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The Organophosphate Pesticide Chlorpyrifos Affects Form Deprivation Myopia

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Purpose. The effects of the anti-cholinesterase organophosphate pesticide chlorpyrifos (CPF) on the refractive development of the eye were examined. Form deprivation was used to induce eye growth to address the previously reported relationship between organophosphate pesticide use and the incidence of myopia.

Methods. Chickens, a well-established animal model for experimental myopia and organophosphate neurotoxicity, were dosed with chlorpyrifos (3 mg/kg per day, orally, from day 2 to day 9 after hatching) or corn oil vehicle (VEH) with or without monocular form deprivation (MFD) over the same period. The set of dependent measures included the refractive state of each eye measured using retinoscopy, axial dimensions determined with A-scan ultrasound, and intraocular pressure.

Results. Dosing with CPF yielded an inhibition of 35% butyrylcholinesterase in plasma and 45% acetylcholinesterase in brain. MFD resulted in a significant degree of myopia in form-deprived eyes resulting from significant lengthening of the vitreal chamber of the eye. CPF significantly reduced the effect of MFD, resulting in less myopic eyes (mean refraction: VEH-MFD = -16.2 ± 2.3 diopters; CPF-MFD = -11.1 ± 1.8 diopters) with significantly shorter vitreal chambers. Nonoccluded eyes were, on average, slightly hyperopic. Treatment with CPF for 1 week in the absence of MFD led to no significant change in ocular dimensions or refraction relative to controls.

Conclusions. The use of form deprivation as a challenge suggests that CPF treatment interferes with the visual regulation of eye growth. (*Invest Ophthalmol Vis Sci.* 1998;39:1290-1294)

An epidemic of ocular toxicity reported in agricultural regions of Japan during the late 1950s, 1960s, and 1970s was attributed to heavy regional use of organophosphate insecticides.[1] A high incidence of myopia was prominent among a variety of other visual symptoms and classical cholinergic signs and symptoms. Subsequent experimental studies in Japan reported degeneration of the retina, optic nerve, and extraocular muscles in addition to myopia.[1] The Japanese reports are controversial, however, because similar cases of ocular toxicity have not been reported elsewhere, and many of the Japanese reports do not meet currently accepted standards of experimental design, methodology, and reporting of results. In support of the Japanese findings, retinal and optic nerve lesions have been seen after the feeding of organophosphate pesticides to rats in studies reported to the US Environmental Protection Agency by pesticide manufacturers.[2] In addition, a variety of biochemical, neuropathologic, and electrophysiological changes have been noted in the retina and optic nerve of adult rats treated with the organophosphate pesticide fenthion.[2,3] To date, we know of no investigations outside of the early Japanese reports that address the issue of myopia after developmental organophosphate pesticide exposure.

The study of experimental myopia has shown that the growth of the axial length of the eye is governed by visual input and that a cholinergic system is involved in the regulation of this process.[4,5] Deprivation of patterned visual input during development results in an increased axial length of the eye (i.e., axial myopia).[6] Treatment with the muscarinic blocking agents atropine or pirenzepine prevents the development of experimental axial myopia independent of effects on accommodation, implicating retinal processes.[5,7] Areas of local pat-

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tern deprivation produce areas of focal ocular elongation, suggesting that cholinergically mediated local trophic factors may be involved in governing the growth of the length of the eye.[8]

Chlorpyrifos (CPF; *O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl)phosphorothioate) is an organophosphate insecticide and is present in many pesticide formulations that are commonly used for household and agricultural applications. Exposure to sufficient dose levels of CPF (like other organophosphate pesticides) causes inhibition of blood and nervous system cholinesterase (ChE) activity, with accompanying cholinergic signs. Although the acute effects of inhibition of ChE enzymes in adult animals are generally considered to be reversible and without long-term consequences, concern has arisen recently regarding developmental exposure to pesticides.[9]

The present study investigates the effects of treatment with CPF on the development of the chick eye. In addition to CPF administration, some of the subjects were treated with monocular pattern deprivation to serve as a positive control for the measures of ocular dimensions, and also to study the influence of CPF on the visual regulation of ocular growth. It was hypothesized that the cholinergic effects of CPF treatment would interfere with the development of the eye and produce an increased axial length, resulting in axial myopia.

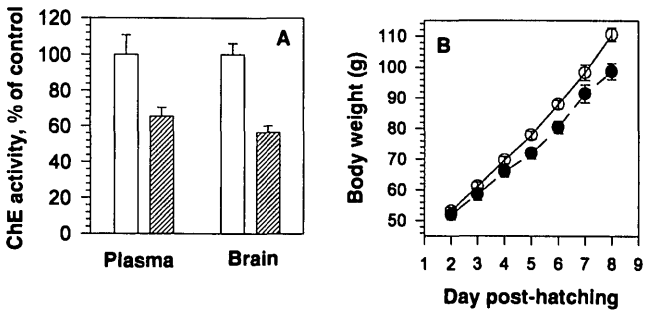

FIGURE 1. (A) Chlorpyrifos (CPF) significantly inhibited cholinesterase (ChE) activity in plasma ($t_{44} = 3.10, P < 0.003$) and whole brain ($t_{41} = 6.39, P < 0.0001$). ChE activity is expressed as a percentage of control. Statistics compared all CPF-dosed animals with all corn oil vehicle (VEH)-dosed animals. Control activity was calculated as the mean of VEH-monocular form deprivation (MFD) and VEH-Open groups. The treated animals showed no overt cholinergic signs, for example, tremor, diarrhea. VEH, *open bar*; CPF, *shaded bars*. (B) The CPF-dosed chicks were slightly, though significantly, lighter than the control chicks and gained weight more slowly than the controls (treatment effect: $F_{1,50} = 6.33, P < 0.02$; day x treatment: $F_{6,300} = 4.08, P < 0.001$).

METHODS

One-day-old broiler chicks (Arbor × Acre), received from the North Carolina State University Poultry Research Farm (Raleigh, NC), were housed in the vivarium of the Duke University Medical Center. All aspects of the animal care and use were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. On arrival, the chicks were placed in a mesh-bottomed box brooder (G.Q.F. Manufacturing, Savannah, GA) at a temperature of 37.8°C. Chicks were housed under fluorescent light on a 12-hour light, 12-hour dark cycle and fed tap water and chick starter food (Ralston Purina, St. Louis, MO) ad libitum.

Treatment of the chicks began on day 2 after hatching and continued for 7 days. The chicks were divided into four groups: two receiving the test compound CPF (Dow Elanco, Midland, MI) and two receiving corn oil vehicle (VEH) only, 2 ml/kg. CPF was dissolved in corn oil and delivered at a dose of 3 mg/kg per day, 2 ml/kg, by oral gavage. This dose is approximately 10% of the acute oral LD_{50} for young chicks as determined in pilot work. The oral route of exposure was selected instead of intravitreal injections, a route sometimes used in pharmacologic investigations of experimental myopia, because the oral route is more toxicologically relevant and also because CPF must be metabolically activated to chlorpyrifos-oxon by microsomal enzymes in the liver to become a potent inhibitor of acetylcholinesterase (AChE).

One VEH group and one CPF group were selected for monocular form deprivation (MFD). Three cohorts of chicks were run to produce the following group sizes: VEH-MFD, $n = 13$; VEH-OPEN, $n = 14$; CPF-MFD, $n = 14$; and CPF-OPEN, $n = 13$.

Form deprivation was accomplished using translucent plastic domes 6 mm in height constructed from 12-mm diameter, round-bottomed, polypropylene test tubes, attached over one eye of each chick in the MFD groups using a cyanoacrylate adhesive. These occluders diffused light and degraded pattern vision. The occluders contained two small triangular notches to allow ventilation of the eye. MFD was maintained for a period of 1 week, concurrent with dosing.

Ocular Measurements. The animals were examined with a battery of ocular tests on the day after the final treatment with CPF or VEH. Anesthesia was induced with 50 mg/kg ketamine (Ketaset; Fort Dodge Laboratories, Fort Dodge, IA) and 3.5 mg/kg xylazine (American Animal Health, Wisner, NE) anesthetic (intramuscularly, 1 ml/kg injection volume), after which the occluders were removed. The animals were assessed for refractive error without cycloplegia using streak retinoscopy (Welch Allyn, Skaneateles Falls, NY). No correction was made for the small eye artifact of retinoscopy.[10] Ocular dimensions of the chicks were measured using A-scan ultrasonography (Echorule Biometer; 3M, Canada) under "aphakic" mode, which used an effective conduction velocity of 1548 m/sec for the whole eye. The ultrasound output yielded measures of anterior chamber depth, lens thickness, and vitreal chamber depth. Three ultrasound measurements were taken for each eye, and the mean was used for further data analysis.

Intraocular pressure (IOP) was measured (Tono-pen XL; Mentor, Norwell, MA) in each eye. For IOP, eyelids were gently manually retracted and the covered applanator was brought into perpendicular contact with the central cornea. Readings were taken until the device recorded four valid readings with a 5% or better statistical reliability. In some cases no reading was possible because of the small size of the eye.

ChE Inhibition. At the end of the experiments, the animals were anesthetized using 0.25% halothane and euthanatized (approximately 24 hours after the final dose). Brains were rapidly removed and homogenized (10% wt/vol) in 50 mM Tris-HCl buffer, pH 8.0, with 0.1 mM EDTA. Blood samples were collected into heparinized tubes and centrifuged at 4°C to separate plasma and red blood cells. ChE activities in plasma and brain homogenates were determined by the method of Ellman et al.[11] Because the majority of ChE activity in chicken plasma is butyrylcholines-

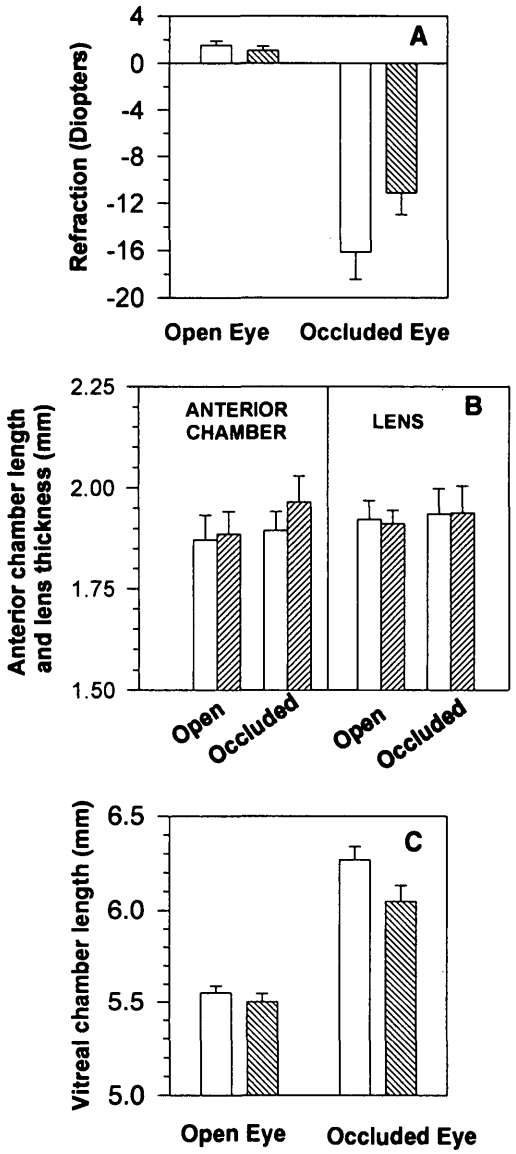

FIGURE 2. (A) Mean refractions from open and occluded eyes of chicks that underwent monocular form deprivation (MFD). MFD resulted in extreme myopia ($F_{1,102} = 190.91$, $P < 0.0001$). The chlorpyrifos (CPF)-treated chicks (*shaded bar*) were significantly less myopic than controls (*open bar*) (group × occlusion interaction, $F_{1,102} = 6.38$, $P < 0.013$; corn oil vehicle [VEH]-MFD versus CPF-MFD: $t_{25} = 3.30$, $P < 0.0013$). There was no effect of dosing on the unoccluded (open) eyes in the MFD animals. Refractions from the unoccluded groups were not significantly different from the open eyes of controls (data not shown). (B) There was no significant difference in length of the anterior chamber or lens thickness caused by MFD or treatment with CPF measured with A-scan ultrasound. (C) The vitreal chamber lengthened significantly because of form deprivation ($F_{1,102} = 68.02$, $P < 0.0001$); elongation was significantly reduced in the CPF-treated animals (*shaded bars*) compared with controls (*open bars*) ($t_{25} = 2.024$, $P < 0.046$). Axial measurements from the unoccluded groups were not significantly different from the open eyes of controls (data not shown).

terase, butyrylthiocholine (0.2 mM) was used as the substrate for testing the plasma. For brain, the predominant ChE enzyme is AChE. Therefore, acetylthiocholine (0.2 mM) was used as the substrate. It should be noted, however, that acetylthiocholine is not specific for AChE activity and that activity numbers may reflect contributions of activity by the other forms of ChE. Protein concentrations for brain and plasma were determined using the bicinchoninic acid method.[12] Enucleated eyes were immersion fixed in 10% buffered formalin and processed routinely for light microscopy.

Statistical Analyses. Ultrasound and refraction data were analyzed using a two-way analysis of variance (treatment, occlusion) to examine the main effects and interactions, and least-squared means *t*-test for breakout paired comparisons of specific groups (GLM procedure; SAS Institute, Cary, NC). Body weight was analyzed using a repeated-measures analysis of variance (treatment, day; GLM procedure). ChE inhibition data were compared using *t*-tests (Sigmaplot; Jandel Scientific, San Rafael, CA).

RESULTS

Cholinesterase Inhibition

The dose of 3 mg/kg per day CPF significantly reduced ChE activity in plasma (65.5% of control) and whole brain (56.5% of control) (Fig. 1A). The treated animals showed no overt cholinergic signs, for example, tremor, diarrhea, pupillary miosis. The CPF-dosed chicks were slightly, though significantly, lighter than the control chicks and gained weight more slowly than the controls (Fig. 1B). This difference in body weight has not been replicated in subsequent experiments with the same dose of CPF.

Refraction

The occluded eyes refracted significantly more myopic than the unoccluded eyes, producing −16.15 ± 2.30 diopters (D) (SEM) of myopia in the VEH-MFD controls and −11.11 ± 1.84 D in the CPF-MFD group (Fig. 2A). The occluded eyes from the CPF-MFD group were significantly less myopic than the VEH-MFD eyes.

The unoccluded eyes measured slightly hyperopic, on average. There was no significant difference in refraction between groups for the unoccluded eyes.

Ultrasound

MFD produced significant increases in axial length ($F_{1,102} = 52.68$, $P < 0.0001$). There were no significant differences caused by occlusion or treatment in anterior chamber length or lens thickness (Fig. 2B). The effect of MFD can be seen in the increase in vitreal chamber depth (Fig. 2C). There was a significant difference between the length of the vitreal chamber in the MFD eyes of the VEH and CPF groups. The vitreal chamber of the VEH-MFD group (6.27 ± 0.07 mm) was significantly longer than the CPF-MFD group (6.05 ± 0.09 mm).

Histology and Intraocular Pressure

IOP for the VEH-MFD animals was a mean of 9.25 mm Hg ±1.07 (SEM) in the unoccluded eyes compared with 10.58 ± 1.58 mm Hg in the occluded eye. In the CPF-MFD chicks, the unoccluded eyes had a mean IOP of 10.07 ± 1.00 mm Hg compared with 12.14 ± 0.93 mm Hg in the occluded eyes. None of these differences were statistically significant. Light microscopy demonstrated no differences between treatment

groups, with scleral, ciliary muscle, and retinal morphology unaffected by occlusion or CPF treatment.

Discussion

The experiment reported here investigated whether treatment with the organophosphate pesticide CPF could alter ocular development in an experimental model of myopia. One week of form deprivation with translucent diffusers produced a measurable myopic state in chicks. Contrary to the a priori hypothesis, oral administration of the organophosphate pesticide CPF yielded a reduction in form deprivation myopia. The occluded eyes of the CPF group were shorter and had a less negative refraction than the corresponding eyes in the VEH control group. In the absence of form deprivation, a 1-week treatment with CPF had no measurable effect on refraction or axial eye dimensions.

The reduction of form deprivation myopia by CPF is surprising, given that AChE inhibition should lead to an increase in cholinergic activity, whereas cholinergic blockers atropine and pirenzepine reduce form deprivation myopia.[5,7] Subconjunctival injections of carbachol, a nonselective cholinergic agonist, had no effect on form deprivation myopia,[5] but there was no independent indication in that study that the administered dose was high enough to have had any ocular effect. This kind of paradoxical effect is not unique to cholinergic compounds, however. The eye has previously been demonstrated to become more myopic or less myopic in response to administration of the same neuroactive compound depending on dose.[13]

There is a discrepancy between the ability of CPF to alter the growth of the occluded eye without changing the growth of the open eye. In this, CPF is similar to many other pharmacologic treatments tested in conjunction with form deprivation.[5,7,14,15] It is known that visual impairment alters refractive eye growth. However, the lack of cholinergic signs and the modest level of ChE inhibition in the present study suggest that CPF was not delivered at a dose level high enough to produce a major impairment of vision in the unoccluded eye. This suggests that the undegraded visual input in the opened eye, which has a powerful influence on the regulation of eye growth, was sufficient to overcome whatever subtle effects, if any, of CPF on vision. In contrast, the occluded eye, lacking strong visual input, may have been more susceptible to modulating influences of the pesticide treatment.

One possibility is that the effects of CPF, metabolized into chlopyrifos-oxon, are mediated through disruption of cholinergic neurotransmission. AChE activity is found throughout the chicken eye,[16,17] including the inner plexiform layer of the retina.[18] The inner plexiform layer is where other pharmacologic agents that affect experimental myopia may also have their effects.[14] While no retinal ChE activity was determined for these chicks, previous work on organophosphate pesticides showed that ChE inhibition in rat brain and retina correlated highly.[3] Another possibility is that chlorpyrifos-oxon had a direct effect, because it binds directly to M2 and M4 muscarinic receptors,[19] but previous evidence points toward a role for the M1 receptors alone in mediating form deprivation myopia.[5]

Another alternative is that CPF may have produced the observed effects through general toxicity, which impaired the maximal possible rate of ocular growth. It should be considered, however, that no overt cholinergic signs were noted in the CPF-dosed animals, nor were there signs of overt retinal toxicity. Although the CPF-dosed animals weighed slightly less than the VEH-dosed animals, it is unlikely that difference in body size accounted for the difference in eye growth, given that the axial length of eye structures in the unoccluded eyes of the CPF groups and the comparable eyes of the VEH groups did not differ. We also note that the observed difference in body weight has not been replicated in subsequent experiments in which a similar reduction of MFD was observed with the same dosing regimen of CPF.

Refraction was done without cycloplegia. Spasm of accommodation resulting from cholinergic action on the ciliary muscle and pupillary miosis occur in poisoning by ChE inhibitors, yielding a more myopic refraction. Our data, however, show the opposite. The occluded eyes of CPF-MFD chicks were less myopic than the occluded eyes of VEH-MFD controls, and no difference was observed between the unoccluded eyes. In addition, biometry yielded no significant difference in lens thickness between groups, suggesting that the accommodative state was similar in treated and control groups. Finally, the lack of overt signs of ChE inhibition along with the use of a systemic anesthetic while taking the measurements suggest that spasm of the accommodative muscles was unlikely.

The findings reported here demonstrate that CPF affects eye growth in an experimental model of form deprivation myopia. Form deprivation myopia is the expected response to an idiosyncratic stimulus condition consisting of a visual image blurred by translucent diffusers. As such, form deprivation can be considered a challenge for which axial elongation is the normal response. CPF partially inhibited the growth response to form deprivation. Although there are well-documented limitations in the applying the chicken model to human myopia,[20] it is clear that in avian and mammalian species there are visually regulated eye growth signals similarly affected by pattern deprivation. Pharmacologic manipulations that modify eye growth in birds also modify eye growth in mammals,[21,22] suggesting that similar neurochemical modulators underlie regulation of eye growth across species.

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