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Maternal pregnancy serum level of heptachlor epoxide, hexachlorobenzene, and β-hexachlorocyclohexane and risk of cryptorchidism in offspring

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Abstract

Prenatal exposure to environmental endocrine disrupters has been postulated to cause adverse effects on male reproductive health. Exposure to organochlorine pesticides with anti-androgenic and estrogenic potency has been shown to interfere with the sex-hormone dependent process of testicular descent in animal models. We examined the relation between serum levels of the pesticides heptachlor epoxide (HCE), hexachlorobenzene (HCB), and β-hexachlorocyclohexane (β-HCCH) in pregnant women, and the occurrence of cryptorchidism in their sons. These three pesticides were previously suggested as risk factors for cryptorchidism. In a nested case-control design, we compared serum levels between mothers of cases (n=219) and controls (n=564), selected from the Collaborative Perinatal Project, a US birth cohort study of pregnancies in 1959−1966. The offspring of mothers with HCE levels above the 90th percentile compared to those below the 10th percentile had an adjusted odds ratio of cryptorchidism of 1.2 (95% confidence interval 0.6−2.6); for β-HCCH the odds ratio was 1.6 (0.7−3.6). For HCB the adjusted odds ratio was near one. These results provide little support for an association of cryptorchidism with exposure to low levels of HCE or HCB. For β-HCCH the findings were somewhat suggestive of an association but were inconclusive.

Keywords

Cryptorchidism; organochlorine pesticides; environment; children; endocrine disruptors

INTRODUCTION

Cryptorchidism (undescended testes) is the most frequent congenital abnormality in newborn boys. In some countries, including the U.S., the prevalence has increased over the past 40 years (Paulozzi, 1999), and among fullterm male births worldwide ~ 1−5% are diagnosed with this condition (Boisen et al., 2004;John Radcliffe Hospital Cryptorchidism Study Group,

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1986;Pierik et al., 2005;Toppari and Kaleva, 1999). Some studies have related cryptorchidism with exposure to endocrine disruptors during fetal life (Sharpe, 2003;Sharpe and Skakkebæk, 1993). Normal male genital development is dependent on androgens, anti-Mullerian hormone, and insulin-like peptide hormone (INSL3) (Klonisch et al., 2004), and it was hypothesized that endocrine disrupters with anti-androgen and estrogen-like activity could result in cryptorchidism by interference with these hormones. Whereas some chemicals (i.e. pesticides, phthalates, and diethylstilbestrol (DES)) possessing estrogen or anti-androgen-like activity show the potential to cause cryptorchidism after fetal exposure in rodents (Emmen et al., 2000;Fisher et al., 2003;Fisher, 2004;Gray et al., 2001;McIntyre et al., 2001;Wilson et al., 2004), epidemiological data are limited in this respect.

Few reports have studied the association between cryptorchidism in humans and foetal exposure to specific environmental pesticides. Studies of the relationship between maternal serum levels of p-p'-DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene) during pregnancy and cryptorchidism in male offspring did not find a statistically significant elevated risk associated with this ubiquitous environmental anti-androgen, a metabolite of the banned pesticide DDT (Bhatia et al., 2005;Longnecker et al., 2002). A recent study reported on the relation between persistent pesticides in breast milk and cryptorchidism (n=62), and although the pesticide levels were higher in cases for 21 of the 27 pesticides, these differences were not statistically significant for any of the individual pesticides (Damgaard et al., 2006). The study by Hosie and co-workers (2000) (n = 48) reported a 2−3 times higher median concentration of heptachloroepoxide (HCE) and hexachlorobenzene (HCB) in fat tissue of cryptorchid boys compared with controls (Hosie et al., 2000). The β-hexachlorocyclohexane (β-HCCH) levels were also nearly twice as high in cases versus controls, but the difference was non-significant. HCE is a metabolite of the pesticide heptachlor, HCB is used as insecticide, plasticizer and rubber additive, and β-HCCH is a constituent of lindane, an insecticide. Although HCE, HCB and β-HCCH have been shown to affect estrogen and androgen metabolism after administration *in vitro* or to rodents (Alvarez et al., 2000;Oduma et al., 1995;Ralph et al., 2003;Schaefer et al., 2000;Shekhar et al., 1997), there is no support for an association with cryptorchidism from animal studies. Nonetheless, because of the suggestive human findings and the combination of both a high prevalence of cryptorchidism and exposure, additional data are needed.

We examined the relationship between HCB, HCE, and β-HCCH exposure and the occurrence of cryptorchidism in a relatively large prospective case-control study nested within a multi-center cohort. Although the increase in prevalence of cryptorchidism (Paulozzi, 1999) may have begun after these cases were diagnosed (1959−1966), they occurred when all three of these organochlorines were still in use.

MATERIALS AND METHODS

Study Subjects

The subjects were enrolled in the Collaborative Perinatal Project (CPP), a prospective study of neurologic disorders and other conditions in children (Niswander and Gordon, 1972). Pregnant women were recruited between 1959 to 1965 from 12 U.S. study centers (in Baltimore, Boston, Buffalo, Memphis, Minneapolis, New Orleans, New York [two], Philadelphia, Portland, Providence, and Richmond). Eleven study centers recruited patients from the participating University Hospital's prenatal clinic, and one study center (Buffalo) recruited patients from private practices. The subject selection method varied across study centers. Women were ineligible if they were incarcerated, if they were planning to leave the area or to give up the child up for adoption, or if they gave birth on the day they were recruited into the study. Records of the number of women who refused to participate at baseline were not kept, though, e.g., participation rates were greater than 99 percent at the Johns Hopkins center (Janet Hardy, personal communication, 2001). The characteristics of women in the

sample were, at registration, essentially the same as those in the sampling frame (Niswander and Gordon, 1972). Four percent of subjects who enrolled were lost to follow-up before delivery. Once enrolled, the mothers' nonfasting blood was collected approximately every eight weeks, at delivery, and six weeks postpartum. Sera were stored in glass at −20° C, with no recorded thaws. Approximately 42,000 women were enrolled, with 55,000 children born in the study. The children were systematically assessed for the presence of birth defects and other outcomes through age seven years. Follow-up to age seven was completed for about 75 percent of subjects born into the study.

We used a nested case-control design to study the association of maternal serum organochlorine levels with risk of cryptorchidism among sons (Longnecker et al., 2002). Eligibility criteria were: a) male, b) live born, c) singleton, and d) availability of a three ml aliquot of third trimester maternal serum. We considered subjects as having cryptorchidism if they were classified as having unilateral or bilateral undescended testicles at any time during the first year of life. Subjects first observed to have an undescended testicle(s) after the first year of life were not considered cryptorchid because they might have had retractile testes. Among the 28,444 boys in the CPP, 267 were not live born, 441 were not singletons, and for 5,389 no maternal blood was available.

Among the eligible 22,347 boys, there were 241 cases with cryptorchidism. From the eligible subjects we selected at random 618 controls. Other than restriction of the study to males, there were no other matching criteria.

Among those diagnosed as cryptorchid during the first year of life, the study records indicated that the testicles were descended at birth in 103. Because the cremasteric reflex (causes retraction of the testicles) is not well developed in the first year of life (John Radcliffe Hospital Cryptorchidism Study Group, 1986), we assumed that these 103 subjects had been misclassified as normal on their birth examination. We considered these subjects separately in a sensitivity analysis, described below, to evaluate this assumption. Among those with at least one undescended testicle at birth (n = 138), all but one also had a subsequent observation of the abnormality on at least one of the three subsequent physical examinations (four months, one year, and seven years of age), or orchidopexy.

Laboratory Assays

Serum levels of HCE, HCB, and β-HCCH, and other organochlorines were measured in 1997−1999 after solid-phase extraction, clean-up, and dual-column gas chromatography using electron capture detection (Brock et al., 1996). The proportion of HCE, HCB, and β-HCCH recovered by extraction averaged 72%, 64% and 73%, respectively. Results were presented without recovery adjustment (Longnecker et al., 2004). The limit of detection and percent of subjects with a level above the detection limit were: HCE, 0.21 μg/L, 81%; HCB, 0.08 μg/L, 78%; and β-HCCH, 0.23, 100%. Concentrations that were reported by the laboratory that were below the limit of detection were included in the analyses (Longnecker et al., 2002). Among the analytical batches that included a case, about 80% included at least one male control from the same center. 87 percent of batches contained an aliquot from a single large pool, used to calculate the between-assay coefficient of variation (CV). The between-assay CV was, for HCE, 19 percent at 0.77 μg/L (n = 290); for HCB, 58 percent at 0.18 μg/L (n = 294); and for β-HCCH, 19 percent at 1.46 μg/L (n = 298). The order of specimens within batch was determined by a random process. The levels of several other organochlorine pesticides, including oxychlordane, dieldrin, p,p'-DDE, and p,p'-DDT were also determined. The laboratory personnel were masked with respect to the type of sample. Serum levels of cholesterol and triglycerides were measured using standard enzymatic methods (Longnecker et al., 2002).

Statistical Methods

To divide subjects into categories based on exposure, we used five percentile-based cutpoints (10th, 25th, 50th, 75th, and 90th) for HCE and <beta>-HCCH. For hexachlorobenzene, more than 10 percent of all subjects had values below the detection limit, so for that compound we used four percentile-based cutpoints (25th, 50th, 75th, and 90th). We estimated the odds of having cryptorchidism in relation to organochlorine level using conditional logistic regression, conditional on study center (12 strata). Serum levels of these organochlorines are proportional to those of lipid, thus serum triglycerides and cholesterol were included as continuous variables in models where the organochlorine level was expressed as unit per volume of serum. In one set of models we expressed organochlorines on a per-unit-serum-lipid basis, and did not include the lipid levels as independent variables. To express organochlorines on a per-unit-serum-lipid basis, the lipid content of serum was estimated using the formula given by Phillips et al. (Phillips et al., 1989) and the level of total cholesterol and triglycerides in each sample.

Confounding was evaluated by comparing the coefficient(s) for a given organochlorine from models including lipids to the coefficient(s) in a model with an additional factor. The organochlorine levels were modeled as a continuous variable and as a categorical variable, and results from both types of models were used to evaluate the change in estimate. If the odds ratio (OR) per μg/L of organochlorine or the OR for the contrast of the highest-to-lowest exposure categories changed by 15% or more, the factor was considered a confounder. The factors considered as potentially confounding were race, season of birth, mother's age, parity, socioeconomic index, body mass index before pregnancy, weight gain during pregnancy, smoking during pregnancy, hyperemesis gravidarum, gestational hypertension, age at menarche, history of infertility, menstrual cycle irregularity, estrogen use during pregnancy, progesterone use during pregnancy, method of delivery, and p,p'-DDE. None of these factors met the criteria. We similarly considered the effect of adjustment for preterm birth, birth weight, small-for-gestational-age, and gestational age, even though these were potentially intermediate variables. Using a similar approach, but with cross-product terms, we evaluated effect modification by maternal age, race, smoking, prepregnancy body mass index, triglycerides, cholesterol, DDE level, gestational hypertension, socioeconomic index, and study center. Evaluation of effect modification by categorical variables with more than two categories was supplemented by comparing the model fit statistics for models with and without the cross-product terms. If the *p*-value associated with the interaction term or likelihood ratio test had a value of 0.10 or less, the degree of potential effect modification was further considered by examining tables stratified by the potentially modifying factor(s). All statistical analyses were conducted using the SAS statistical software package, Version 9.1 (SAS Institute Inc., Cary, NC).

For HCE, a laboratory result was not obtained for 91 of the potentially eligible subjects (27 cases, 64 controls), mainly because the measured value did not meet the quality control criteria for acceptance; for HCB and β-HCCH, the respective figures were 89 (25 cases, 64 controls), and 76 (22 cases and 54 controls). For all three compounds, the proportion of subjects with missing data or without an acceptable level was similar for cases and controls, e.g., for HCE these were 11% of cases and 10% of controls. Thus, in the final analysis, for the HCE analysis there were 214 boys with cryptorchidism and 554 controls; for HCB the respective numbers were 216 and 554; and for β-HCCH the numbers were 219 and 564.

RESULTS

Table 1 shows the general characteristics of the study population. Based on the controls, the majority was White (47%) or Black (48%), with the remainder of the population being predominantly Hispanic and Asian. The higher proportion of Whites among cryptorchidism cases was not statistically significant. The median age of mothers of cryptorchidism cases was

slightly greater than that of controls. The mothers were relatively young and the socioeconomic index of the subjects was just under that for the United States as a whole (median = 5.0 in the 1960s). The median birth weight and gestational age in controls were typical of healthy births. A previous paper has already described the study population in more detail (Longnecker et al., 2002).

The adjusted odds ratio for the highest category of HCB compared with the lowest was 1.24 (95% CI 0.59−2.64); for HCE the odds ratio was 1.06 (0.57−1.98); and for β-HCCH was 1.60 (0.72−3.55)(Table 2). For β-HCCH, those with levels between the 50^{th} and 90^{th} percentiles had odds ratios of about 2 that were statistically significant. The trend-test for a dose-response over the exposure categories did not show a dose-response between the organochlorine levels and the occurrence of cryptorchidism. The crude odds ratios were similar to the adjusted values (data not shown). The results obtained with the organochlorines expressed on a lipid-basis were not appreciably different than those shown, as were those obtained using recovery-adjusted levels of organochlorines. Further adjustment of results for factors that may be considered as intermediary (preterm birth, birth weight, small-for-gestational-age, or gestational age) had no appreciable effect on the results (not shown). The evaluation of potential effect modification revealed nothing remarkable (results not shown).

When similarly adjusted analyses were done for 4 other organochlorines, the odds ratios (high compared with low exposure) and trend test results were as follows: oxychlordane: OR 1.12, 95% CI 0.61−2.07, trend p = 0.87; dieldrin: OR 0.97, 95% CI 0.49−1.93, trend p = 0.97; p,p'-DDE: OR 1.33, 95% CI 0.70−2.50, trend p = 0.54; and p,p'-DDT: OR 1.27, 95% CI 0.66−2.41, trend p = 0.29.

We repeated the analyses with exclusion of cases who had a reported normal location of the testes at birth. The results were essentially the same at those in Table 2.

DISCUSSION

These results provide little support for an association of cryptorchidism with exposure to low levels of HCE or HCB. For β-HCCH the findings were somewhat suggestive of an association but were inconclusive. Hosie et al. (2000) reported that among the 20 compounds tested, statistically significant associations with cryptorchidism were found only with HCE and HCB. In that study, which was based on 18 cases, β-HCCH levels were about 2-fold greater among cases than controls. In a related recent study of cryptorchidism, higher breast milk levels of many organochlorines were found in cases (n=62) versus controls (n=68) (Damgaard et al., 2006), but the differences were not statistically significant. The latter study particularly focused on the 8 most abundant pesticides: HCE, HCB and β-HCCH, cis-chlordane, oxychlordane, dieldrin, p,p`-DDT and p,p-DDE, for which levels in cases were higher than in controls. Seven of these eight compounds were previously analyzed in the Collaborative Perinatal Project, and for 6 out of 7 (HCE, HCB, β-HCCH, oxychlordane, p,p'-DDT and p,p'-DDE) the odds ratios (high:low exposure categories) were above 1, but none were statistically significant. In other studies, an increased risk of cryptorchidism was found in offspring of parents who were occupationally exposed to pesticides (Pierik et al., 2004;Weidner et al., 1998). However, pesticide exposure was estimated based on job-title or self-report, and not confirmed by biomonitoring of pesticides or their metabolites in blood or urine samples.

To our knowledge the effect of β-HCCH on testicular descent in animal models has not been evaluated. β-HCCH, however, was found to have estrogenic activity in some studies (Zou and Matsumura, 2003), even at low levels of exposure (Ulrich et al., 2000). Estrogens have been shown to interfere with testicular descent in animal models (Nef et al., 2000). Therefore some

basis exists for a biologically plausible mechanism for the association between β-HCCH and cryptorchidism suggested in these data.

Strengths of the current study were the prospective design, and measurement of exposure during the relevant period of development. Laboratory personnel who performed the analyses of organochlorines had no access to the cryptorchidism status of the participating subjects. Compared with the 2 previous studies (Damgaard et al., 2006;Hosie et al., 2000), the median level of HCE and β-HCCH in the CPP was more than 15 times higher, and for HCB was about 40% higher (Hosie et al., 2000) or 400% higher (Damgaard et al., 2006). Use of lindane, which contains about 10% β-HCCH, has been banned in 52 countries, and agricultural use was recently banned in the U.S. (CDC, 2003). The primary use is in agriculture, as a treatment for seeds; much smaller quantities are used medicinally (CDC, 2003). Previous studies have shown that general population serum levels of β-HCCH, HCE, and HCB have been declining since the 1970s (CDC, 2003;Solomon and Weiss, 2002). The study was relatively large compared to previous studies, and provided enough statistical power to detect modest associations. Our single measurement of organchlorine levels provided a good estimate of exposure during pregnancy owing to the long half-lives of the compounds (ATSDR, 2005;Jung et al., 1997;Sala et al., 1999). While precise data on the latter are scant, for β-HCCH the half-life was reported to be 7 years (Jung et al., 1997), and for HCB, between 3 and 4 years (Sala et al., 1999). Data on other organochlorines suggest that levels track well over pregnancy (Longnecker et al., 1999).

Our study had some limitations. The high between-batch coefficient of variation (CV) for HCB means that our results for that compound may not be very informative. The CV was however determined in a pooled sample with a HCB level close to the assay detection limit, whereas the median level in our subjects was well above the LOD. Since the CV should be lower at higher analyte levels (Cantor et al., 2003), the CV in our study was probably lower than the reported 58%. If specifically the missing cryptorchidism cases had high maternal serum levels of HCB, HCE, and β-HCCH, we may have underestimated a positive association, but we have no reason to believe this would occur. Also, we cannot rule out residual confounding. In a study of stored breast milk specimens, organochlorine levels measured before and after 10 years of storage at −20°C showed no decline, which is consistent with the known extreme stability of these compounds (Lunden and Noren, 1998;Noren, 1988), suggesting degradation during storage may have had little effect on our results.

There may have been misclassification affecting the diagnosis of cryptorchidism. The independence of exposure and outcome assessment implies that misclassification would be nondifferential, so that it would result in a bias of the odds ratio toward unity. As indicated, of the cases who were diagnosed before the age of 1 year, the records stated that testes were descended at birth in 103/241. Because the cremasteric reflex is not well developed in the first year of life we do not consider these cases as having retractile testes. We assumed that these 103 cases were missed on their birth examination, which may have caused misclassification. However, an analysis in which these 103 subjects were excluded gave essentially the same results. Testicular ascendance or acquired cryptorchidism is the phenomenon in which a previously normally located testis spontaneously migrates to a non-scrotal position, and may be another explanation for the 103 cases that were normal at birth (Barthold and Gonzalez, 2003). However, acquired cryptorchidism (testicular ascent) will mostly be excluded from this study since they are typically identified in mid childhood (Barthold and Gonzalez, 2003;Rusnack et al., 2002). Since all boys in the Collaborative Perinatal Project were systematically examined for cryptorchidism during the first year of life, we expect to have captured most cases. The prevalence of 1.1% cryptorchidism in the Collaborative Perinatal Project is higher than the rate in 2 registry-based studies around the same time period (1970) in the US (Paulozzi, 1999), which is expected knowing that registries generally report lower

prevalences of congenital abnormalities since not all subjects are systematically examined (Pierik et al., 2005;Toppari et al., 2001). Another study in the US reported a cryptorchidism prevalence identical to that in the CPP in the first year of life, after systematic examination of a birth cohort (n=6935) in the late 1980s (Berkowitz et al., 1993). Finally, cryptorchidism is a phenotype comprising conditions that may have different etiologies (e.g., gonads that will eventually spontaneously descend, ectopic testes, inguinal or abdominal testes); treating these as one entity could make an etiologic factor affecting only one of these difficult to detect.

In summary, these data provide evidence that low levels of HCE and HCB do not increase risk of cryptorchidism, and raise the possibility that additional data on β-HCCH and the occurrence of cryptorchidism may be useful.

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Table 1

Characteristics of the subjects and their mothers, according to the subject's case-control status. Values are percentages or medians (and quartiles)

Characteristic	Cases (n=219)[a]	Controls (n=564)[a]
Race (%)		
White	57.5	47.0
Black	41.6	47.5
Other	0.9	5.5
Gestation (weeks)	39.0 (37.0, 41.0)	39.0 (38.0, 40.0)
Preterm birth (%)	16.6	14.1
Birth weight (grams)	3260.0 (2863.0, 3629.0)	3260.0 (2948.0, 3600.0)
Small for gestational age (%)	9.1	5.4
Maternal age (years)	24.0 (20.0, 30.0)	22.0 (20.0, 28.0)
Parity*		
None	26.5	31.3
1	22.8	22.4
2+	50.7	46.4
Socioeconomic index[b]	4.7 (3.3, 6.3)	4.7 (3.3, 6.0)
Prepregnancy BMI (kg/m^2)	22.2 (20.4, 25.0)	22.0 (20.0, 24.9)
Gestational hypertension (%)	5.9	6.4
Total cholesterol (ug/L)	232.0 (190.0, 273.0)	234.0 (196.0, 279.0)
Triglycerides (ug/L)	202.0 (161.0, 252.0)	204.0 (158.5, 259.5)
Study center (%)		
Boston	32.9	24.5
Buffalo	7.3	3.9
New Orleans	6.4	4.6
New York-Columbia	2.3	3.0
Baltimore	5.9	7.6
Richmond	7.3	6.2
Minneapolis	3.7	5.3
New York-Medical College	1.4	7.4
Portland	4.1	7.3
Philadelphia	15.1	17.7
Providence	10.5	5.3
Memphis	3.2	7.1
DDE (ug/L)	23.6 (15.9, 35.3)	24.3 (16.7, 37.2)
Heptachlor Epoxide (ug/L)	0.3 (0.2, 0.6)	0.4 (0.2, 0.7)
Hexachlorobenzene (ug/L)	0.3 (0.2, 0.4)	0.2 (0.1, 0.4)
B-Hexachlorocyclohexane (ug/L)	1.5 (1.1, 2.0)	1.4 (2.0, 2.0)

[a] The number of subjects given is for the B-HCCH analysis. In the HCE analysis there were 214 cases and 554 controls; in the HCB analysis there were 216 cases and 554 controls. The percentages and medians for those in the HCE and HCB analyses were nearly identical to those shown.

[b] The socioeconomic index calculated for subjects in the CPP was the mean of three percentile scores (for education, occupation, and family income), where education was that of the head of the household, occupation was that of the head of the household or the chief wage-earner, and the score used to calculate the percentile for an occupation was based on the percentiles of education and income among those with the same occupation.

Table 2

Adjusted odds ratios and 95% confidence intervals for cryptorchidism in relation to concentration of organochlorines in the mother's serum (ug/L). Male children in the Collaborative Prenatal Project, 1959−1966

Organochlorine (ug/L)	Exposure	No. of cases	No. of controls	Adjusted OR[a]	95% Confidence Interval		P value
Heptachlor epoxide	<0.13	20	52	1.00			
	0.13−0.23	36	81	1.01	0.58	1.78	
	0.24−0.36	59	142	1.03	0.60	1.77	
	0.37−0.67	51	134	0.98	0.56	1.70	
	0.68−1.22	25	91	0.82	0.41	1.65	
	>=1.23	23	54	1.24	0.59	2.64	
	Trend test[b]	214	554				0.56
Hexachlorobenzene	<0.11	45	136				
	0.11−0.23	42	134	0.93	0.61	1.43	
	0.24−0.37	76	139	1.14	0.77	1.70	
	0.38−0.58	32	87	0.85	0.52	1.40	
	>=0.59	21	58	1.06	0.57	1.98	
	Trend test[b]	216	554				0.96
Hexachlorocyclohexane	<0.75	14	65	1.00			
	0.75−0.98	31	84	1.37	0.73	2.60	
	0.99−1.38	60	143	1.68	0.93	3.03	
	1.39−1.96	62	129	2.01	1.10	3.67	
	1.97−3.40	36	80	2.08	1.08	4.01	
	>=3.41	16	63	1.60	0.72	3.55	
	Trend test[b]	219	564				0.35

[a] Results are from conditional logistic regression models (conditional on study center), with adjustment for triglycerides and cholesterol as continuous variables.

[b] Ordinal test across categories of exposure, based on the median value within each category.