Human Exposure to Endocrine-Disrupting Chemicals and Prenatal Risk Factors for Cryptorchidism and Hypospadias: A Nested Case–Control Study

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Background: Exposure to xenoestrogens during pregnancy may disturb the development and function of male sexual organs.

Objective: In this study we aimed to determine whether the combined effect of environmental estrogens measured as total effective xenoestrogen burden (TEXB) is a risk factor for male urogenital malformations.

Methods: In a case–control study, nested in a mother–child cohort (n = 702) established at Granada University Hospital, we compared 50 newborns with diagnosis of cryptorchidism and/or hypospadias with 114 boys without malformations matched by gestational age, date of birth, and parity. Controls did not differ from the total cohort in confounding variables. TEXB and levels of 16 organochlorine pesticides were measured in placenta tissues. Characteristics of parents, pregnancy, and birth were gathered by questionnaire. We used conditional and unconditional regression models to estimate odds ratios (ORs) and 95% confidence intervals (CIs).

Results: TEXB from organohalogenated compounds was detectable in 72% and 54% of case and control placentas, respectively. Compared with controls, cases had an OR for detectable versus nondetectable TEXB of 2.82 (95% CI, 1.10–7.24). More pesticides were detected in cases than in controls (9.34 ± 3.19 vs. 6.97 ± 3.93). ORs for cases with detectable levels of pesticides, after adjusting for potential confounders in the conditional regression analysis, were *o,p′*-DDT (OR = 2.25; 95% CI, 1.03–4.89), *p,p′*-DDT (OR = 2.63; 95% CI, 1.21–5.72), lindane (OR = 3.38; 95% CI, 1.36–8.38), mirex (OR = 2.85; 95% CI, 1.22–6.66), and endosulfan alpha (OR = 2.19; 95% CI, 0.99–4.82). Engagement of mothers in agriculture (OR = 3.47; 95% CI, 1.33–9.03), fathers' occupational exposure to xenoestrogens (OR = 2.98; 95% CI, 1.11–8.01), and history of previous stillbirths (OR = 4.20; 95% CI, 1.11–16.66) were also associated with risk of malformations.

Conclusions: We found an increased risk for male urogenital malformations related to the combined effect of environmental estrogens in placenta.

Key words: cryptorchidism, endocrine-disrupting chemicals, environmental estrogens, hypospadias, occupational exposure, risk factors. *Environ Health Perspect* 115(suppl 1):8–14 (2007). doi:10.1289/ehp.9351 available via *http://dx.doi.org/* [Online 8 June 2007]

Ten years ago, it was hypothesized that exposure of the developing male fetus to environmental estrogens may be responsible for anomalies of sexual maturation and reproductive function in adult life (Anonymous 1995). Male sexual differentiation and reproductive functioning are critically dependent on a balanced androgen:estrogen ratio. In this regard, two common male reproductive-tract malformations—cryptorchidism (failure of one or both testicles to descend into scrotum) and hypospadias (urethral opening on ventral side of penis)—are birth defects of prenatal origin that may be related to *in utero* exposure to estrogens/androgens.

Animal (Edwards et al. 2006) and human data (Nurminen 2001) point toward a causal relationship between exposure to pesticides during pregnancy and the development of congenital malformations. In fact, parental involvement in agricultural work and/or parental exposure to pesticides has been associated with higher risk of a wide range of congenital malformations (Kristensen et al. 1997). For example, in Spain, maternal involvement in agricultural activity during the month before conception and the first trimester of pregnancy was followed by a 3-fold increase in the risk of bearing a child with a malformation (Garcia et al. 1999). Moreover, an ecologic investigation into variations in orchidopexy rates in the Spanish province of Granada found an association between exposure to pesticides and the risk of cryptorchidism (García-Rodriguez et al. 1996). A later retrospective case–control study in the same geographic area suggested that cryptorchidism was related to the father's employment in agriculture (Rueda-Domingo et al. 2001).

Because of their persistence in the environment, pesticides are common contaminants in soil, water, and wildlife and are present in tissues of mothers and children, especially in regions devoted to intensive agriculture (Botella et al. 2004; Cerrillo et al. 2006; Olea et al. 1999). Their ubiquity supports the plausibility of embryo-fetal exposure during pregnancy, although mutagenic or epigenetic parental germ cell damage cannot be ruled out. Some organochlorine pesticides are endocrine disrupting chemicals (EDCs) (Soto et al. 1995), defined as exogenous substances or mixtures with the ability to disrupt hormonal homeostasis, alter endocrine system functions, and consequently cause adverse health effects in an intact organism or its progeny or subpopulations. EDC pesticides are now considered to include not only chemicals with estrogenic and androgenic properties but also those with antihormonal and enzymatic/metabolic properties (Almstrup et al. 2002).

Maternal exposure to pesticides has been associated with urogenital malformations, semen quality impairment, and testicular, prostate, ovarian, and breast cancer (Koifman et al. 2002). Thus, an excess of cryptorchidism but not of hypospadias was reported in sons of women working in farming, especially horticulture (Weidner et al. 1998), whereas the occupation of the father had no influence on the risk of either cryptorchidism or hypospadias. Kristensen and coworkers (1997), who studied the association of different birth defects with farm purchases of pesticides and tractor pesticide spraying equipment, found a positive relationship with cryptorchidism and a moderate association with hypospadias. However, when exposure assessment was based on parental occupation (farmers vs. other jobs), no significant differences were observed. Occupational exposure of the father was associated with cryptorchidism in a nested case–control study conducted by Pierik et al. (2004), and with hypospadias in an investigation by Irgens et al. (2000). In contrast with the above studies, other authors found no association between cryptorchidism and maternal exposure to pesticides during pregnancy

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(Restrepo et al. 1990), between hypospadias and occupational exposure to EDCs by the mother (Aho et al. 2000; Vrijheid et al. 2003), or between serum levels of dichlorodiphenyldichloroethene (DDE) in the third trimester of pregnancy and risk of malformations (Longnecker et al. 2002). These inconsistent results led to the conclusion that epidemiologic studies do not provide sufficient grounds to support a role for environmental estrogens in urogenital malformations, and that a more focused exposure assessment methodology is required, with more specific markers of embryo-fetal exposure (Dolk and Vrijheid 2003; Silva et al. 2002; Vidaeff and Sever 2005).

The need for data to support the endocrine disruptor hypothesis prompted the European Community to support a prospective multicenter cohort study in five European countries (Denmark, England, Finland, France, and Spain) to explore the possible association between exposure of the male fetus to endocrine disruptors and sex differentiation disorders. A prospective mother–child cohort was established in Granada (southern Spain), in which a case–control study was nested for investigation of the main risk factors for male urogenital malformations. In the present study, we examined the relationship between cryptorchidism or hypospadias and environmental factors, with special emphasis on exposure to xenoestrogens, estimated by assessment of the combined estrogenic effects of chemicals extracted from placentas.

Methods

Design and participants. From October 2000 to July 2002, we recruited male newborns registered at the San Cecilio University Hospital (one of the two reference public hospitals serving Granada province in southern Spain), excluding delivering mothers with serious chronic diseases, such as diabetes, hypertension, or thyroid disease, those who developed any pregnancy complication that could affect fetal growth or development, and nonresidents in the hospital referral area. A total cohort of 702 mother–son pairs was established. All boys with urogenital malformations (cryptorchidism and/or hypospadias) born in the study period were included. This study was approved by the Institutional Ethical Committee of the San Cecilio University Hospital, and all participating mothers signed informed consent.

All mothers were of Caucasian origin. All boys in the cohort were examined at birth (within 2 days), and those diagnosed with cryptorchidism and/or hypospadias were reexamined at 1 month of age. Only boys with congenital malformations at reexamination were considered cases. The examination technique and definition of cryptorchidism and hypospadias followed recommendations of a Danish–Finnish study (Boisen et al. 2004) developed by Scorer (1964). In brief, all examinations were done by two physicians in warm conditions (room temperature 20–24°C) with the child in supine position. Testicular position was recorded after manipulation of the testis to the most distal position along the pathway of normal descent using firm traction. Fifty boys were diagnosed with cryptorchidism and/or hypospadias; two of these were excluded from the investigation because their parents refused to participate. For each case, three controls were selected by gestational age (± 1 week), date of birth (± 7 days), and parity (primiparous/multiparous). Although the case–control ratio was 1:3, only 114 controls met the matching criteria. Cases were 27 boys born with undescended testes (19 unilaterally, 8 bilaterally) that persisted until 30 days of age, 19 boys born with hypospadias, and 2 boys born with both malformations. Among cryptorchidism, 17 were severe and 12 were mild cases, classifying nonpalpable inguinal and/or suprascrotal cases as severe and high scrotal cases as mild.

Information on potential confounding variables related to parents, pregnancy and delivery, and activities with potential for pesticide exposure were gathered from structured face-to-face interviews with the mother held within the first 48 hr after delivery. Both mother and interviewer were blinded to the case or control status of the child.

Occupational exposure was derived from questions on paid employment and jobs focusing on chemicals with possible endocrine activity or previously described as male reproductive toxicants (van Tongeren et al. 2002). Questionnaires were completed for 47 (96%) cases and 105 (92%) controls.

Preliminary statistical analysis demonstrated similar characteristics between boys in the mother–child cohort and those selected as controls in the case–control study, except for a small difference in father's age (cohort, 33.6 ± 5.7 years vs. controls, 31.8 ± 5.3 years; p = 0.01) and in gestational age in days (cohort, 276.6 ± 9.2 days vs. controls, 272.6 ± 11.6 days; p = 0.002), although not when expressed in weeks (39.5 ± 1.3 weeks vs. 38.9 ± 1.6 weeks, p = 0.9).

Laboratory analyses. Samples of placenta without deciduas basalis and chorionic plate were collected at the time of delivery from 36 (75%) cases and 109 (95.6%) controls and sent to the Laboratory of Medical Investigation for analysis. They were immediately frozen at –70°C and stored. Before analysis, the placenta was defrosted and mechanically homogenized. The laboratory was blinded to the case–control status of samples. Bioaccumulated compounds were extracted from samples by a previously described method (Fernandez et al. 2004) with slight modifications. Briefly, 1.6 g placenta homogenate were dissolved in hexane and eluted in a glass column filled with Alumine Merck 90 (70–230 mesh no. 1097; Merck, Darmstadt, Germany) that had been dried at 600°C for 4 hr, followed by the addition of 5% water. The eluate obtained was concentrated at reduced pressure under nitrogen stream to a volume of 800 µL and then injected (4 × 200 µL) into the preparative high-pressure liquid chromatography (HPLC) (Waters model 501 Millipore apparatus with ultraviolet/visible detector model 490; Millipore, Marlborough, MA, USA). One microliter of HPLC chromatography fraction was dried, dissolved in *n*-hexane, and then injected into a gas chromatography apparatus.

The presence of aldrin, dieldrin, endrin, lindane, methoxychlor, endosulphan I and II, mirex, *o,p′*-DDT (dichlorodiphenyltrichloroethane), *p,p′*-DDT, *o,p′*-DDD (dichlorodiphenyldichloroethane), *p,p′*-DDE, endosulfan diol, sulfate, lactone, and ether was determined by gas chromatography with electron-capture detection, using *p′*-dichlorobenzophenone as internal standard, and mass spectrometry. Standard solutions of organochlorine compounds were analyzed previously to determine retention times and calibration curves of these chemicals. The calibration linearity of all chemicals in pure and processed standards was > 0.98. The recovery of studied chemicals, measured by spiking placenta samples with pure chemicals, ranged from 84 to 102%. The operational quality control and limits of detection and quantification were previously reported (Lopez-Espinosa et al. 2006; Moreno-Frias et al. 2001). The limit of detection (LOD) ranged from 0.1 to 3 ng/mL. The reproducibility of the process was established by running 10 placenta samples 10 times. The gas chromatography apparatus (Varian-3350; Varian Inc., Walnut Creek, CA, USA) with Millennium Chromatography Manager software was equipped with a methyl silicone column (length 30 m) and dual Ni-63 electron capture detectors. The gas chromatography-mass spectrometry apparatus (Saturn 2000; Varian Inc.) with automatic injector was equipped with a DB5-MS capillary column (30 m × 0.25 mm). Results are presented with recovery correction.

We estimated the total effective xenoestrogen burden (TEXB) to measure the exposure to xenoestrogens in placenta extracts. Previously, an HPLC method was developed to separate natural estrogens (β fraction) from more lipophilic xenoestrogens (α fraction) without destroying either (Fernandez et al. 2004; Soto et al. 1997). Extensive testing demonstrated that the pesticides DDT and metabolites and dieldrin, aldrin, and lindane, among other organochlorines, all elute in the HPLC α-fraction, along with other chlorinated and/or brominated organohalogenated

chemicals. The estrogenicity of the α-fraction, which contains no endogenous sex-hormones, can be considered a marker of the TEXB of environmental organohalogenated estrogens (Ibarluzea et al. 2004). The combined estrogenic effect is analyzed from its proliferative effect on MCF-7 human breast cancer cells and expressed as estradiol equivalent units. Duplicated dry pooled α and β fractions (eluted from 0 to 11 min and from 13 to 30 min, respectively) were resuspended in charcoal-dextran serum and tested in the E-SCREEN bioassay for estrogenicity using a slight modification of the originally described technique (Soto et al. 1995). Each sample was assayed in triplicate with a negative (vehicle) and positive (estradiol) control in each plate. The proliferative effect of the fractions was referred to the maximal effect obtained with estradiol transformed into estradiol equivalent units (Eeq), by reading from a dose–response curve prepared using estradiol (concentration range 0.1 pM to 10 nM), and was expressed as total effective xenoestrogen burden (TEXB-α and TEXB-β) in Eeq per gram of tissue and per gram of lipid (Ibarluzea et al. 2004).

Statistical analysis. We performed statistical analysis to determine associations between predictors of interest and the presence of cryptorchidism and/or hypospadias and between exposure to environmental pesticides and these urogenital malformations. Descriptive statistics are reported as arithmetic mean ± SD. All statistical analyses were performed using SPSS statistical software for Windows version 11.0 (SPSS Inc., Chicago, IL, USA). We computed odds ratios (ORs) for malformation and 95% confidence interval (CI) by unconditional and conditional logistic regression. We adjusted for potential confounders and matching variables. Potential confounding variables were selected on the basis of their significant association with outcomes in the univariate models. In addition, exposure variables of interest from the literature were included. The modifying effect of these variables and their association with organochlorine levels and TEXB values were investigated. In the final adjusted model, only maternal age and newborn birth weight had substantial effect on results. We also tested interactions of all variables for significance. Differences between groups were tested with Pearson´s chi-square test or Fisher´s exact test, when appropriate. Pesticide levels and TEXB were also analyzed using an analysis of variance test.

Results

There was a 1.8% prevalence of cryptorchidism plus hypospadias in our cohort. Considered separately, the prevalence of cryptorchidism at 1 month of age was 1.1%, and the prevalence of hypospadias at delivery was 0.74%.

Mean age (± SD) of the mothers was 29 ± 4.96 years, 9.2% of the mothers were illiterate, and 14.5% had university studies. More than half of the women lived in rural areas (53.9%), but only 13.2% stated work in agriculture. One of four of the women were overweight or obese before pregnancy, according to World Health Organization criteria (World Health Organization 1998) [body mass index (BMI) > 25]. Table 1 shows the relationships between selected characteristics and urogenital malformations. After testing a large number of more complex stratifications, we categorized maternal occupation as agricultural or other. An almost 3.5-fold increase in risk for urogenital

Table 1. Selected characteristics of parents, pregnancy, delivery, and infants in relation to urogenital malformations according to case/control status of newborn.

Variable	Cases [*n* (%)]	Controls [*n* (%)]	*p*-Value	OR[a] (95% CI)
Place of residence[b]				
Urban	9 (29.0)	22 (71.0)	0.75	1
Rural	39 (32.0)	87 (68.0)		0.91 (0.48–2.72)
Mother´s occupation				
Other	37 (26.1)	105 (73.9)	0.01	1
Agriculture	11 (55.0)	9 (47.9)		3.47 (1.33–9.03)
Father's occupation exposure[c]				
Low	7 (20.6)	27 (79.4)	0.03	1
Medium	15 (25.4)	44 (74.6)		0.76 (0.47–3.64)
High	24 (43.6)	31 (56.4)		2.98 (1.11–8.01)
Mother's age (years)[d]				
≤ 30	33 (37.1)	56 (62.9)	0.09	1
> 30	15 (24.2)	47 (75.8)		0.54 (0.26–1.06)
Father´s age (years)[d]				
≤ 31	29 (35.8)	52 (64.2)	0.18	1
> 31	18 (25.7)	52 (74.3)		1.61 (0.79–3.25)
BMI before pregnancy[d]				
≤ 23	19 (25.3)	56 (74.7)	0.11	1
> 23	28 (37.3)	47 (62.7)		1.57 (0.28–1.14)
Previous pregnancy				
Yes	18 (29.5)	43 (70.5)	0.75	1
No	29 (31.9)	62 (68.1)		1.12 (0.56–2.26)
Previous stillbirths				
No	31 (26.3)	87 (73.7)	0.02	1
Yes	6 (60.0)	4 (40.0)		4.20 (1.11–16.66)
Problems during pregnancy				
No	23 (30.3)	53 (69.7)	0.83	1
Yes	19 (28.8)	47 (71.2)		0.98 (0.46–1.89)
Hyperemesis gravidarium				
No	19 (27.9)	49 (72.1)	0.64	1
Yes	23 (31.1)	51 (68.9)		1.22 (0.58–2.59)
Bleeding during pregnancy				
No	7 (35.0)	13 (65.0)	0.97	1
Yes	35 (28.7)	87 (71.3)		1.21 (0.54–2.70)
Pregnancy weight gain (kg)[d]				
< 12	26 (36.6)	45 (63.4)	0.07	1
≥ 12	16 (22.9)	54 (77.1)		0.56 (0.26–1.06)
Oral contraceptive before pregnancy				
No	31 (32.6)	64 (67.4)	0.56	1
Yes	16 (28.1)	41 (71.9)		0.81 (0.36–1.65)
Prenatal smoking				
Nonsmoker	36 (34.0)	70 (66.0)	0.22	1
Smoker	11 (23.9)	35 (76.1)		0.61 (0.28–1.34)
Method of delivery				
Spontaneous	22 (25.9)	63 (74.1)	0.14	1
Instrumental	2 (12.5)	14 (87.5)		0.51 (0.20–1.26)
Cesarean	11 (40.7)	16 (59.3)		2.44 (0.51–11.62)
Fetal presentation				
Normal (cephalic)	32 (27.8)	83 (72.2)	0.88	1
Abnormal (breech/transverse)	3 (30.0)	7 (70.0)		0.90 (0.22–3.69)
Gestational age (weeks)				
≥ 37	35 (26.7)	96 (73.3)	0.10	1
< 37	13 (41.9)	18 (58.1)		1.98 (0.88–4.46)
Birth weight (g)				
≥ 2,500	43 (28.3)	109 (71.7)	0.14	1
< 2,500	5 (50.0)	5 (50.0)		2.54 (0.69–9.17)
Season of birth				
Spring	11 (23.4)	36 (76.6)		1
Summer	6 (23.1)	20 (76.9)	0.08	1.12 (0.00–8.08)
Autumn	12 (30.8)	27 (69.2)		1.11 (0.98–3.41)
Winter	14 (35.0)	26 (65.0)		1.28 (1.19–9.12)
Length at birth (cm)[d]				
< 50.9	28 (41.8)	39 (58.2)	0.04	1
≥ 50.9	15 (25.0)	45 (75.0)		0.46 (0.22–0.99)
Head circumference (cm)[d]				
≤ 34.6	26 (36.1)	46 (63.9)	0.54	1
> 34.6	17 (30.9)	38 (69.1)		1.26 (0.59–2.66)
Minor incidences[e]				
No	21 (43.8)	46 (29.8)	0.08	1
Yes	27 (56.3)	38 (70.2)		1.83 (0.95–3.67)

[a]Unadjusted; an OR of 1 denotes the reference category. [b]Urban, > 10,000 inhabitants; rural, ≤ 10,000 inhabitants. [c]We derived occupational exposure from generic questions on paid employment and jobs focusing on chemicals that may have endocrine activity or that have previously been described as male reproductive toxicants (van Tongeren et al. 2002). [d]Mother's age, father´s age, BMI [weight (kg)/height (m)^2], pregnancy weight gain (kg), length, and head circumference at birth were categorized below and above the median value of the case–control study. [e]Epispadia, phymosis, hydrocele, and/or micropenis.

malformation was observed when the mother reported taking part in agricultural activities. In contrast, when the father's work was categorized as agricultural or other, no association was found. However, when a different approach was taken and fathers were asked about specific work tasks and chemical exposures, a 2.98-fold increased risk of urogenital malformation was found for the highest versus lowest occupational exposure level. Effects of an urban or rural setting on mother–child exposure were examined by categorizing their place of residence as having a population of more (urban) or less (rural) than 10,000 inhabitants, but no association was found.

A history of spontaneous abortion was associated with an increased risk of cryptorchidism and/or hypospadias (OR= 4.20; 95% CI, 1.11–16.66). Other maternal characteristics that were studied but showed no association with cryptorchidism or hypospadias were problems during pregnancy (i.e., hyperemesis, episodes of bleeding, and loss of amniotic fluid), use of oral contraceptive before pregnancy, and smoking habit (Table 1). No association was seen with maternal weight, although when BMI was categorized below and above the mean value of the study population (23 kg/m^2), a higher BMI was borderline significantly associated with risk of cryptorchidism and/or hypospadias in the conditional regression analysis adjusted for maternal age and birth weight [adjusted OR (AOR) = 1.89; 95% CI, 0.84–4.26].

A weight gain of ≥ 12 kg during pregnancy appeared to have a protective effect (OR = 0.56; 95% CI, 0.26–1.06). Interestingly, mothers of cases gained significantly ($p = 0.07$) less weight during pregnancy (11.3 ± 4.7 kg) compared with mothers of controls (13.9 ± 5.5 kg) (Table 1).

Effects on risk of urogenital anomaly of type of delivery, fetal presentation, weeks of gestation, child weight and length, head size, presence of other malformation, and season of birth are also shown in Table 1. The risk increased with decreasing birth length ($p < 0.05$) but not with decreasing birth weight (OR = 2.54; 95% CI, 0.69–9.17), although the latter was close to statistical significance ($p = 0.1$). Interestingly, the mean weight of cases (3110.63 ± 614.67 g) was significantly ($p = 0.03$) lower than the mean weight of controls (3304.39 ± 449.13 g).

Weeks of gestation, with category boundary established at 37 weeks, was associated with risk of malformation, with a more frequent presence of cryptorchidism and/or hypospadias in the group of lower gestational age (OR = 1.98; 95% CI, 0.88–4.46), and this risk was higher in the conditional logistic analysis [crude OR (COR) = 4.18; 95% CI, 0.75–23.34; AOR = 2.29; 95% CI, 0.38–13.74]. Delivery by cesarean was more frequent in the cryptorchidism/hypospadias group (OR = 2.44; 95% CI, 0.51–11.62).

The season of birth had an effect in our study population. Taking spring as category reference, more boys with malformations were born in another season, especially in winter (COR = 1.28; 95% CI, 1.19–9.12), although without statistical significance in the conditional logistic analysis adjusted for maternal age and birth weight (AOR = 5.42; 95% CI, 0.56–15.42). Finally, the risk of cryptorchidism and/or hypospadias was associated with presence of minor disorders of the urogenital tract such as phymosis, hydrocele, epispadia, and/or micropenis (OR = 1.83; 95% CI, 0.91–3.67).

All placentas studied were positive for at least one chemical (mean residue number 7.6 ± 3.9). Detectable concentrations of *p,p′*-DDE were found in 84.8% of placenta samples, with a mean value of 9.21 ng/g of placenta. Lindane and endosulfan-α, and ether followed DDE in frequency of presence and were detected in 61.4, 52.4, and 53.1%, respectively, although at lower concentrations (Table 2). A higher number of pesticides were detected in cases than in controls (9.3 ± 3.2 vs. 6.9 ± 3.1, respectively; $p = 0.002$), but there were no statistically significant differences between cases and controls in the mean concentration of any pesticide except for dieldrin (1.19 vs. 0.33 ng/g placenta, $p < 0.05$) and lindane (0.72 vs. 0.36 ng/g placenta, $p = 0.007$). Residues of *o,p′*-DDT, *p,p′*-DDT, lindane, endosulfan-α, and mirex were more frequently detected in cases than in controls, and the unconditional regression analysis associated malformation risk with the presence of these same chemicals, as follows: *o,p′*-DDT (OR = 2.25; 95% CI, 1.03–4.89); *p,p′*-DDT (OR = 2.63; 95% CI, 1.21–5.72), lindane (OR = 3.38; 95% CI, 1.36–8.38), mirex (OR = 2.85; 95% CI, 1.22–6.66), and endosulfan-α (OR = 2.19; 95% CI, 0.99–4.82). Table 3 lists these findings and results of the conditional regression analysis with crude and adjusted ORs for malformations in relation to the presence of these five selected pesticide residues.

Seventy-two percent of placentas from cases and 54% of those from controls were positive for estrogenicity of the α fraction. Mean values of estrogenicity, measured as the TEXB of the α fraction, were 2.92 Eeq pM/g of placenta for cases versus 1.45 Eeq pM/g of placenta for controls. In the unconditional regression analysis, the TEXB of the α fraction was borderline associated with the risk of malformation, with an OR of 2.02 (95% CI,

Table 2. Concentrations of organochlorine pesticides (ng/g of lipid) in placenta samples of cases and controls.

	Cases ($n = 48$)						Controls ($n = 114$)					
	No.	Mean ± SD	25th	50th	75th	Range	No.	Mean ± SD	25th	50th	75th	Range
o,p′-DDD	19	28.7 ± 43.7	1.0	14.1	52.4	1.0–169.6	44	64.9 ± 158.3	1.0	11.6	62.4	1.0–997.0
p,p′-DDE	31	10.8 ± 28.0	2.6	4.0	8.9	0.5–158.1	92	8.7 ± 16.0	1.8	3.5	7.7	0.5–97.7
o,p′-DDT	23	3.6 ± 8.3	1.0	1.1	1.9	1.0–38.3	48	5.8 ± 10.7	1.0	1.5	4.8	1.0–40.5
p,p′-DDT	18	2.0 ± 5.2	1.0	1.0	1.0	1.0–22.2	30	9.1 ± 25.1	1.0	1.0	2.7	1.0–127.0
ΣDDTs	35	28.7 ± 47.3	4.4	10.2	29.6	1.0–206.9	104	39.9 ± 110.2	2.9	8.0	34.9	1.0–1025.1
E ether	20	0.2 ± 0.2	0.1	0.2	0.4	0.1–1.01	57	0.3 ± 0.3	0.1	0.2	0.4	0.1–1.0
E lactone	15	19.7 ± 15.6	1.2	21.5	28.4	0.1–55.3	33	23.7 ± 22.1	1.0	19.2	34.1	0.1–74.7
E diol	16	5.3 ± 4.4	1.5	4.1	8.8	0.5–13.8	55	8.1 ± 9.8	2.5	4.2	10.7	0.5–45.6
E sulf	26	5.6 ± 7.0	1.0	2.2	6.5	0.5–21.9	80	3.6 ± 6.0	1.2	2.0	3.5	0.5–44.6
E I	24	3.4 ± 6.1	0.5	0.5	4.9	0.5–28.3	52	4.9 ± 6.6	0.5	2.4	6.15	0.5–26.7
E II	22	4.1 ± 10.4	2.0	2.0	2.0	2.0–46.2	49	2.5 ± 2.7	2.0	2.0	2.2	2.0–10.7
ΣE	36	20.8 ± 25.0	2.7	8.0	36.3	0.14–103	103	19.7 ± 29.7	3.0	7.0	26.4	0.5–189.5
Aldrin	18	0.63 ± 0.2	1.0	1.0	1.2	1.0–1.0	39	1.9 ± 7.14	1.0	1.0	1.1	1.0–45.2
Dieldrin	11	3.8 ± 4.2	1.0	2.2	4.8	1.0–12.5	19	1.9 ± 2.5	1.0	1.0	1.8	1.0–9.6
Endrin	22	5.0 ± 4.8	3.0	3.8	6.5	3.0–19.7	48	7.4 ± 11.9	2.2	3.5	7.2	3.0–67.0
Lindane	29	0.9 ± 0.8	0.5	0.7	1.0	0.5–3.2	60	0.7 ± 1.0	0.2	0.3	1.0	0.5–6.87
M-chlor	19	1.2 ± 1.1	1.0	1.0	1.7	1.0–4.7	39	2.3 ± 6.2	1.0	1.2	1.4	1.0–34.5
Mirex	13	1.4 ± 1.1	0.1	0.1	2.5	1.0–3.0	18	3.7 ± 3.7	1.0	2.8	4.5	1.0–15.1
Chlordane	4	276 ± 355	41	139.8	511.5	3.0–791.9	11	138.4 ± 112.3	63	92.2	165.9	3.0–383.9
HCB	22	6.9 ± 11.6	1	2.7	4.3	0.5–41.6	55	9.49 ± 12.7	2.0	4.2	10.6	0.5–60.5

Abbreviations: E, endosulfan; HBC, hexachlorobenzene; M-chlor, methoxychlor. 25th, 50th, and 75th are percentiles.

0.84–4.80) that increased to an OR of 2.82 (95% CI, 1.10–7.24) in the conditional regression analysis adjusted for maternal age and birth weight (Table 3). The TEXB of the β fractions was more frequently positive and higher in cases versus controls but without reaching statistical significance. Neither the TEXB-α nor -β fraction showed association with any of the 16 pesticides quantified. The relationship between the estrogenicity of α fractions (TEXB-α) and the variables most frequently related to malformations was investigated. The variables associated with TEXB-α levels were maternal age, educational level, BMI before pregnancy, and parent occupation. All placentas from mothers working in agriculture were positive (≥ LOD).

When variables were simultaneously examined in a multivariate regression model, the main predictors of urogenital malformations were pregnancy weight gain, dieldrin levels, and presence of mirex or lindane (Table 4).

Discussion

We used a novel method to measure exposure of the embryo-fetus to xenoestrogens by estimating the estrogenicity resulting from the combined effect of chemicals extracted from placentas (TEXB measurement) (Fernandez et al. 2004; Ibarluzea et al. 2004). Three of four placentas from boys with cryptorchidism and/or hypospadias and one of two placentas from control boys had a measurable level of estrogenicity due to xenoestrogens (TEXB of the α fraction). We detected a statistically significant relationship between the risk of malformation and xenoestrogen exposure in the study population. Therefore, we are able to report the first demonstration of a significant relationship between male urogenital malformation and the estrogenicity of the α-fraction—the estrogenicity due to bioaccumulated organohalogenated xenoestrogens. Compared with controls, cases had an OR for detectable versus nondetectable TEXB-α of 2.82 (95% CI, 1.10–7.24).

These results support the environmental working hypothesis for male sexual differentiation in humans. This process is known to depend on a balanced steroid ratio and is therefore highly sensitive to disruption by exogenous estrogens; exposure to EDCs would provoke an unbalanced androgen/estrogen ratio, leading to an inadequate maturation of Sertoli and Leydig cells (de Kretser 2003; Skakkebaek et al. 2001). Disagreements in the scientific community about adverse trends in male reproductive health may be partly related to difficulties in comparing studies across time, to the selection of study populations, and to differences in clinical definitions and diagnostic procedures for these diseases. Moreover, epidemiologists traditionally analyze the incidence and risk factors separately for each disorder. Skakkebaek and co-workers (2001) proposed that sperm counts, demand for assisted reproduction, testicular cancer, hypospadias, and undescended testes are all symptoms of a single underlying entity, the so-called testicular dysgenesis syndrome. They concluded that environment and lifestyle factors are among the most likely causes, with minor participation of the genomic background.

Following proposals of separate genetic but common environmental components of risk for cryptorchidism and hypospadias (Akre et al. 1999; Weidner et al. 1999), cryptorchidism and hypospadias were considered comparable entities in terms of their etiology, grouping together boys with one or both malformations in the statistical analysis. The prevalence of cryptorchidism and/or hypospadias was 1.8% in our series, within the range reported by studies in other geographic areas (Boisen et al. 2004, 2005; Paulozzi 1999; Preiksa et al. 2005; Toppari et al. 2001). When the two malformations were considered separately, the prevalence of cryptorchidism at 1 month of age was 1.1%, and the prevalence of hypospadias at delivery was 0.74%. The cohort study conducted by Preiksa et al. (2005) detected a lower frequency of cryptorchidism at birth in Lithuanian boys (5.7%) in comparison with Danes (9.0%) and a higher frequency in comparison with Finns (2.4%). Highly variable prevalences of cryptorchidism have been reported in Western countries on the basis of registry studies (Boisen et al. 2004; Paulozzi 1999; Toppari et al. 2001). However, comparisons in the incidence rate of urogenital malformations among countries may be limited by differences in study populations (registry versus cohort studies), case definitions, and examination techniques. The Spanish Collaborative Study of Congenital Malformations (ECEMC) reported (Martínez-Frias et al. 2004) that the frequency of hypospadias in Spain was 1 of 284 male births (0.35%) and had remained at this level for the past few decades until after 1996, when a decreasing frequency of severe forms was recorded. The authors suggested that this effect was probably caused by a radical change in exposure that affected the whole country. Surprisingly, this change was not observed in the prevalence of any other congenital malformations.

All placentas had measurable concentrations of at least one of the 16 organochlorine pesticides quantified, reflecting the ubiquity of exposure in the population, although the number of quantifiable residues was significantly higher in cases than in controls. No single chemical could be positively and significantly associated with the biologic

Table 3. Crude and adjusted ORs (95% CIs) for urogenital malformations among male offspring in relation to the presence in placenta samples of specific endocrine disruptors and the TEXB, according to the case/control status of newborn.

Variable	Cases [*n* (%)]	Controls [*n* (%)]	*p*-Value	COR (95% CI)	AOR[a] (95% CI)
o,p′-DDT					
< LOD	12 (16.7)	60 (83.3)	0.047	1	1
≥ LOD	23 (33.3)	46 (66.7)		2.25 (1.03–4.89)	2.17 (0.96–5.00)
p,p′-DDT					
< LOD	17 (18.1)	77 (81.9)	0.017	1	1
≥ LOD	18 (38.3)	29 (61.7)		2.63 (1.21–5.72)	2.17 (0.95–5.00)
Endosulfan I					
< LOD	11 (16.4)	56 (83.6)	0.025	1	1
≥ LOD	24 (32.4)	50 (67.6)		2.19 (0.99–4.82)	2.49 (0.99–6.24)
Lindane					
< LOD	6 (11.1)	48 (88.9)	0.002	1	1
≥ LOD	29 (33.3)	58 (66.7)		3.38 (1.36–8.38)	9.48 (2.43–36.96)
Mirex					
< LOD	23 (20.7)	88 (79.3)	0.023	1	1
≥ LOD	12 (40.0)	18 (60.0)		2.85 (1.22–6.66)	3.42 (1.19–9.77)
TEXB-α fraction					
< LOD	10 (18.2)	45 (81.8)	0.031	1	1
≥ LOD	25 (30.9)	56 (69.1)		2.02 (0.84–4.80)	2.82 (1.10–7.24)
TEXB-β fraction					
< LOD	11 (19.6)	45 (80.4)	0.069	1	1
≥ LOD	24 (30.0)	56 (70.0)		1.75 (0.75–1.00)	2.31 (0.94–5.70)

Results were obtained from conditional logistic regression models.
[a]Adjusted for mother's age at delivery and infant weight at birth.

Table 4. Predictors of urogenital malformations in multivariate analysis.

Variables	Beta	*p*-Value	OR
Lindane			
< LOD			1
≥ LOD	1.011	0.046	2.748
Dieldrin	0.179	0.079	1.196
Mirex	1.176	0.016	
< LOD			1
≥ LOD			3.243
Pregnancy weight gain (kg)	–0.096	0.027	0.908
Constant	–0.896	0.178	0.408

All variables were simultaneously entered into the model for prediction of urogenital malformations.

effect measured by TEXB-α. There may be several reasons for this lack of concordance. Thus, the estrogenic effect measured in the E-SCREEN bioassay is a consequence of the combined effect of measured organohalogens; and the estrogenic effect may be caused by other chemicals not quantified, either other organochlorine pesticides or other lipophilic compounds.

Over the past three decades, published research into the presence of organochlorine pesticides in placenta tissue has been limited. The highest concentrations detected in placentas in our study were those of DDT, isomers, and metabolites, followed by endosulfans, with *p,p′*-DDE showing both highest mean concentration and frequency. These findings are far below the mean values reported in placenta tissue of women in a neighboring region of southeast Spain (Falcon et al. 2004). In the latter series, recruited from 1998 to 2000, the mean total DDT value found was similar to findings in placenta in a Romanian population in 1996–1997 (Hura et al. 1999). The present *p,p′*-DDE results are similar to those recently reported in placentas collected from 1997 to 2001 in Finland (Shen et al. 2005). In our view, caution must be taken in comparing concentrations of lipophilic endocrine disruptor chemicals in adipose tissue with data derived from lipid-adjusted concentrations in blood/serum or placenta.

Nevertheless, our results showed an excess risk of malformation associated with the presence of five pesticides (*o,p′*-DDT, *p,p′*-DDT, endosulfan-α, lindane, and mirex). The ORs were higher than unity, reaching 9.48 (95% CI, 2.43–36.96) for lindane in the conditional regression analysis adjusted for maternal age and birth weight. In the multivariate regression model, presence of mirex or lindane was a predictor of urogenital malformations.

A significant association was observed between maternal involvement in agriculture activities and an increased frequency of congenital malformations in the offspring, although agricultural employment by fathers was not associated with a significant increase in risk. Other authors have reported a higher risk of congenital anomalies at birth according to occupation (Dolk et al. 1998; Garcia et al. 1999; Hosie et al. 2000; Irgens et al. 2000; Kristensen et al. 1997; Pierik et al. 2004; Vrijheid et al. 2003; Weidner et al. 1998). In fact, pesticide exposure is likely in agricultural work even when direct handling of chemicals is not reported (Garcia et al. 1999). Thus, entry into a greenhouse after spraying can be a major source of exposure, as can be the use of fertilizers and crop-preserving chemicals. Moreover, factors related to residence in an agricultural setting may be an important source of inadvertent exposure to pesticides (Olea et al. 1999).

No effect of parental age on urogenital malformations was observed, in agreement with most (Preiksa et al. 2005; Weidner et al. 1999) but not all (Fisch et al. 2001) of the previous studies. An increased risk of these malformations was, however, significantly associated with lower birth weight, as previously documented (Berkowitz et al. 2003; Weidner et al. 1999), and also appeared to be related to low gestational age although significance was not reached, unlike in some other reports (Berkowitz et al. 2004; Preiksa et al. 2005). In our region, Lopez-Espinosa et al. (2006) found that a higher number of organochlorine pesticide residues in placenta was associated with lower birth weight, confirming these observations.

A 4-fold increased risk of malformation in the sons of women with a history of stillbirth was noted (OR = 4.2; 95% CI, 1.11–16.66), confirming previous observations (Weidner et al. 1999). Although cryptorchidism and hypospadias have frequently been described in associated congenital malformations (Mayr et al. 1999), only minor concomitant diseases of the urogenital tract were observed in our series, confirming findings by other authors (Biggs et al. 2002; Preiksa et al. 2005).

Other variables had less or no influence on the risk in our study population. An increase in malformation has been associated with cesarean delivery (Aschim et al. 2004; Rueda-Domingo et al. 2001), but no significant association was observed in the present study (OR = 2.44; 95% CI, 0.51–11.62).

Seasonal cyclicity in the month of delivery/conception in relation to these malformations has been reported. In agreement with some of these authors (e.g., Mayr et al. 1999), a seasonal cyclicity was also observed in the present study, with a peak of cases born between January and March (COR = 1.28; 95%CI, 1.19–9.12). If the first trimester is considered the most vulnerable time for the embryo-fetus, and if spring is the season during which most of the pesticides were applied, this additional information may strengthen the results. Unfortunately, we do not have accurate data on the application of pesticides in spring versus other seasons.

Finally, maternal weight before pregnancy, categorized below and above the median value of the BMI (23 kg/m^2), showed close to a significant association. We have no explanation for this finding, but it would be interesting to consider it together with the observation of a lower body weight increase during pregnancy in mothers of cases versus mothers of control boys (11.3 ± 4.7 kg vs. 13.9 ± 5.5 kg).

In conclusion, although the complexity of human biology makes it very difficult to establish a relationship between EDC exposure and male congenital malformations, our data suggest that environmental chemicals with estrogenic activity play a role in the risk of cryptorchidism and/or hypospadias. Unfortunately, few comparable studies have addressed this issue (e.g., Vidaeff and Sever 2005), and these were limited to a small number of EDCs (Hosie et al. 2000; Longnecker et al. 2002; Vrijheid et al. 2003). However, the present study illustrates the utility for exposure assessment of a biomarker that evaluates the combined effects of bioaccumulated xenoestrogens in placentas.

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