax, maximal rod response was calculated. In addition, the ERG response evoked to the brightest flash delivered in scotopic conditions (the mixed rod-cone response) was also included in the analysis. Two-way repeated measures ANOVA (P < 0.05) with Bonferroni post-tests were used to determine the effect of hyperoxia and trolox C treatment on the different parameters of the ERG and on retinal histologic structures, with maturation (30 and 60 days) as the repeated factor and treatment group as the independent factor. Data are presented as the mean ± 1 SD.

Figure 1 shows representative scotopic (rod Vmax, mixed rod-cone ERGs, and mixed rod-cone OPs) and photopic (cone ERG and cone OPs) retinal responses obtained from four of the eight study groups. The first group illustrated in Figure 1 consists of rats raised in room air (normoxic control). They are compared with rats raised in room air while receiving 600 µg/kg trolox C from postnatal day 5 through postnatal day 14 (normoxia-treated), with rats raised in a hyperoxic environment from birth through postnatal day 14 (hyperoxic control), and with rats exposed to postnatal hyperoxia for 14 days from birth while receiving 600 µg/kg trolox C (hyperoxia-treated) from postnatal day 5 through postnatal day 14. ERG responses were obtained twice, at 30 days (upper tracings) and at 60 days (lower tracings) of age. Amplitude measurements are reported in Table 1.
Amplitude attenuation of the scotopic and photopic b-waves, scotopic a-wave, and OPs can be observed in the hyperoxic control rat compared with the normoxic control rat. As shown in Table 1, amplitudes of the rod $V_{\text{max}}$ and the scotopic and photopic SOPs measured from hyperoxic rats treated with 600 $\mu$g/kg trolox C were nearly double the size of those measured from hyperoxic control rats ($P < 0.05$). There was also a slight, but not significant, enhancement ($P > 0.05$) of the rod-cone b-wave and photopic b-wave amplitudes, whereas the amplitude of the a-wave was unaltered by trolox C treatment (Table 1).

Results presented in Table 1 also suggest that trolox C might alter the functioning of the normal (unexposed) retina. The scotopic ERG responses (rod $V_{\text{max}}$ and rod-cone b-wave) of control treated rats were larger (but not significantly; $P > 0.05$) than those of untreated controls, whereas the photopic SOPs were significantly smaller ($P < 0.05$) than untreated controls. Consequently, to dissociate the protective effect that trolox C exerted on the retinas of newborn rats exposed to hyperoxia from its retinoactive effect observed in the normal retina, we normalized ERG amplitude measurements obtained from each normoxia-treated rat (trolox C 300-, 600-, and 900-$\mu$g/kg)

### Table 1. Group Data

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Age (d)</th>
<th>Control</th>
<th>Control+Trolox (600 $\mu$g/kg)</th>
<th>Oxygen</th>
<th>Oxygen+Trolox (600 $\mu$g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scotopic a-wave</td>
<td>30</td>
<td>485.2 ± 78.3</td>
<td>468.5 ± 78.5</td>
<td>363.1 ± 74.4</td>
<td>376.9 ± 72.0</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>358.4 ± 61.5†</td>
<td>317.8 ± 42.8†</td>
<td>294.1 ± 50.7†</td>
<td>282.8 ± 86.9†</td>
</tr>
<tr>
<td>Rod $V_{\text{max}}$</td>
<td>30</td>
<td>688.0 ± 96.7</td>
<td>859.3 ± 80.3‡</td>
<td>229.2 ± 125.9§</td>
<td>390.6 ± 123.8§</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>600.7 ± 71.9</td>
<td>701.4 ± 117.5‡</td>
<td>214.4 ± 110.4§</td>
<td>347.7 ± 117.2*</td>
</tr>
<tr>
<td>Rod-cone b-wave</td>
<td>30</td>
<td>1142.3 ± 171.7</td>
<td>1147.5 ± 169.3‡</td>
<td>435.9 ± 134.7*</td>
<td>599.5 ± 144.6*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>913.9 ± 102.4†</td>
<td>922.1 ± 122.5§</td>
<td>407.5 ± 137.6*</td>
<td>530.1 ± 162.3*</td>
</tr>
<tr>
<td>Scotopic SOPs</td>
<td>30</td>
<td>469.4 ± 60.0</td>
<td>456.8 ± 62.0‡</td>
<td>140.2 ± 87.7*</td>
<td>271.6 ± 92.2§</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>354.9 ± 66.6‡</td>
<td>352.8 ± 68.3‡</td>
<td>120.8 ± 54.7†</td>
<td>199.3 ± 82.6*</td>
</tr>
<tr>
<td>Photopic b-wave</td>
<td>30</td>
<td>262.0 ± 30.0</td>
<td>241.6 ± 37.0‡</td>
<td>86.3 ± 31.8*</td>
<td>111.2 ± 26.5*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>229.0 ± 20.0</td>
<td>161.9 ± 21.9†</td>
<td>78.7 ± 28.9*</td>
<td>110.2 ± 38.1†</td>
</tr>
<tr>
<td>Photopic SOPs</td>
<td>30</td>
<td>112.7 ± 26.0</td>
<td>81.0 ± 18.8‡</td>
<td>27.9 ± 15.5*</td>
<td>43.7 ± 13.2§</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>100.3 ± 15.7</td>
<td>34.9 ± 8.1†</td>
<td>32.0 ± 10.0</td>
<td>39.5 ± 14.9*</td>
</tr>
</tbody>
</table>

Data were obtained in scotopic (rod-cone a-wave, rod $V_{\text{max}}$, rod-cone b-wave, rod SOPs) and photopic (b-wave and SOPs) conditions from rats raised in normoxic and hyperoxic conditions, with or without treatment with 600 $\mu$g/kg of trolox C. Amplitudes are reported in mean microvolts ± SD.

* Significant difference from the control group.
† Significant difference from 30-day results.
‡ Significant difference from the oxygen+Trolox (different from $O_2$+respective dose).
§ Significant difference from the oxygen-exposed group.
groups, respectively) to the mean amplitude of the normoxic control group (taken as 100%). Similarly, ERG amplitude measurements obtained from each hyperoxia-treated rat (trolox C 300-, 600-, and 900-μg/kg groups, respectively) were normalized to the mean amplitude of the hyperoxia control group (also taken as 100%). Results obtained from this data manipulation are shown in Figure 2, in which data obtained at postnatal days 30 and 60 are also compared. Our results confirm our initial impression that, despite the lack of an effect on the a-wave (Figs. 2A, 2B), treatment with trolox C does indeed have an impact on retinal function. Amplitude ratios generated from hyperoxia-treated rats always tended to be greater than those obtained from normoxia-treated rats, suggesting that the therapeutic effect of trolox C outweighed its (normal) retinoactive effect. In fact, when normoxia-treated and hyperoxia-treated rats are compared, significant differences favoring the therapeutic effect of trolox C are seen for the photopic b-wave at postnatal days 30 and 60 and for scotopic and photopic SOPs at postnatal days 30 and 60 (Figs. 2G–L). Similar tendencies can also be seen for the rod V_{max} and the rod-cone b-waves. As mentioned, OPs have been shown to be particularly susceptible to postnatal hyperoxia. The dose-dependent effect observed on the SOPs after the administration of trolox C is, therefore, not surprising. Once normalized to their respective controls, scotopic and, more important, photopic SOPs measured in the hyperoxia-treated group are significantly different from those obtained from the normoxia-treated rats, indicating that the OPs clearly benefited from the therapeutic effect of trolox C. This holds true for groups that received as little as 300 μg/kg (Figs. 2J–L).

In previous studies, we show that the amplitude of ERG responses decreases with age when data obtained from 30- and 60-day-old rats are compared.21,26 This was further exemplified in Figure 3, with its comparison of the maturation-induced ERG amplitude decrease in normoxic and hyperoxic rats regardless of whether they received trolox C treatment. To facilitate the comparison, we devised a maturation index that represented the average of the percentage difference between ERGs obtained at 30 days and those obtained at 60 days for each rat as measured with the ERG a-wave and scotopic (combined rod V_{max} and mixed rod-cone) and photopic b-waves. Figure 3A shows the index of maturation for the a-wave, which was similar for normoxic control and hyperoxic control rats (23.19% vs. 24.18%; P = 0.05). Furthermore, despite some variability, responses obtained from animals raised in either environment while receiving trolox C, irrespective of concentration, were not significantly different from those of respective controls, suggesting that oxygen exposure alone or with concomitant trolox C treatment did not result in maturation-induced attenuation of the a-wave that exceeded what was considered the expected normal range. This result further supported a previous claim of ours that,
compared with the other ERG components, the a-wave is minimally affected after postnatal hyperoxia. On the other hand, the scotopic b-wave (including the rod \( V_{\text{max}} \) and mixed rod-cone responses) appeared to have matured differently depending on whether the rats were exposed to the hyperoxic environment or not, as shown with the maturation indices in Figure 3B. The index of maturation obtained from the normoxic control rats (13.49% \( \pm 16.12\% \)) was similar (\( P > 0.05 \)) to that obtained from normoxia-treated rats, irrespective of concentration, indicating that trolox C did not impair the normal maturational process. In contrast, the negative (\(-14.73% \pm 88.95\%\)) index of maturation found for the hyperoxic control rats suggested that the hyperoxic environment tended, albeit not significantly (\( P > 0.05 \)), to alter the normal course of the maturation process. Interestingly, however, a positive (normoxic-like) index of maturation was reinstated if the hyperoxic rats were treated with 600 and 900 mg/kg trolox C but not with the 300-mg/kg dose. A similar trend was also observed with the photopic b-wave index of maturation (Fig. 3C), though in these conditions, the lowest dose, 300 \( \mu \)g/kg, already tended to return the index of maturation closer to normal, generating results similar to what was obtained using 600 \( \mu \)g/kg, whereas the 900-\( \mu \)g/kg dose yielded a maturation index identical with that measured in normoxic control rats.

**Effect of Trolox on the Retinal Cytoarchitecture**

Illustrated in Figure 4 are representative cross-sections of retinas obtained from control (with and without 600 \( \mu \)g/kg trolox C supplementation) and oxygen-exposed (with and without 600 \( \mu \)g/kg trolox C supplementation) rats at 60 days of age. We had previously demonstrated that rats exposed to hyperoxic conditions undergo gradual thinning of the OPL and reduction in the number of horizontal cells. Retinal sections shown in Figure 4 confirm these findings. OPL thickness is significantly reduced in the hyperoxic control group (Fig. 4C) compared with that in the normoxic control rats (Fig. 4A). Hyperoxia-treated rats (600 \( \mu \)g/kg trolox C; Fig. 4D) showed a better-
preserved OPL than was observed in the hyperoxia control rats (Fig. 4C). In contrast, no changes in OPL thickness were observed in the normoxia-treated group (Fig. 4B). Measurements of OPL thickness and horizontal cell counts are graphically presented in Figure 5. Hyperoxic control rats showed a significant \( P < 0.05 \) 55% reduction in the thickness of the OPL that was accompanied by a significant \( P < 0.05 \) 60% reduction in horizontal cell count. Concomitant treatment of oxygen-exposed rats with 600 \( \mu g/kg \) trolox C limited the reduction of the OPL thickness to 75% of control \( P < 0.05 \) but had no impact on the horizontal cell count. It should be noted that trolox C alone (without hyperoxia), though not altering the thickness of the OPL layer, significantly reduced the number of horizontal cells by 30%.

**DISCUSSION**

Our findings suggest that trolox C represents a valid therapeutic alternative in limiting the structural and functional damages intrinsic to the rat model of OIR. When retinal function is considered, our results (Fig. 2) show that the a-wave was not impaired by hyperoxic exposure or by trolox C treatment (irrespective of dosage), the therapeutic effect of trolox C (especially at higher doses) outweighed its retinoactive effect, and the OPs are the ERG components that benefit most from the therapeutic effects of trolox C. Furthermore, results presented in Figure 2 also suggest that the photopic (cone-mediated) function benefits significantly more than the scotopic (rod-mediated) function from the therapeutic effects of trolox C. Given that the cone-mediated function was previously shown to be relatively more impaired than the rod-mediated function after postnatal hyperoxia, a feature also evidenced in the present study (Table 1), our finding of a more potent effect of trolox C therapy on cone-mediated signals indicates that the treatment is targeted to where it is needed. The therapeutic efficacy of trolox C is further evidenced by the fact that it never modified the ERG a-wave, a component that was impaired by hyperoxic exposure or by trolox C treatment (irrespective of dosage).

Interestingly, the effect that trolox C exerted on the ERG signal appeared to depend on whether ROS were present. In the hyperoxic group, trolox C treatment enhanced the cone- and rod-mediated signals (compared with the untreated hyperoxic group), whereas in the normoxia-treated group, its use enhanced the rod response but reduced the cone-mediated ERG (com pared with the normoxia-untreated group). Trolox C has been reported to have a dual mode of action—antioxidant and prooxidant—depending on the method by which peroxidation is induced. For example, when the oxidative stress is induced by \( Cu^{2+} \) or \( Fe^{3+} \), trolox C acts preferentially as a toxic prooxidant and is converted to an \( \alpha \)-tocopherol radical as cellular functions deteriorate. In contrast, trolox C exerts its antioxidant role after metal-independent, peroxyl radical–induced oxidation. This dual mode of action could apply to our model of OIR by which, in the presence of free radicals and, after exposure to postnatal hyperoxia, trolox C would carry out its antioxidant role. In the normal unexposed retina, in the absence of free radicals, trolox C could take on its more toxic role as a prooxidant. As with the mechanism by which cone function is more susceptible than rod function to postnatal hyperoxia (in the absence of any antioxidant), as previously reported by us, the toxic effect of trolox C, as a prooxidant in the normal retina, could also be more devastating to cone function.

In addition, it has been shown that different mechanisms, namely apoptosis and necrosis, could be implicated in the pathophysiology of OIR and its human counterpart ROP. Recent in vitro studies carried out on cultured bursal cells and neurons in which cell death occurred resulting from oxidative stress suggest that trolox C could have a protective effect against necrosis but none against apoptosis. Other studies, however, have concluded that trolox C was successful, for example, in reducing apoptosis in mouse thymocytes, rabbit myocytes, and anterior pituitary cells. It will be useful to investigate the presence of apoptotic and necrotic markers in OIR to better understand the predominant mode of neuronal cell death taking place in this model and, more specifically, the implications on rod and cone pathways. Future studies in this area will also help to further elucidate the role of trolox C on these mechanisms of cellular death. Finally, though the present study indicates a beneficial effect on retinal structure, retinal function was spared to a lesser degree in animals that receive trolox C, suggesting that changes in retinal vasculature, retinal...
structure, and retinal function after exposure to postnatal hyperoxia might have proceeded in a cascade of events. For example, as previously described, postnatal exposure to hyperoxia generates a reduction in vasculature because of vasoconstruction followed by vaso-obliteration. Consequently, this inadequate blood supply could have made it difficult for the inner retina to receive adequate nourishment to carry out its proper function. It is possible that we observed loss or retraction of synapses that would lead to a thinning of the OPL only after cell function was lost. This could suggest a lag time between functional and structural impairment and perhaps an important role for vasoconstruction and vaso-obliteration as the trigger of the pathogenesis of OIR because this appeared to be the stage at which the cascade began. Given that each phase appeared to be interdependent (beginning with early changes in retinal vasculature and ending with changes in retinal cytoarchitecture and function), it seems logical to target the first step of this pathologic cascade. This would further support the use of a free radical scavenger such as trolox C. Furthermore, it is possible that though trolox C aids in facilitating vasocgenesis under hyperoxic conditions (thereby improving retinal function), its ability to take on the role of an antiapoptotic mediator prevents synaptic death that leads to a thinning of the OPL.

In summary, though we observed a therapeutic effect of trolox C, its use could not fully prevent oxygen-induced retinal damage from occurring. This could suggest, as previously proposed, that free radical formation is likely not the sole cause of the damage in retinal structure and function observed in OIR or that a significant proportion of the retinal damage induced by ROS occurs in hydrophobic domains in which trolox C would lose its efficacy because of its water solubility. For example, trolox C could act in limiting the circulating (plasmatic) ROS before lipid peroxidation. Should that be the case, a combination of liposoluble and hydrosoluble free radical scavengers (such as vitamin E [at a nontoxic level] + trolox C) could represent an interesting therapeutic alternative for further investigation.

References


