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The effects of D-penicillamine on a murine model of oxygen-induced retinopathy

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Abstract

PURPOSE—To determine the effect of intraperitoneal and intravitreal D-penicillamine (DPA) on retinal neovascularization in a murine model of oxygen-induced retinopathy.

METHODS—On postnatal day 7, 16 mice were injected intraperitoneally with 300 mg/kg/day DPA for 3 days followed by 50 mg/kg/day for 7 days. A different group of 7 mice were injected intraperitoneally with 600 mg/kg/day DPA for 3 days followed by 100 mg/kg/day for 7 days. A third group of 14 mice were injected with 1,500 mg/kg/day DPA for 2 days; a control cohort of 17 mice received intraperitoneal phosphate-buffered saline (PBS). An additional 15 mice underwent intravitreal injection of 1 μ L of 100 mg/mL DPA in the right eye and 1 μ L PBS intravitreally in the left eye as a control. All groups were placed in a 75% oxygen chamber for 7 days then room air for 3 days before being sacrificed and enucleated. The retinas were stained and flat-mounted to determine the severity of retinal neovascularization by quantifying neovascular buds.

RESULTS—After intraperitoneal injection, the mean number of glomeruli and tubules was similar in the DPA and PBS groups ($P = 1.0$), regardless of DPA dosage. The dosage of 1,500 mg/kg/day proved to be uniformly lethal. After intravitreal injections, the mean number of glomeruli ($P = 0.16$) and tubules ($P = 0.7$) were similar in the DPA and PBS groups.

CONCLUSIONS—Neither intraperitoneal nor intravitreal injection of DPA inhibits retinal neovascularization in a murine model of oxygen-induced retinopathy.

Retinopathy of prematurity (ROP) is a vasoproliferative disorder in which the incompletely vascularized anoxic retina produces angiogenic factors with subsequent extraretinal neovascular proliferation. Our understanding of the molecular events associated with the development of ROP is incomplete, but the up-regulation of vascular endothelial growth factor (VEGF) and other cytokines¹ made by mesenchymal spindle cells is one of the mechanisms thought to be responsible.^{2–5} Oxidative stress has also been linked to the development of ROP through several mechanisms.^{2–5}

A growing body of research shows a correlation between oxidative stress and ROP, bronchopulmonary dysplasia, necrotizing enterocolitis, and periventricular leukomalacia.^{6,7} Oxygen-free radicals may potentially damage developing retinal capillaries in premature infants; antioxidants may have a protective effect by scavenging and altering oxygen-free radicals.

Forty years ago, Hungarian physicians used systemic D-penicillamine (DPA) to treat neonatal hyperbilirubinemia.⁸⁻¹² Coincidentally, they noted a very low incidence of ROP in these same infants. This observation led to the investigation of the use of DPA to prevent ROP.¹⁰ DPA, also known as 3-mercapto-D-valine, is a chelator of pro-oxidant heavy metals, such as copper. Although DPA is a metabolite of penicillin, it has no antibiotic properties.

Two studies from Lakatos and colleagues^{8,10} indicated that intravenous DPA prevented ROP in preterm infants.^{8,10} In this 1986 randomized controlled trial, 300 mg/kg/day DPA was administered intravenously in 204 preterm infants (birthweight <1,500 g) for 3 days, beginning 12 hours after birth, reduced thereafter to 50 mg/kg/day for up to 2 weeks. ROP did not occur in any of the 71 treated survivors (0%) or in 6 of the 70 control survivors (9%; $P=0.013$). No acute toxicity was reported in either group during hospitalization.

One year later, Lakatos and colleagues⁹ randomized 77 infants in a similar fashion. There was no ROP found in 27 treated survivors, and there were 3 infants with ROP in the control group of 32. Although the mechanism of action remains unclear, the authors postulated that a reduction of free radical activity and alteration of disulfide bonds in mesenchymal tissues of premature infants could arrest ROP progression and that the reduction in copper levels induced by DPA retards angiogenesis. The purpose of our study was to evaluate, in a murine model, the potential inhibition of retinal neovascularization by DPA, via both intraperitoneal and intravitreal routes.

Methods

All experimental procedures involving animals were performed according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and conformed to the principles of our Institutional Animal Research Committee.

An animal model of proliferative retinopathy was obtained by placing litters of Friend Virus B × Balb/C newborn mice and their mothers in a room air chamber for the first 6 days of life. On postnatal day 7 (P7), the mice pups were exposed to 75% oxygen for 7 days in a controlled chamber and then returned to room air for 3 days, producing retinal ischemia with subsequent retinal neovascularization by P17.

There were 4 subgroups in the treatment arm of this study. In each subgroup, the mice were minimally sedated with ketamine for the injections. The DPA used in our study was in the form of a white crystalline powder, stable at room temperature, mixed with sterile phosphate buffered solution to obtain 1 mg/1 mL, so that a volume between 0.5 mL and 1.5 mL was injected. Treated mice were injected in the intraperitoneal space with DPA (Sigma-Aldrich, St-Louis, MO) in phosphate-buffered saline (PBS) starting on P7. The dosages used for DPA injections were the same as the dosages in the studies of Lakatos and colleagues.⁸ Group 1 was injected with 300 mg/kg/day for 3 days, from P7 through P9, followed by 50 mg/kg/day for the next 7 days, P10 through P16. Group 2 was injected with twice the dosage used in the study by Lakatos and colleagues⁸: 600 mg/kg/day from P7 through P9, followed by 100 mg/kg/day for the next 7 days, P10 through P16. Group 3 was injected with 5 times the dose given by Lakatos and colleagues⁸: 1,500 mg/kg/day for 2 days, P7 and P8.

A control group for study groups 1, 2, and 3 was created by use of the same animal model of proliferative retinopathy. Mice were injected daily with PBS from P7 through P17. A total of 17 control slides were analyzed.

Group 4, the intravitreal arm of the study, used the same animal model of retinopathy. On P7, anesthetized mice underwent mechanical eyelid separation and intravitreal injection of 1

μL of 100 mg/mL of DPA in PBS in the posterior vitreous of the right eye using a 33-gauge Hamilton syringe (Hamilton Company USA, Reno, NV). A control group for group 4 used the same animal model, and they underwent intravitreal injection of 1 μL of plain PBS in the posterior vitreous of the left eye. A total of 13 control slides were analyzed. The eyelids were resealed with Derma-Bond (Ethicon Inc, San Angelo, TX). The mice were then placed in the 75% oxygen chamber for 7 days according to the animal model of proliferative retinopathy described above.

On P17 all mice in all 4 subgroups and both control groups were killed. Both eyes were enucleated and fixated in 10% neutral-buffered formalin overnight. The following day, the retinas were carefully dissected and blocked with 10% horse serum for 1 hour, followed by an initial overnight incubation with primary antibody Biotinylated Lectin I (Vector Laboratories, Burlingame, CA). The next day, a second and final staining was performed with secondary antibody Streptavidin Alexa Fluor 488 conjugate (Invitrogen, Carlsbad, CA), which was allowed to stain each retina for 2 hours. The retinas were then flat-mounted on slides by creating four separate leaflets to allow better exposure and visualization of the neovascularization.

We quantified retinal neovascularization by manually counting glomeruli and tubules found on each retina with the use of a Nikon Epifluorescence Microscope (Nikon Corporation, Tokyo, Japan). Glomeruli represent early neovascularization or balls of endothelial cells (Figure 1, white arrow); tubules represent more developed neovascularization or tubes of endothelial cells (Figure 1, arrowhead). Each leaflet of each retina was analyzed, and each glomeruli and tubule counts were recorded separately. Each slide was reviewed 3 times by one blinded observer. An average of each count was calculated for each slide.

The primary analysis used data from mice for which paired penicillamine injection and control measures were available. The median measures were compared between penicillamine injection and control eyes with a paired Wilcoxon signed-rank test. As a secondary analysis, given the loss of data when pairing was accounted for, a nonparametric Wilcoxon rank-sum test was used to compare the measurement distributions between the injection and control eyes without regard to pairing within mouse, meaning the result of the right and left eyes were considered separately. More sophisticated methods of analysis, such as accounting for the unbalanced pairing of penicillamine injection and control measures across the mice, were not feasible, given the limited number of observations and skewed distributions. Data were analyzed by the use of SAS (SAS System for Windows, version 9.1, SAS Institute Inc, Cary, NC).

Results

In Group 1, a total of 16 retinas treated with intraperitoneal DPA were analyzed. In Group 2, 7 treated retinas were analyzed. In Group 3, where mice received 1,500 mg/kg/day of DPA, 5 times the dose given by Lakatos and colleagues,⁸ all died before completion of the induction of retinopathy. In Group 4, we analyzed 15 flat-mounted retinas injected with DPA intravitreally. If any part of a retinal leaflet was missing or not stained appropriately, the slide was discarded and not considered in the results.

Mice in Groups 1 and 2 demonstrated a similar number of neovascular glomeruli and tubules compared with PBS-treated controls as depicted in Table 1. Mice in Group 3 died before a retinal count was possible. In Group 4, the number of tubules and glomeruli measurements in the group treated with intravitreal DPA was lower than the control group, as noted in Table 2, but this was not statistically significant.

Given the skewed distribution of the data and the limited sample size, we used a nonparametric Wilcoxon rank-sum test to compare the median values between the treatment groups. There was no significant difference in median values between the treatment groups ($P = 0.2$). Results were similar when a parametric 2-sample t test is used. There was no significant difference in mean levels between the groups ($P = 0.2$).

Discussion

The World Health Organization's "Vision 2020" program targets ROP as an "avoidable disease" that requires early detection and treatment to prevent blindness and its significant costs to the patient and society as a whole.¹³ Advances in modern neonatology have allowed for the survival of immature infants, which has led to an increase in the number of children who have ROP. The ideal prophylactic treatment for ROP would be a single, low-dose, locally administered medication. Our study was undertaken in order to better define the effect of DPA on an animal model of retinopathy of prematurity.

DPA is a white, crystalline degradation product of penicillin mainly used as a chelating agent. It has the potential of reducing free radical activity and altering intermolecular disulfide bonds necessary for angiogenesis controlled by vascular endothelial cell growth factor (VEGF). Increased expression of VEGF plays a central role in the development of retinal neovascularization in ischemic retinopathies, including ROP.¹⁴

Although Lakatos and colleagues⁸ concluded that DPA lowered the incidence of ROP without significant acute side effects on neonates, in 2005, Christensen and colleagues^{15,16} designed a nonrandomized clinical trial to evaluate the administration of a prophylactic potential of an enteral form of DPA to preterm neonates but failed to replicate his results.

During a 15-month period, researchers carried out an open-label nonrandomized trial of 15 preterm neonates who received a 14-day course of enteral DPA via nasogastric tube within 48 hours of birth. The control group included 34 neonates of similar gestational age and birth weight as the treated group. The treated groups received a total of 300 mg/kg for 3 days divided in 3 doses, and then 50 mg/kg once daily for 11 additional days.

Of the 14 treated survivors, 3 (21%) were diagnosed with ROP, and all 3 progressed to high-risk disease requiring laser surgery. Of the 30 control survivors, 18 (60%) developed ROP ($P = 0.01$), with 7 (23%) of them meeting the criteria for laser surgery. The study end point of "ROP requiring surgery" was defined as retinopathy of a severity equal to or greater than any one of the following: (1) threshold disease as defined by the cryotherapy for retinopathy of prematurity study (CRYO-ROP); (2) type 1 prethreshold disease as defined by the early treatment for ROP (ETROP) study; and (3) aggressive posterior ROP as defined by the International Committee.¹⁶

There was no short-term DPA toxicity observed. This finding led Christensen and colleagues¹⁶ to conclude that DPA reduced the likelihood of developing any ROP but had no effect on the prevalence of ROP severe enough to require surgery.

Our study was divided in 2 major parts. First, we reproduced the dosages given by Lakatos⁸ and Christensen¹⁶ and their colleagues to provide intraperitoneal injections of DPA to an animal model of retinal neovascularization. Our results demonstrated that, in an animal model, DPA was not associated with a statistically significant reduction in retinal neovascularization. In addition, we found no statistically significant decrease in the formation of neovascularization after intravitreal administration of DPA.

There were no side effects noted in our lower intraperitoneal dosage injection groups, but there was uniform death of all mice injected with 1,500 mg/kg of DPA. It is unclear to us what lead to the demise of the mice in this group, but it is possible that renal or hepatic toxicity resulted from the high dose of penicillamine used. In the pediatric population, the current dosage of penicillamine for treatment of cystinuria or Wilson disease is up to 40 mg/kg/day, although Lakatos and colleagues⁸ used 300 mg/kg/day during his experiment.

The most notable side effect of the intravitreal injection group was the high rate of retinal detachment in both control and treated groups. There were also mice found to have significant lens disruption, with nuclear debris noted in the vitreous. This was explained by the difficulty in placing 1 μ L of DPA in the posterior vitreous space of a 7-day-old mouse.

The weaknesses of our intervention include the fact that the biological behavior of animal model may not fully simulate human disease. One disadvantage of the oxygen-induced ischemic retinopathy model is that increased expression of VEGF is transient, leading to spontaneous regression of neovascularization, which makes it difficult to test for drug-induced regression. In addition, the numbers of retinas we analyzed limit the power of this study, although we found no differences among groups that even approached statistical significance. A sample size of 129 observations per group would be required for 80% power to detect a difference between means of 15 for the intervention group and 25 for the control group, assuming a standard deviation of 20 and 35, respectively, using a 2-sided 0.05 alpha level. Alternatively, with the chosen sample sizes of 30 intervention and 25 control observations, a difference in means of 21 could be detected with 80% power assuming a 2-sided 0.05 alpha level and a common standard deviation of 27. The technical difficulties of the intravitreal injections and the variability in quantifying the neovascularization created additional limitations of this study. Finally, our numbers were too small for analysis without combining both eyes.

Nevertheless, we have demonstrated that neither intraperitoneal nor intravitreal injection of DPA significantly inhibits retinal neovascularization in a murine model of oxygen-induced retinopathy. This study does not support further investigation of this compound in human subjects.

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FIG 1.
Depiction of glomeruli (arrow) and tubules (arrowhead) for quantification of neovascularization.

Table 1

Intraperitoneal penicillamine: Number of retinal glomeruli and tubules in mice after intraperitoneal penicillamine (Group 1: 300 mg/kg/day; Group 2: 600 mg/kg/day) or phosphate-buffered saline (Control Group) injection

	Treatment groups		
	Control (PBS)	Group 1 (DPA)	Group 2 (DPA × 2)
No. of retinas	17	16	7
Mean no. of glomeruli ($P=1.0$)	37 (± 21)	36 (± 13)	42 (± 49)
Mean no. of tubules ($P=1.0$)	3 (± 2)	3 (± 2)	5 (± 5)

DPA, D-penicillamine; PBS, phosphate-buffered saline.

Table 2

Intravitreal penicillamine: Group 4 versus Control group. Number of retinal glomeruli and tubules in mice after IV DPA versus controls (intravitreal PBS injection)

	Treatment groups	
	Control	IV DPA
No. of retinas	13	15
Mean no. glomeruli ($P = 0.16$)	27 (± 21)	18 (± 18)
Mean no. tubules ($P = 0.7$)	5 (± 5)	4 (± 4)

IV DPA, intravitreal penicillamine injection; *PBS*, phosphate-buffered saline.