Dexamethasone and Critical Effect of Timing on Retinopathy

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PURPOSE. Administration of corticosteroids soon after birth has been reported to have deleterious, protective, and no effect on retinopathy of prematurity. Conflicting results may be due to timing of corticosteroid administration. The goal of this study was to determine effects of pretreatment and late dexamethasone on retinopathy in a mouse model.

METHODS. The C57BL/6 mouse model of oxygen-induced retinopathy (by placing animals in 75% oxygen from postnatal days 7 through 12) was used to create retinal neovascularization. Dexamethasone at 0.5 mg/kg per day was administered from day 1 through day 5 in the pretreatment group. The late-treatment group received 5 days of dexamethasone at the same dose beginning on day 12. Mice were killed at days 17 through 20, and retinal vasculature was assessed by a retinal scoring system of wholemount preparation after high-molecular-weight fluorescein-labeled dextran perfusion. In addition, retinal neovascularization was assessed by quantification of extraretinal neovascular nuclei in retinal sections. Statistical significance was defined as \( P < 0.05 \) and was determined by the Kruskal–Wallis test, Mann–Whitney test, and Student's \( t \)-test.

RESULTS. Oxygen-exposed animals that received treatment with dexamethasone before oxygen exposure had an improvement in retinopathy, with a median score of 6 (5.7; 25th, 75th quartiles) compared with 10 (8,11) in the untreated oxygen-exposed (\( P < 0.05 \)). The group treated late (after oxygen exposure) with dexamethasone had a median score of 10 (9,11). Pretreatment reduced extraretinal vascularization, when assessed by quantification of neovascular nuclei, to a mean \( \pm \) SEM of 19 \( \pm \) 9, significantly less than in the untreated oxygen-exposed group (55 \( \pm \) 12; \( P < 0.05 \)). No difference was observed in the late-treatment group when compared with the untreated oxygen-exposed group. Significant growth retardation, indicated by body weight, was observed in the pretreatment (\( P < 0.01 \)) and late-treatment (\( P < 0.05 \)) groups when compared with the control group.

CONCLUSIONS. Timing of dexamethasone administration was critical to the inhibition of development of retinopathy in the mouse model. Degree of growth retardation, measured by body weight, also appeared to be time dependent. These data may explain the different results of clinical observations with respect to corticosteroid treatment, timing, and development of retinopathy. (Invest Ophthalmol Vis Sci. 2000;41:3095–3099)

Birth weight, gestational age, and duration of supplemental oxygen have been found to be the leading risk factors for retinopathy of prematurity (ROP).\(^1\)–\(^5\) Corticosteroid treatment has been used with increasing frequency in neonatology. Antenatal corticosteroid administration is now a recommended treatment to promote lung maturity in premature delivery at gestational ages of 24 to 34 weeks.\(^6\) Higgins et al.,\(^7\) Kennedy,\(^8\) and the Italian ROP study,\(^9\) reported decreasing severity of ROP in infants born to mothers who had received antenatal steroid treatment. Postnatal administration of corticosteroids has also been studied extensively with the goal of decreasing the incidence of chronic lung disease, duration of mechanical ventilation, and supplemental oxygen requirement.\(^10\) There are still no clear-cut guidelines for postnatal administration of steroids. With some, treatment begins as early as 24 hours after birth and with others, after 2 to 3 weeks of life. Results are controversial. ROP has also been evaluated after postnatal corticosteroid therapy, because it has anti-inflammatory and angiostatic effects. At least four studies\(^11\)–\(^14\) have focused on the association between corticosteroid treatment and ROP, with contradictory results. Wright and Wright\(^11\) reported that there is no association, Sobel and Philip\(^12\) demonstrated that prolonged use of steroids beginning on a mean of day 23 after birth reduces the need for cryotherapy, and Batton et al.\(^13\) and Ramanathan et al.\(^14\) both reported that postnatal steroid therapy is associated with severe ROP and requirement for cryotherapy. Regression analyses to control for degree of systemic illness of preterm infants were performed and confirmed no association with ROP\(^11,12\) and, in contrast, a significant increase in risk\(^13\) for ROP in infants treated with dexamethasone.

Timing and underlying systemic disease may explain the different observations. Rotchild et al.\(^15\) reported the protective
effect of dexamethasone administered concurrently with oxygen exposure in a mouse model. We designed this study to assess the effect of timing of dexamethasone, before and after oxygen exposure, in a mouse model of oxygen-induced retinopathy.

**METHODS**

**Animal Model and Dexamethasone Administration**

The protocol was approved by Georgetown University Animal Care and Use Committee. C57BL6 mice were obtained from Taconic Farms (Germantown, NY). Mice were placed with their nursing mothers in an infant incubator (Ohmeda, Columbia, MD) with 75% oxygen from postnatal day (P)7 through P12, as previously described16 and used in our laboratory. 17 Oxygen concentration was measured using an oxygen analyzer (Hudson Ventronics, Temecula, CA) and was checked at least twice daily during the period of oxygen exposure. Animals were returned to room air on P12.

Twenty-nine litters (n = 118 animals) were assigned to either the room air-reared group or the oxygen-reared group. Within individual litters, animals were randomly assigned to receive no treatment, sham injection of normal saline (sham-treatment group), pretreatment with dexamethasone (pretreatment group), or late treatment with dexamethasone (late-treatment group). Pretreatment with dexamethasone from P1 through P5 was selected to expose animals to dexamethasone before oxygen-induced injury to the retinal vessels to attempt to simulate antenatal corticosteroid administration or very early postnatal administration.

Late dexamethasone treatment was used to simulate administration of dexamethasone, because it is commonly used in neonatal intensive care nurseries to facilitate weaning from mechanical ventilation. A single dose of 0.5 mg/kg per day for 5 days was selected based on a previous study15 and based on doses used clinically. 11-14 Dexamethasone-treated animals were divided into two groups: pretreatment and late-treatment groups. Animals assigned to the pretreatment group were given a single daily dose of 0.5 mg/kg per day of dexamethasone (American Regent Laboratories, Shirley, NY) subcutaneously in the nape of the neck for 5 days from P1 to P5 before exposure to oxygen. The late-treatment group was given the same dose of dexamethasone for 5 days, beginning on P12, after the mice were removed from oxygen and returned to room air. The animals were killed by lethal intraperitoneal injection of sodium pentobarbital (Abbott Laboratories, North Chicago, IL) at P17 through P20. P17 through P20 was chosen as the time of death, because maximal retinal neovascularization has been reported at these time points. 16,17 A sham group received normal saline injection at the same volume and for the same periods as the dexamethasone treatment groups and was also divided into groups according to exposure and nonexposure to oxygen. The weights of the animals were recorded at P1, P7, P12, and on the day killed (P17 through P20).

**Fluorescein Dextran Perfusion of the Retinal Blood Vessels**

To study the retinal vascular pattern, systemic perfusion was performed19 using high-molecular-weight (MW = 2,000,000) fluorescein-conjugated dextran (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS; Gibco, Grand Island, NY). Briefly, animals were given a lethal dose of sodium pentobarbital, and a median sternotomy was performed. The left ventricle of the heart was identified and perfused with 1 ml fluorescein-conjugated dextran (50 mg/ml in 4% PBS) using a 1-milliliter tuberculin syringe with a 27-gauge needle. Eyes were then enucleated and placed in 4% paraformaldehyde (Sigma) in PBS for 4 to 24 hours. Under a dissecting microscope, the retina was removed, and a flatmount was prepared by making radial cuts. A coverslip was applied over the retinas after placement of a drop of 2% gelatin (Sigma). The edge of the coverslip was sealed with transparent nail polish. The scoring of retinal wholemounts was performed using fluorescence microscopy. Each retina was scored by two investigators working independently in a masked fashion and using the retinopathy scoring system17 shown in Table 1, and the average retinopathy score (average of two eyes and two investigators) for each animal was used for the statistical analysis.

**PAS Stain of Retinal Sections**

Mice were killed as indicated. The eyes were enucleated, placed immediately in optimal cutting temperature (OCT) embedding compound (Sakura Fine Tek, Torrance, CA), and frozen at −70°C. Serial sections (7–9 μm thick) over a minimum of 450 μm were cut in a sagittal plane through the cornea,

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**Table 1. Retinopathy Scoring System**

<table>
<thead>
<tr>
<th>Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood vessel growth</td>
<td>Complete</td>
<td>Incomplete outer third</td>
<td>Incomplete middle third</td>
<td>Incomplete inner third</td>
<td>9–12 Clock hours</td>
</tr>
<tr>
<td>Blood vessel tufts</td>
<td>None</td>
<td>Few, scattered &lt;3 clock hours</td>
<td>3–5 Clock hours</td>
<td>6–8 Clock hours</td>
<td>9–12 Clock hours</td>
</tr>
<tr>
<td>Extraretinal neovascularization</td>
<td>None</td>
<td>Mild, &lt;3 clock hours</td>
<td>Moderate, 3–6 clock hours</td>
<td>Severe, &gt;6 clock hours</td>
<td></td>
</tr>
<tr>
<td>Central vasoconstriction</td>
<td>None</td>
<td>Mild, early zone 1 (inner 50% of zone 1)</td>
<td>Moderate throughout zone 1 (outer 50% of zone 1)</td>
<td>Severe, extending to zone 2</td>
<td></td>
</tr>
<tr>
<td>Retinal hemorrhage</td>
<td>Absent</td>
<td>Present</td>
<td>Moderate, 3–6 clock hours</td>
<td>Severe, &gt;6 clock hours</td>
<td></td>
</tr>
<tr>
<td>Blood vessel tortuosity</td>
<td>None</td>
<td>Mild, &lt;3 clock hours</td>
<td>Moderate, 3–6 clock hours</td>
<td>Severe, &gt;6 clock hours</td>
<td></td>
</tr>
</tbody>
</table>

parallel to the optic disc. Tissue sections were stained with periodic acid–Schiff (PAS) reagent and hematoxylin.19 Multiple sections from individual eyes were scored in a masked fashion under light microscopy by counting all nuclei extending beyond the inner limiting membrane into the vitreous, as previously described.16 A minimum of six sections at least 50 µm apart were evaluated and counted per eye and averaged. The mean number of neovascular nuclei per section for each eye was used in the statistical analyses.

Statistical Analyses
Analysis of variance using the Kruskal–Wallis test was performed to test for differences in retinopathy score among the various treatment groups. Mann–Whitney tests were used to compare the total retinopathy scores between individual groups of animals. Student’s t-tests were used to compare the mean number of neovascular nuclei on retinal sections between individual groups. Student’s t-tests were also used to compare animal weight between the room air–reared group and the various other groups at the various time points. Statistical significance was defined as $P < 0.05$.

RESULTS

Total Retinopathy Scores
Pretreatment with dexamethasone ($n = 9$, from seven litters) before oxygen exposure significantly decreased the total retinopathy score to 6 (5.7; 25th,75th quartiles) compared with the nontreated oxygen-exposed group ($n = 14$, from seven litters) with a score of 10, (8,11; $P < 0.05$). Late dexamethasone treatment ($n = 12$, from six litters) did not have a significant effect on the total retinopathy score, with a median score of 10 (9,11) when compared with the nontreated oxygen-exposed group. Sham-treated animals had similar scores to animals reared in oxygen alone: for P1 through P5 ($n = 2$ from one litter) 9.5 (8.25,10.25) and for P12 through 16 ($n = 4$ from 3 litters) 10.5 (7.75, 11.25). Figure 1 shows representative fluorescein-conjugated dextran–perfused retinal wholemounts.

All room air control groups had median retinopathy scores of 0 (0.0 or 0.1), whether untreated ($n = 12$, nine litters), sham-treated ($n = 6$ from four litters), pretreated with dexamethasone ($n = 9$, from seven litters) before oxygen exposure, or sham treated ($n = 6$, from four litters), injected or not, before oxygen exposure.
extraretinal nuclei count also decreased significantly in the pretreatment group (18.9 ± 8.9) compared with the nontreated oxygen-exposed group (55.0 ± 12.1; P = 0.04). There was no significant change in nuclei count between the late-treatment group (30.8 ± 0.79) compared with the nontreated oxygen-exposed group (Fig. 2). All room air-reared animals had nuclei counts similar to those shown in Figure 2.

Growth Suppression

Dexamethasone significantly decreased the growth as indicated by body weight of the animals (Table 2). The maximum effect of growth suppression was found in the pretreatment group.

DISCUSSION

Timing of treatment with dexamethasone is a critical factor in this mouse model of oxygen-induced retinopathy. Pretreatment with dexamethasone before oxygen exposure significantly reduced total retinopathy score and the number of extraretinal neovascular nuclei on PAS-stained retinal sections compared with the non-treated oxygen-exposed group. Late dexamethasone treatment did not show any effect on the severity of retinopathy, according to both retinopathy score and the number of neovascular nuclei. Although a beneficial effect was observed in the development of retinopathy in the mice, dexamethasone treatment caused significant growth retardation, measured by body weight. The effect of growth suppression was more prominent in the pretreatment group when compared with the late-treatment group. Rotschild et al.15 reported a protective effect of dexamethasone at 0.5 mg/kg per day when administered concurrently with oxygen exposure beginning on P7 and continuing for 5 days.

Dexamethasone reduced both the retinopathy score and neovascular nuclei in that study. Barks et al.20 demonstrated that timing and dose of dexamethasone were important with modulation of injury: pretreatment 24 hours before unilateral cerebral hypoxia–ischemia prevented infarction. Higgins et al.7 Kennedy,16 and the Italian ROP study17 reported decreased severity of ROP associated with antenatal dexamethasone treatment.

Several studies focused on postnatal corticosteroids and their effect on ROP.11–14 Ehrenkranz21 commented on some of these data12,13 and speculated that the difference in association between corticosteroids and ROP may be due to the difference in age at initiation, dosage, length of treatment, and indications for treatment with corticosteroids. An animal model, as used in this study, would be able to demonstrate the effect of inter-

Table 2. Animal Weight

<table>
<thead>
<tr>
<th>Age</th>
<th>Room Air</th>
<th>Oxygen</th>
<th>Room Air + Pretreated Dexamethasone</th>
<th>Oxygen + Pretreated Dexamethasone</th>
<th>Room Air + Late Dexamethasone</th>
<th>Oxygen + Late Dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>1.33 ± 0.14</td>
<td>1.37 ± 0.10</td>
<td>1.30 ± 0.21</td>
<td>1.42 ± 0.22</td>
<td>1.41 ± 0.16</td>
<td>1.46 ± 0.18</td>
</tr>
<tr>
<td>P7</td>
<td>4.40 ± 0.78</td>
<td>4.48 ± 0.60</td>
<td>2.55 ± 0.28**</td>
<td>5.08 ± 0.37**</td>
<td>4.49 ± 0.42</td>
<td>4.57 ± 0.51</td>
</tr>
<tr>
<td>P12</td>
<td>6.42 ± 0.90</td>
<td>6.09 ± 0.60</td>
<td>3.28 ± 0.71**</td>
<td>4.51 ± 0.82**</td>
<td>6.65 ± 0.39</td>
<td>6.21 ± 0.82</td>
</tr>
<tr>
<td>P17–P20</td>
<td>8.38 ± 1.06</td>
<td>7.90 ± 0.99</td>
<td>4.60 ± 1.00**</td>
<td>5.45 ± 0.95**</td>
<td>7.71 ± 0.67*</td>
<td>7.36 ± 1.00*</td>
</tr>
<tr>
<td>n</td>
<td>27</td>
<td>27</td>
<td>12</td>
<td>16</td>
<td>18</td>
<td>18</td>
</tr>
</tbody>
</table>

Weights in grams ± SD are shown. Sham injected animals are included in the room air and oxygen groups.

* P < 0.05; ** P < 0.01 when compared with room air group by Student’s t-test.
vention without confounding factors present in clinical investigation. Dexamethasone, when administered before oxygen exposure, improves retinopathy during oxygen exposure, but has no effect when administered after oxygen exposure.

The obvious side effect of dexamethasone was growth retardation in the mouse, which was also found by Rotschild et al. Growth retardation is a known side effect of corticosteroids in clinical practice. It is currently premature to plan a clinical trial based on the current data, because of the significant growth retardation caused by dexamethasone. The dosage of dexamethasone should be determined to minimize the side effect of poor weight gain and maximize the protective effect against retinopathy.

The timing of dexamethasone administration is critical as we demonstrated in this study. These animal model data may help to explain the controversy among observations of effects of corticosteroids on ROP in clinical practice.

References