

Association Between In Utero Organophosphate Pesticide Exposure and Abnormal Reflexes in Neonates

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Abstract

The detrimental effects of organophosphate pesticide (OP) exposure on neurodevelopment have been shown in animals. The present study aimed to assess the relationship between in utero and early postnatal OP exposure and neonatal neurobehavior in humans, as measured by seven clusters (habituation, orientation, motor performance, range of state, regulation of state, autonomic stability, and reflex) on the Brazelton Neonatal Behavioral Assessment Scale (BNBAS). We assessed 381 infants ≤ 2 months old and born to women participating in the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) study, a longitudinal, birth cohort study of low-income, Latina women living in the agricultural community of the Salinas Valley, California. Exposure to OP pesticides was determined by urinary levels of dialkylphosphate (DAP) metabolites, including dimethyl and diethylphosphate metabolites, measured twice during pregnancy ($M = 14$ and 26 weeks gestation) and once post-delivery ($M = 7$ days postpartum). The relationship between exposure and BNBAS performance was examined for the entire sample and stratified by the median age at assessment, 3 days. We observed a significant association between exposure and the reflex cluster for the entire sample and for infants >3 days old ($n = 184$). Among the >3 day old infants, increasing average prenatal urinary metabolite levels were associated with both an increase in number of abnormal reflexes (total DAP: adjusted $\beta = 0.53$, 95% CI = 0.23, 0.82; dimethyls: adjusted $\beta = 0.41$, 95% CI = 0.12, 0.69; diethyls: adjusted $\beta = 0.37$, 95% CI = 0.09, 0.64), and the proportion of infants with more than three abnormal reflexes (total DAP: adjusted OR = 4.9, 95% CI = 1.5, 16.1; dimethyls: adjusted OR = 3.2, 95% CI = 1.1, 9.8; diethyls: adjusted OR = 3.4, 95% CI = 1.2, 9.9). No detrimental associations were found between postnatal urinary metabolite levels and any of the BNBAS clusters for infants ≤ 3 or >3 days old at assessment. Whether neonatal reflex functioning is predictive of neuropsychological functioning as the child matures will continue to be evaluated in this birth cohort.

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INTRODUCTION

Pesticide use is widespread in the United States, with more than one billion pounds of pesticides used annually (Donaldson et al., 2000). Recent studies have indicated that individuals working in agriculture, as well as their children, may be at higher risk of pesticide

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exposure than the general population (Curl et al., 2002; Fenske et al., 2002; Lu et al., 2000; McCauley et al., 2001; O'Rourke et al., 2000; Simcox et al., 1999).

Links between exposure to organophosphate pesticides (OPs) and sub-optimal neurodevelopment are found in numerous animal studies in the literature (Eskenazi et al., 1999). These studies have shown that animals experiencing in utero OP exposure demonstrate decreased balance (Muto et al., 1992), increased righting reflex time and poorer cliff avoidance (Chanda et al., 1995; Chanda and Pope, 1996). It has been suggested that OP exposure may contribute to poorer neurobehavioral functioning in young animals by producing cellular deficits in their developing brains, particularly in regions rich with cholinergic projections (Campbell et al., 1997; Eskenazi et al., 1999). Reasonable evidence exists that these deficits may result even with lower level exposure if it occurs during critical periods of brain development (Eskenazi et al., 1999). A recent study in which the commonly-used diethyl organophosphate insecticide, chlorpyrifos, was administered to neonatal rats further indicated that neonatal exposure produces numerous and persistent deficiencies in cholinergic synaptic function which can continue into adulthood (Slotkin et al., 2001).

The Brazelton Neonatal Behavioral Assessment Scale (BNBAS) (Brazelton and Nugent, 1995) is a widely used assessment tool of neonatal neurodevelopment. Over the past 30 years, many researchers have used this scale to measure the influence of various in utero environmental exposures on newborn neurobehavioral capacities. These exposures include cocaine (Black et al., 1993; Chasnoff et al., 1985; Coles et al., 1992; Datta-Bhutada et al., 1998; Delaney-Black et al., 1996; Dreher et al., 1994; Eisen et al., 1991; Eyler et al., 1998; Mayes et al., 1993; Morrow et al., 2001; Neuspil et al., 1991; Tronick et al., 1996), marijuana (Dreher et al., 1994), obstetric medications (Lieberman et al., 1979; Wittels et al., 1990), lead (Emory et al., 1999; Ernhart et al., 1986), polychlorinated biphenyls (PCBs) (Stewart et al., 2000), and dichlorodiphenyldichloroethylene (DDE) (Rogan et al., 1986). To date, no research has been published exploring the relationship between in utero OP exposure and neurobehavioral outcomes in neonates.

The current analysis aims to assess whether in utero and early postnatal OP exposure has a detrimental impact on neurobehavioral functioning, as assessed by the BNBAS, of full-term neonates born to a cohort of pregnant women living in the Salinas Valley, California. The Salinas Valley, commonly referred to as the "nation's salad bowl", is one of the major centers of

agricultural production in the United States with approximately 500,000 pounds of OPs applied annually (California EPA, 2002).

MATERIALS AND METHODS

Participants and Recruitment

Pregnant women initiating prenatal care at Natividad Medical Center, a county hospital located in the city of Salinas, or at Clínica de Salud del Valle de Salinas located in the Salinas Valley, were recruited to participate in the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) project. CHAMACOS is a longitudinal birth cohort study assessing the impacts of pesticides and other environmental exposures on the health of pregnant women and their children. The recruitment sites largely serve a low-income population of which a substantial proportion are agricultural workers.

Women considered eligible for the study were less than 20 weeks gestation, aged 18 years or older, Medicaid eligible, fluent in English and/or Spanish, and planning to deliver at Natividad Medical Center. A total of 601 women were enrolled between October 1999 and October 2000, with 528 followed through delivery of a live born infant. The BNBAS was performed on 421 infants. Of these 421 infants, eight twins and 27 preterm infants were excluded from analysis. As administration of the BNBAS is appropriate up to the second month of life (Brazelton and Nugent, 1995), an additional five infants whose assessments were performed more than 2 months (62 days) after delivery were further excluded from this analysis. Thus, the final sample consisted of 381 full-term, singleton infants, who were ≤ 62 days old at the time the BNBAS was administered. Institutional Review Boards at participating institutions approved this study and investigators obtained written informed consent from all mothers.

Interview, Medical Record Abstraction, and BNBAS Procedures

Participants were interviewed twice during pregnancy, at an average of 14 ± 8 and 26 ± 2 weeks gestation, and again after delivery, at an average of 7 ± 17 days postpartum (76% occurred within 1 week of delivery). Bilingual, bicultural interviewers conducted the interviews in English or Spanish and ascertained family demographic information; work histories

of household members; maternal behaviors such as smoking, alcohol, and drug use; and maternal medical history data, including information on previous pregnancies. Medical records from prenatal visits and delivery were abstracted by a registered nurse.

The BNBAS was administered once to each infant and in accordance with the BNBAS protocol, by four examiners trained to reliability by a BNBAS certified-trainer. Examiners were blind to the infant's exposure status. Approximately 30% of the assessments included in the final sample were completed prior to discharge from the hospital; the remainder was performed at the CHAMACOS research office or in the participant's home. Assessments were performed away from the mother in a private room with low-level light and noise control.

Pesticide Exposure Measurement

Exposure was assessed by measurement of organophosphate dialkylphosphate (DAP) metabolites in urine. Six organophosphate DAP metabolites were measured in maternal urine: three dimethylphosphate metabolites (dimethylphosphate (DMP), dimethyldithiophosphate (DMDTP), dimethylthiophosphate (DMTP)); and three diethylphosphate metabolites (diethylphosphate (DEP), diethyldithiophosphate (DEDTP), and diethylthiophosphate (DETP)). These six metabolites represent the by-products of approximately 80% of OPs used in the Salinas Valley (California EPA, 2002).

Maternal urine specimens were generally collected at the time of the prenatal and post-delivery interviews. The post-delivery urines were collected within 1 week of delivery for 73% of the sample, with the remainder up to 176 days afterwards. Given the very brief half-life of OPs, metabolite levels in postnatal urines more likely reflected early postnatal rather than in utero exposure. Urine specimens were aliquoted and stored at -80°C until shipment to the Centers for Disease Control and Prevention (CDC) for analysis of the metabolite levels. Metabolite levels were measured using gas chromatography–tandem mass spectrometry and quantified using isotope dilution calibration (Bravo et al., 2002). A detailed description of urine sample collection, analysis, and quality control procedures is provided elsewhere (Eskenazi et al., 2004).

Outcome Definition

The BNBAS consists of 28 behavioral items scored on a nine-point scale and 18 reflex items scored on a

four-point scale. For analysis, the 28 behavioral scores and 18 reflex items were reduced to seven clusters in accordance with the widely used, conceptually and empirically-based scoring method originally developed by Lester et al. (1982). These seven clusters include: habituation (light, rattle, bell, pin-prick); orientation (inanimate visual, inanimate auditory, inanimate visual–auditory, animate visual, animate auditory, animate visual–auditory, alertness); motor performance (tonus, maturity, pull-to-sit, defense, activity); range of state (peak of excitement, rapidity of build-up, irritability, lability of state); regulation of state (cuddliness, consolability, self-quieting, hand-to-mouth); autonomic stability (tremors, startles, skin color); and reflex (plantar, babinski, ankle clonus, rooting, sucking, glabella, passive resistance—legs, passive resistance—arms, palmar, placing, standing, walking, crawling, incurvation, tonic deviation of head and eyes, nystagmus, tonic neck reflex, moro reflex).

The six behavioral cluster scores are calculated by recoding the original BNBAS items where necessary such that higher scores represent more optimal functioning. Individual items within each cluster are then averaged. The seventh cluster, the reflex cluster score, is the total number of reflexes coded as abnormal. Thus, higher scores on the behavioral clusters indicate more optimal functioning while higher scores on the reflex cluster indicate less optimal functioning.

Data Analysis

In order to assess the relationship between urinary metabolite levels and neonatal performance on the BNBAS, separate regression models were fit for each of the seven Lester clusters. Error distributions for the regression models predicting performance on the behavioral clusters were assumed Gaussian. In the case of the reflex cluster, regression diagnostics were more supportive of a Poisson error distribution. Thus, Poisson regression was used to model the association between urinary metabolite levels and number of abnormal reflexes.

Total DAP metabolite level was defined as the sum of the molar concentrations of the six DAP metabolites, dimethylphosphate metabolite level as the sum of the molar concentrations of the three dimethylphosphate metabolites only, and diethylphosphate metabolite level as the sum of the molar concentrations of the three diethylphosphate metabolites only. Total DAP, dimethyl, and diethylphosphate levels were determined for each participant for each of the two pregnancy urine samples and for the post-delivery urine sample.

Individual metabolite levels below the limit of detection (LOD) were assigned a value of the LOD divided by the square root of two (Hornung and Reed, 1990) and this value was included in each sum. For 10 participants, the level of one of the six metabolites was not readable for one of the time points due to analytic interference. As individual metabolite levels within either the diethyl or dimethylphosphate groups were highly correlated, missing values were imputed using regression to predict the missing metabolite level as a function of the other known metabolites for a participant at that time point. The total DAP, dimethyl, and diethylphosphate metabolite levels were then transformed to the \log_{10} scale.

Models relating metabolite levels to each cluster score were initially fit including two variables: the average of the two logged pregnancy measurements (taking the average reduced large observed within-person variability), and, as a separate variable, the post-delivery measurement, with all models adjusted for covariates. If either of the pregnancy urines were missing, the one remaining was used to represent the average. Eighteen mothers were missing one of the two pregnancy urines. Sixteen mothers were missing the post-delivery urine. In order not to exclude those with missing post-delivery urines from the statistical models, results were re-examined with the variable representing post-delivery metabolite level excluded from all models. Results were further re-examined with metabolite levels adjusted for creatinine.

Missing individual BNBAS items for the six behavioral clusters were imputed as the median value of known scores within the cluster associated with the missing item. The cluster score was subsequently calculated with this imputed value. If all items in a particular cluster were missing, then the cluster score was considered missing. The requirement that the infant be asleep immediately prior to the assessment of the items in the habituation cluster resulted in a large number of infants missing all four items comprising this cluster. Fifty-four percent of the sample was missing all habituation items and an additional 22% required at least one imputation. Forty percent of the sample required at least one imputation of the four items comprising the regulation of state cluster, with 13, 25, and 0.5% of the sample requiring one, two, and three items imputed, respectively. Individual items with the most missing values for the regulation of state cluster were consolability and self-quieting, both of which required the infant to be in a crying state for at least 15 s. For the autonomic, motor, orientation, and range of state clusters, 1, 3, 4, and 9% of the sample

required at least one imputation, respectively. Two infants were missing all items comprising the orientation cluster score.

Missing individual reflex items comprising the BNBAS reflex cluster were not imputed; thus, number of abnormal reflexes was calculated based on non-missing values only. Reflexes with the most missing values included incurvation (9%), nystagmus (7%), and tonic neck reflex (7%). Missing values did not exceed 4% for the remaining reflex items.

Covariates were initially selected based on their predictive value of BNBAS performance reported in the literature. Maternal characteristics included: age, pre-pregnancy body mass index (BMI), any smoking during pregnancy, any alcohol use during pregnancy, any caffeine use during pregnancy, any drug use during pregnancy, any vitamin use during pregnancy, gestational age at which prenatal care was initiated, total number of prenatal care visits, mean blood pressure over pregnancy (diastolic and systolic), parity (0 versus ≥ 1), method of delivery (Cesarean versus vaginal), any general anesthesia used during delivery, breastfeeding initiated after delivery, and poverty level (at or below poverty, 200% poverty, >200% poverty). Poverty level was calculated by dividing household income by the number of people supported by that income and comparing this value to federal poverty thresholds (U.S. Census Bureau, 2000). Other covariates included: infant sex, age in days at BNBAS, minutes since last fed at BNBAS, and BNBAS examiner.

Covariates from this list were selected for final regression models if they were related to each of the seven cluster scores, unadjusted for other variables ($p < 0.15$). Missing covariate values were imputed by random selection of an individual amongst all participants with known values for the covariate and assigning the missing value the selected participant's value. Covariates with the largest percentage of items requiring imputation were reported breastfeeding after delivery (9%) and poverty level (6%). Other covariates requiring imputations for 2% or less of the sample included: pre-pregnancy BMI, any smoking during pregnancy, any alcohol use during pregnancy, any caffeine use during pregnancy, any drug use during pregnancy, any vitamin use during pregnancy, and minutes since last fed at BNBAS.

Some studies have recently suggested that in utero pesticide exposure may be associated with birth weight or gestational age (Berkowitz et al., 2004; Eskenazi et al., 2004; Whyatt et al., 2004). Therefore, it is possible that fetal growth and length of gestation may be on the causal pathway between the exposure

and neurobehavioral outcome. For this reason, regression models for the full sample were refit both with and without birth weight and gestational age as covariates.

While the BNBAS can be appropriately administered up through the first 2 months of life, there is some question as to whether estimated associations would be impacted by the wide variation in age of infants at administration, ranging from 0 to 62 days post-delivery. All regression models included age at administration as a covariate; however, in an attempt to assess differences in the acute and delayed postnatal impacts of in utero OP exposure, we separately examined infants with BNBAS administration occurring *within* the first 3 days of life ($n = 197$) and infants with assessments performed *after* this point ($n = 184$). Three days was the median age at administration for the sample of 381 infants. Further, a number of investigators have restricted BNBAS assessment during the early neonatal period to the third day, or within the first 3 days, of life (Black et al., 1993; Chasnoff et al., 1985; Coles et al., 1992; Dreher et al., 1994; Lieberman et al., 1979; Neuspil et al., 1991; Wittels et al., 1990).

RESULTS

Table 1 presents demographic characteristics of the sample. The mean maternal age was 26 ± 5 years. Most women were at or below the poverty line (64%), born in Mexico (85%), multiparous (68%), married or living as married (81%), and had not completed high school (81%). Few women smoked (5%) or drank alcohol (16%) during pregnancy, with a much higher percentage reporting caffeine use (75%). Only 1% of the sample reported any illicit drug use during pregnancy. Cesarean delivery was performed in 24% of the births with 3% requiring general anesthesia. Most women reported initiating breastfeeding after delivery (93%). The sample consisted of a nearly equal number of male and female infants.

Table 2 shows the distributions for total DAP, dimethyl, and diethylphosphate urinary metabolite levels. Median metabolite levels for the average of the two pregnancy measurements of total DAP, dimethyl, and diethylphosphates in maternal urine were 132, 97, and 21 nmol/L, respectively. Median post-delivery measurements were higher at 222, 160, and 27 nmol/L, for total DAP, dimethyl, and diethylphosphates, respectively. Urinary metabolite levels measured at the two points during pregnancy were not significantly correlated with each other or with the post-delivery measurement, with all estimated correla-

Table 1
Demographic characteristics of analysis sample (CHAMACOS study, Salinas Valley, California, 2000–2001, $n = 381^a$)

	<i>N</i>	%
Maternal education		
Less than 6th grade	165	43.3
7th through 12th grade	145	38.1
Completed High school	71	18.6
Marital status		
Married/living as married	309	81.1
Single	72	18.9
Parity		
0	123	32.3
1+	258	67.7
Country of birth		
Mexico	324	85.0
US	49	12.9
Other	8	2.1
Poverty		
At or below poverty level	228	63.5
200% Poverty level	116	32.3
Above 200% poverty level	15	4.2
Smoked during pregnancy		
Yes	20	5.3
No	358	94.7
Alcohol use during pregnancy		
Yes	59	15.6
No	320	84.4
Caffeine use during pregnancy		
Yes	284	74.9
No	95	25.1
Cesarean delivery		
Yes	91	23.9
No	290	76.1
General anesthesia		
Yes	10	2.6
No	371	97.4
Breastfeeding initiated after delivery		
Yes	301	92.6
No	24	7.4
Infant sex		
Female	200	52.5
Male	181	47.5

^a Total number of observations vary due to missing data (table entries do not include imputed values).

tions below 0.1 for total DAP, dimethyl, and diethylphosphate metabolite levels.

The median age at administration of the BNBAS was 3 days, with an interquartile range between 1 and 26 days post-delivery, and a mean of 18.0 ± 17.5 min since the last feeding. The four examiners administered 236 (62%), 114 (30%), 23 (6%), and 8 (2%) of the 381 assessments. Sample mean values, standard deviations,

Table 2

Number of women with measurements, median values, and ranges of dialkylphosphate metabolite levels^a during pregnancy and post-delivery (CHAMACOS Study, Salinas Valley, CA, 2000–2001)

Marker of exposure	Parent compounds or class	N	Median	Range	Measured in
Dialkylphosphate metabolites (nmol/L)					
Total dialkylphosphates	Sum of below				Urine
Average pregnancy		381	132	12–4,177	
Post-delivery		365	222	7–21,867	
Dimethylphosphates	Malathion, oxydemeton-methyl, dimethoate, naled, methidathion ^b				Urine
Average pregnancy		381	97	5–4152	
Post-delivery		365	160	5–21,857	
Diethylphosphates	Diazinon, chlorpyrifos, disulfoton				Urine
Average pregnancy		381	21	2–680	
Post-delivery		365	27	2–666	

^a Urinary metabolites: average of two pregnancy measurements, post-delivery measurement. Not adjusted for creatinine.

^b Only parent compounds with annual usage in Salinas Valley exceeding 10,000 lb are listed.

Table 3

BNBAS Lester cluster scores for study sample (CHAMACOS Study, Salinas Valley, California, 2000–2001)

	N	Mean	S.D.	Range
Habituation	175	6.6	1.1	3.5–8.8
Orientation	379	7.5	1.4	1.6–9.0
Motor	381	5.8	0.7	4.0–7.2
Range of state	381	3.4	1.0	1.3–5.3
Regulation of state	381	5.7	1.3	1.3–8.8
Autonomic stability	381	7.0	0.9	4.3–8.0
Reflexes	381	2.2	1.8	0–10

and ranges for each of the seven cluster scores are presented in Table 3.

Table 4 presents adjusted regression coefficients and 95% confidence intervals, based on the entire sample, for each of the seven cluster scores regressed separately

on average total DAP, dimethyl, and diethylphosphate metabolite levels measured during pregnancy. A significant association was estimated between increasing total DAP, dimethyl, and diethylphosphate metabolite levels during pregnancy and increasing number of abnormal reflexes. No significant associations between urinary metabolite levels and BNBAS performance were observed based on the other clusters.

The results presented in Table 4 reflect models that do not include the post-delivery urine as a separate variable. Results based on models including this variable (not shown) were nearly identical to those presented. Notably, none of the models demonstrated an association between post-delivery urinary metabolite measurements and BNBAS performance. Estimated associations were somewhat reduced after adjustment

Table 4

Association between average urinary metabolites of organophosphate pesticides measured during pregnancy and seven BNBAS Lester cluster scores (CHAMACOS Study, Salinas Valley, California, 2000–2001)

	Dialkylphosphate metabolites ^a (nmol/L—log ₁₀ scale)			
	N	Total dialkylphosphates β (95% CI)	Dimethylphosphates β (95% CI)	Diethylphosphates β (95% CI)
Habituation ^b	175	0.03 (–0.34, 0.40)	–0.06 (–0.39, 0.27)	0.33 (–0.06, 0.72)
Orientation ^c	379	–0.17 (–0.50, 0.17)	–0.12 (–0.43, 0.19)	–0.32 (–0.66, 0.03)
Motor performance ^d	381	–0.03 (–0.19, 0.14)	–0.05 (–0.20, 0.10)	0.10 (–0.06, 0.27)
Range of state ^e	381	0.09 (–0.16, 0.34)	0.08 (–0.15, 0.32)	–0.02 (–0.27, 0.24)
Regulation of state ^f	381	–0.07 (–0.39, 0.24)	–0.05 (–0.34, 0.24)	–0.15 (–0.47, 0.17)
Autonomic stability ^g	381	–0.16 (–0.36, 0.05)	–0.17 (–0.35, 0.02)	0.06 (–0.15, 0.27)
Reflexes ^h	381	0.23 (0.05, 0.41)*	0.18 (0.02, 0.34)*	0.22 (0.04, 0.40)*

^a Urinary metabolite levels are not adjusted for urinary creatinine concentration.

^b Adjusted for age at BNBAS, smoking, alcohol, method of delivery, minutes since fed at BNBAS, and BNBAS interviewer.

^c Adjusted for age at BNBAS, BNBAS interviewer, and number of prenatal care visits.

^d Adjusted for age at BNBAS, poverty level, gestational age at initiation of prenatal care, and BNBAS interviewer.

^e Adjusted for age at BNBAS, number of prenatal care visits, gestational age at initiation of prenatal care, alcohol, and BNBAS interviewer.

^f Adjusted for age at BNBAS, pre-pregnancy BMI, infant sex, parity, caffeine use, and BNBAS interviewer.

^g Adjusted for age at BNBAS, infant sex, parity, vitamin use, minutes since fed at BNBAS, BNBAS interviewer, and illicit drug use during pregnancy.

^h Adjusted for age at BNBAS, maternal age at delivery, smoking, vitamin use, BNBAS interviewer, and mean diastolic and systolic blood pressure.

* $p < 0.05$.

Table 5

Association between average urinary metabolites of organophosphate pesticides measured during pregnancy and seven BNBAS Lester cluster scores^a (stratified by age at BNBAS assessment (≤ 3 days vs. > 3 days post-delivery; CHAMACOS Study, Salinas Valley, California, 2000–2001))

	Dialkylphosphate metabolites ^b (nmol/L—log ₁₀ scale)			
	<i>N</i>	Total dialkylphosphates β (95% CI)	Dimethylphosphates β (95% CI)	Diethylphosphates β (95% CI)
≤ 3 days				
Habituation	109	0.10 (–0.40, 0.60)	–0.04 (–0.49, 0.40)	0.47 (–0.05, 0.99)
Orientation	197	–0.02 (–0.53, 0.49)	–0.08 (–0.54, 0.39)	–0.11 (–0.65, 0.43)
Motor performance	197	0.04 (–0.20, 0.28)	0.03 (–0.19, 0.24)	0.08 (–0.17, 0.33)
Range of state	197	0.11 (–0.21, 0.43)	0.17 (–0.12, 0.46)	–0.21 (–0.54, 0.12)
Regulation of state	197	–0.07 (–0.50, 0.36)	–0.06 (–0.45, 0.33)	–0.08 (–0.52, 0.37)
Autonomic stability	197	–0.09 (–0.38, 0.20)	–0.15 (–0.42, 0.11)	0.31 (0.01, 0.61)*
Reflexes	197	–0.01 (–0.24, 0.22)	–0.00 (–0.21, 0.20)	0.08 (–0.16, 0.32)
> 3 days				
Habituation	66	0.06 (–0.54, 0.66)	0.04 (–0.50, 0.58)	0.20 (–0.43, 0.83)
Orientation	182	–0.13 (–0.54, 0.27)	0.01 (–0.37, 0.38)	–0.33 (–0.73, 0.08)
Motor performance	184	–0.07 (–0.28, 0.15)	–0.11 (–0.31, 0.09)	0.17 (–0.05, 0.38)
Range of state	184	–0.02 (–0.44, 0.40)	–0.12 (–0.51, 0.27)	0.20 (–0.21, 0.62)
Regulation of state	184	–0.10 (–0.58, 0.37)	–0.06 (–0.50, 0.39)	–0.24 (–0.72, 0.24)
Autonomic stability	184	–0.19 (–0.49, 0.12)	–0.14 (–0.43, 0.14)	–0.16 (–0.47, 0.14)
Reflexes	184	0.53 (0.23, 0.82)*	0.41 (0.12, 0.69)*	0.37 (0.09, 0.64)*

^a Covariates for all models are identical to those reported in Table 4.

^b Urinary metabolite levels are not adjusted for urinary creatinine concentration.

* $p < 0.05$.

for creatinine but remained in the same direction (results not shown). Including birth weight and gestational age did not have a substantial impact on the exposure coefficient, in terms of direction or precision, for any of the models presented in Table 4.

Table 5 presents results analogous to those presented in Table 4, but stratified by age at assessment. For infants assessed *within* the first 3 days of life ($n = 197$), only diethylphosphate metabolite levels and the autonomic cluster were significantly associated; however, the direction of this association was contrary to the hypothesis of a harmful exposure effect. However, similar to results for the entire sample, findings based on infants assessed *after* the first 3 days ($n = 184$) demonstrated a detrimental association between exposure, as measured by total DAP, diethyl, and dimethylphosphate metabolite levels, and reflex functioning.

The most common primitive reflexes rated as abnormal in the infants assessed *after* 3 days of age included: rooting (23%), passive resistance-legs (39%), walking (25%), incurvation (40%), and Moro reflex (18%). According to the Lester scheme (Lester et al., 1982), a reflex is rated abnormal if it is not elicited despite several attempts, or the reflex response is either hypoactive or hyperactive. In nearly all cases, reflexes were rated as abnormal due to failure to elicit a response or a hypoactive response. The 18 reflex items of the BNBAS, while not designed to provide

a neurological diagnosis, can potentially identify gross neurologic abnormalities and more than three abnormally rated reflexes may be clinically relevant (Brazelton and Nugent, 1995). Of the infants older than 3 days at assessment, 17% had more than three abnormal reflexes.

Table 6 presents the proportion of infants older than 3 days at assessment with more than three abnormal reflexes by quintiles of average log₁₀ total DAP, dimethyl, and diethylphosphate metabolite levels during pregnancy. For total DAP, dimethyl, and diethylphosphate metabolites, there is a significant trend of increasing proportion of more than three abnormal reflexes with increasing metabolite levels. A logistic regression model was also fit to assess this association. We found that the odds of having more than three abnormal reflexes significantly increased with average urinary metabolite levels during pregnancy, after adjustment for covariates (identical to those in the models for reflexes in Table 4). Specifically, we found that, based on a one-unit change on the log₁₀ scale, or equivalently, a 10-fold increase such as from 10 to 100 nmol/L (the range of exposure is over 2 log₁₀ units), the adjusted odds ratio for more than three abnormal reflexes versus three or less was 4.9 for total DAP (95% CI = 1.5, 16.1), 3.2 for dimethylphosphate (95% CI = 1.1, 9.8), and 3.4 for diethylphosphate (95% CI = 1.2, 9.9) metabolite levels during pregnancy.

Table 6

Proportion of infants^a with more than three abnormal reflexes by quintiles of urinary metabolite levels^b (CHAMACOS Study, Salinas Valley, California, 2000–2001)

Level (nmol/L—log ₁₀)	N	Proportion
Total DAP		
1.07–1.65	37	0.08
1.65–1.83	37	0.14
1.83–2.07	37	0.16
2.08–2.30	37	0.14
2.31–3.17	36	0.33
$\chi^2_{\text{trend}} = 6.7, p = 0.01$		
Dimethyls		
0.68–1.48	37	0.08
1.48–1.69	37	0.14
1.70–1.95	37	0.16
1.95–2.19	37	0.24
2.19–3.15	36	0.22
$\chi^2_{\text{trend}} = 4.7, p = 0.03$		
Diethyls		
0.51–0.90	37	0.16
0.90–1.10	37	0.05
1.11–1.27	37	0.16
1.28–1.58	37	0.14
1.58–2.35	36	0.33
$\chi^2_{\text{trend}} = 4.0, p = 0.05$		

^a Within the group of infants assessed *after* 3 days post-delivery ($n = 184$).

^b Average of the two pregnancy measurements.

Results for the stratified analyses presented in Table 5 are based on models that are unadjusted for post-delivery urinary metabolite levels. Associations between average pregnancy levels and BNBAS functioning adjusted for post-delivery levels (not shown) were nearly identical to those presented in Table 5. For infants assessed *within* 3 days of birth, significant associations were observed between postnatal total DAP levels and the orientation cluster, as well as between postnatal dimethylphosphate metabolite levels and the orientation and regulation of state clusters; however, the directions of these associations were opposite to a priori hypotheses of a detrimental effect of OP exposure. As in analyses based on the full sample, post-delivery urinary metabolite levels were not associated with any of the seven clusters in the group of infants assessed *after* 3 days post-delivery.

DISCUSSION

Results of analyses based on this sample of 381 singleton, full-term infants, with age at BNBAS assessment ranging from day of delivery to 62 days

post-delivery, are suggestive of a detrimental impact of in utero OP exposure, as measured by total DAP, dimethylphosphate, and diethylphosphate metabolites, on reflex functioning, particularly in those infants assessed *after* 3 days of life. This association with increasing urinary metabolite levels was observed for both increase in number of abnormal reflexes and the proportion of infants with more than three abnormal reflexes, a number that may be clinically relevant. Rogan et al. (1986) similarly reported higher proportions of more than three abnormal reflexes based on the BNBAS reflex items amongst infants with higher exposure to PCBs and DDE, with both chemicals associated with hyporeflexia, and neither with hyperreflexia. They reported similar proportions of infants with more than three abnormal reflexes in the highest exposure groups to those reported in the present study.

We also found significant associations that were contrary to a priori hypotheses of a detrimental impact of OP exposure on neonatal neurobehavioral functioning. Whether these results are worthy of further investigation or simply the result of multiple testing is unclear, particularly as there is no ready explanation for a biological protective effect of OP exposure on newborn neurobehavior.

Our results indicate a difference in the association between prenatal urinary metabolite levels and reflex functioning based on age at administration of the BNBAS. Differences in associations between in utero toxicants and BNBAS performance by age at administration have been found in other studies as well. Dreher et al. (1994) assessed a group of Jamaican infants exposed to marijuana in utero on the BNBAS at 3 days and on the same infants again at 1-month old. In this study, no significant differences were reported at 3 days while at 1 month, exposure was significantly associated with autonomic stability, orientation, and reflexes. Tronick et al. (1996) used the BNBAS at 2 days and/or 17 days post-delivery to assess the impact of prenatal cocaine exposure on neonatal functioning and, similarly, reported no significant findings based on the earlier assessment, yet found an association of exposure with the regulation of state cluster based on the later assessment. Contrary to these findings, Black et al. (1993), comparing infants with in utero exposure to cocaine to unexposed infants on the BNBAS in four administrations (the second or third day of life, 2, 4, and 6 weeks after birth), found significantly less optimal neurodevelopment in drug-exposed infants soon after birth based on the orientation, regulation of state, and autonomic stability clusters. Significant associations with exposure remained

only for autonomic stability at the 2- and 6-week assessments with no differences based on exposure reported at 4 weeks post-delivery.

Various explanations may account for discrepancies in estimated associations between in utero exposures and performance on the BNBAS at different ages of administration in these studies, as well as the present study. Specifically, differences in findings may be due to a delayed impact of in utero exposures on neuro-behavioral functioning, or that the neurologic abnormalities, which the BNBAS reflex items aim to identify, may only be observed with maturity. Conversely, one potential hypothesis is that postnatal environmental exposures, such as take-home exposure from a farm worker parent, inhalation, or possibly by consumption of contaminated breast milk, may explain observed associations between abnormal reflex functioning and prenatal exposure biomarkers; however, we found adjustment for postnatal urinary metabolite levels did not substantially impact estimates of the association of prenatal metabolite levels with reflex functioning or the other clusters. Further, the postnatal metabolite levels themselves were not significantly associated with any of the clusters.

The present study had the disadvantage of only assessing each infant once. Thus, differences in findings across age strata cannot clearly be attributed to differences in the impact of exposure on functioning by age at assessment. The possibility remains that differences may be, at least in part, due to inherent differences between those infants assessed earlier in the neonatal period and those assessed later. However, infants assessed within 3 days and infants assessed after this point were generally similar on important measures of newborn health including: Apgar scores at 1 min ($M = 8.0 \pm 0.9$ and 7.8 ± 1.1 , respectively); Apgar scores at 5 min ($M = 8.9 \pm 0.3$ and 8.8 ± 0.5); birth weight ($M = 3510 \pm 430$ g and 3519 ± 420 g); and gestational age at delivery ($M = 39 \pm 1.2$ weeks and 39 ± 1.2 weeks).

Another limitation of the present study is that misclassification of exposure may have hindered detection of significant adverse associations between exposure and neonatal neurobehavioral outcomes. OPs and their metabolites have a brief residence time in the body (Abu-Qare et al., 2001; Garfitt et al., 2002; Griffin et al., 1999; WHO, 1996). Large within-person variability of DAP metabolite levels suggests that even multiple isolated measurements over pregnancy may not represent prenatal exposure, particularly when exposure is transient and highly variable. Further, while the DAP metabolites reflect exposure to approxi-

mately 80% of the OPs used in the Salinas Valley, metabolite measurements in urine may also reflect exposure to the metabolite itself, not only to the parent compound (Lu et al., in press; Duggan et al., 2003; Wilson et al., 2003). Presently, there are no better methods of exposure assessment to capture exposure to multiple organophosphate compounds.

In summary, the present study was suggestive of a detrimental association between prenatal OP exposure, as measured by urinary metabolite levels, and abnormal reflexes, as measured by the BNBAS, particularly in infants assessed after the first 3 days of life. Despite limitations, this study is the first reported examination of the impacts of organophosphate pesticide exposure on human neurodevelopment using an exposure biomarker in a population with high potential for exposure. Whether neonatal reflex functioning as measured by the BNBAS is predictive of neuropsychological functioning as the child matures will continue to be evaluated in this birth cohort.

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References

- Abu-Qare AW, Abdel-Rahman AA, Ahmad H, Kishk AM, Abou-Donia MB. Absorption, distribution, metabolism and excretion of daily oral doses of [¹⁴C]methyl parathion in hens. *Toxicol Lett* 2001;125:1–10.
- Berkowitz GS, Wetmur JG, Birman-Deych E, Obel J, Lapinski RH, Godbold JH, et al. In utero pesticide exposure, maternal paraoxonase activity, and head circumference. *Environ Health Perspect* 2004;112:388–91.
- Black M, Schuler M, Nair P. Prenatal drug exposure: neurodevelopmental outcome and parenting environment. *J Pediatr Psychol* 1993;18:605–20.
- Bravo R, Driskell WJ, Whitehead RD Jr, Needham LL, Barr DB. Quantitation of dialkyl phosphate metabolites of organophosphates in urine. *Toxicol Lett* 2004;152:1–10.

- sphate pesticides in human urine using GC–MS–MS with isotopic internal standards. *J Anal Toxicol* 2002;26:245–52.
- Brazelton TB, Nugent K. Neonatal behavioral assessment scale. 3rd ed. London: MacKeith Press; 1995.
- California EPA. Pesticide Use Reporting 2001 Summary Data, 2002. Available: http://www.cdpr.ca.gov/docs/pur/pur01rep/01_pur.htm. Accessed February 11, 2004.
- Campbell CG, Seidler FJ, Slotkin TA. Chlorpyrifos interferes with cell development in rat brain regions. *Brain Res Bull* 1997;43:179–89.
- Chanda SM, Harp P, Liu J, Pope CN. Comparative developmental and maternal neurotoxicity following acute gestational exposure to chlorpyrifos in rats. *J Toxicol Environ Health* 1995;44:189–202.
- Chanda SM, Pope CN. Neurochemical and neurobehavioral effects of repeated gestational exposure to chlorpyrifos in maternal and developing rats. *Pharmacol Biochem Behav* 1996;53:771–6.
- Chasnoff IJ, Burns WJ, Schnoll SH, Burns KA. Cocaine use in pregnancy. *N Engl J Med* 1985;313:666–9.
- Coles CD, Platzman KA, Smith I, James ME, Falek A. Effects of cocaine and alcohol use in pregnancy on neonatal growth and neurobehavioral status. *Neurotoxicol Teratol* 1992;14:23–33.
- Curl CL, Fenske RA, Kissel JC, Shirai JH, Moate TF, Griffith W, et al. Evaluation of take-home organophosphorus pesticide exposure among agricultural workers and their children. *Environ Health Perspect* 2002;110:787–92.
- Datta-Bhutada S, Johnson HL, Rosen TS. Intrauterine cocaine and crack exposure: neonatal outcome. *J Perinatol* 1998;18:183–8.
- Delaney-Black V, Covington C, Ostrea E Jr, Romero A, Baker D, Tagle MT, et al. Prenatal cocaine and neonatal outcome: evaluation of dose–response relationship. *Pediatrics* 1996;98:735–40.
- Donaldson D, Kiely T, Grube A. Pesticides Industry Sales and Usage, 1998 and 1999 Market Estimates. Washington, DC: U.S. Environmental Protection Agency, Office of Prevention, Pesticides, and Toxic Substances, Office of Pesticide Programs, 2002.
- Dreher MC, Nugent K, Hudgins R. Prenatal marijuana exposure and neonatal outcomes in Jamaica: an ethnographic study. *Pediatrics* 1994;93:254–60.
- Duggan A, Charnley G, Chen W, Chukwudebe A, Hawk R, Krieger RI, et al. Di-alkyl phosphate biomonitoring data: assessing cumulative exposure to organophosphate pesticides. *Regul Toxicol Pharmacol* 2003;37:382–95.
- Eisen LN, Field TM, Bandstra ES, Roberts JP, Morrow C, Larson SK, et al. Perinatal cocaine effects on neonatal stress behavior and performance on the Brazelton scale. *Pediatrics* 1991;88:477–80.
- Emory E, Pattillo R, Archibold E, Bayorh M, Sung F. Neurobehavioral effects of low-level lead exposure in human neonates. *Am J Obstet Gynecol* 1999;181:S2–11.
- Ernhart CB, Wolf AW, Kennard MJ, Erhard P, Filipovich HF, Sokol RJ. Intrauterine exposure to low levels of lead: the status of the neonate. *Arch Environ Health* 1986;41:287–91.
- Eskenazi B, Bradman A, Castorina R. Exposures of children to organophosphate pesticides and their potential adverse health effects. *Environ Health Perspect* 1999;107(Suppl 3):409–19.
- Eskenazi B, Harley K, Bradman A, Weltzien E, Jewell NP, Barr D, et al. Association of in utero organophosphate pesticide exposure and fetal growth and length of gestation in an agricultural population. *Environ Health Perspect* 2004;112:1116–24.
- Eyler FD, Behnke M, Conlon M, Woods NS, Wobie K. Birth outcome from a prospective, matched study of prenatal crack/cocaine use: II Interactive and dose effects on neurobehavioral assessment. *Pediatrics* 1998;101:237–41.
- Fenske RA, Lu C, Barr D, Needham L. Children's exposure to chlorpyrifos and parathion in an agricultural community in central Washington State. *Environ Health Perspect* 2002;110:549–53.
- Garfitt SJ, Jones K, Mason HJ, Cocker J. Exposure to the organophosphate diazinon: data from a human volunteer study with oral and dermal doses. *Toxicol Lett* 2002;134:105–13.
- Griffin P, Mason H, Heywood K, Cocker J. Oral and dermal absorption of chlorpyrifos: a human volunteer study. *Occup Environ Med* 1999;56:10–3.
- Hornung RW, Reed LD. Estimation of average concentration in the presence of nondetectable values. *Appl Occup Environ Hyg* 1990;5:46–51.
- Lester BM, Als H, Brazelton TB. Regional obstetric anesthesia and newborn behavior: a reanalysis toward synergistic effects. *Child Dev* 1982;53:687–92.
- Lieberman BA, Rosenblatt DB, Belsey E, Packer M, Redshaw M, Mills M, et al. The effects of maternally administered pethidine or epidural bupivacaine on the fetus and newborn. *Br J Obstet Gynaecol* 1979;86:598–606.
- Lu C, Bravo R, Caltabiano LM, Irish RM, Weerasekera G, Barr DB. The presence of dialkylphosphates in fresh fruit juices: implications on organophosphorus pesticide exposure and risk assessments. *J Toxicol Environ Health*, in press.
- Lu C, Fenske RA, Simcox NJ, Kalman D. Pesticide exposure of children in an agricultural community: evidence of household proximity to farmland and take home exposure pathways. *Environ Res* 2000;84:290–302.
- Mayes LC, Granger RH, Frank MA, Schottenfeld R, Bornstein MH. Neurobehavioral profiles of neonates exposed to cocaine prenatally. *Pediatrics* 1993;91:778–83.
- McCauley LA, Lasarev MR, Higgins G, Rothlein J, Muniz J, Ebbert C, et al. Work characteristics and pesticide exposures among migrant agricultural families: a community-based research approach. *Environ Health Perspect* 2001;109:533–8.
- Morrow CE, Bandstra ES, Anthony JC, Ofir AY, Xue L, Reyes ML. Influence of prenatal cocaine exposure on full-term infant neurobehavioral functioning. *Neurotoxicol Teratol* 2001;23:533–44.
- Muto MA, Lobelle F Jr, Bidanset JH, Wurlpel JN. Embryotoxicity and neurotoxicity in rats associated with prenatal exposure to DURSBN. *Vet Hum Toxicol* 1992;34:498–501.
- Neuspiel DR, Hamel SC, Hochberg E, Greene J, Campbell D. Maternal cocaine use and infant behavior. *Neurotoxicol Teratol* 1991;13:229–33.
- O'Rourke MK, Lizardi PS, Rogan SP, Freeman NC, Aguirre A, Saint CG. Pesticide exposure and creatinine variation among young children. *J Expo Anal Environ Epidemiol* 2000;10:672–81.
- Rogan WJ, Gladen BC, McKinney JD, Carreras N, Hardy P, Thullen J, et al. Neonatal effects of transplacental exposure to PCBs and DDE. *J Pediatr* 1986;109:335–41.
- Simcox NJ, Camp J, Kalman D, Stebbins A, Bellamy G, Lee IC, et al. Farmworker exposure to organophosphorus pesticide residues during apple thinning in central Washington State. *Am Ind Hyg Assoc J* 1999;60:752–61.

- Slotkin TA, Cousins MM, Tate CA, Seidler FJ. Persistent cholinergic presynaptic deficits after neonatal chlorpyrifos exposure. *Brain Res* 2001;902:229–43.
- Stewart P, Reihman J, Lonky E, Darvill T, Pagano J. Prenatal PCB exposure and neonatal behavioral assessment scale (NBAS) performance. *Neurotoxicol Teratol* 2000;22:21–9.
- Tronick EZ, Frank DA, Cabral H, Mirochnick M, Zuckerman B. Late dose–response effects of prenatal cocaine exposure on newborn neurobehavioral performance. *Pediatrics* 1996;98:76–83.
- U.S. Census Bureau. Poverty Thresholds 2000, Current Population Survey, 2000. Available: <http://www.census.gov/hhes/poverty/poverty00/pv00thrs.html>. Accessed 3/5/2003.
- WHO. Biological monitoring of chemical exposures in the workplace. Geneva: World Health Organization; 1996.
- Whyatt RM, Rauh V, Barr DB, Camann DE, Andrews HF, Garfinkel R, et al. Prenatal insecticide exposures and birth weight and length among an urban minority cohort. *Environ Health Perspect* 2004;112:1125–32.
- Wilson NK, Chuang JC, Lyu C, Menton R, Morgan MK. Aggregate exposures of nine preschool children to persistent organic pollutants at day care and at home. *J Expo Anal Environ Epidemiol* 2003;13:187–202.
- Wittels B, Scott DT, Sinatra RS. Exogenous opioids in human breast milk and acute neonatal neurobehavior: a preliminary study. *Anesthesiology* 1990;73:864–9.