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ORIGINAL ARTICLE *Reproductive endocrinology*

Influence of prenatal organohalogen levels on infant male sexual development: sex hormone levels, testes volume and penile length

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BACKGROUND: Prenatal exposure to endocrine disruptors, like organohalogen compounds (OHCs), might be responsible for the increased aberrations in human male sexual development (hypospadias, cryptorchidism, testicular cancer and fall in sperm count) observed over the past decades. This development is established during fetal life, and reflected in sex hormone levels, testes volume and penile length post-partum. The present study investigates the correlation between prenatal OHC levels and male sexual development outcomes.

METHODS AND RESULTS: Levels of eight neutral [2,2′-bis-(4-chlorophenyl)-1,1′-dichloroethene (4,4′-DDE), 2,2′,4,4′,5,5′-hexachlorobiphenyl, 2,2′,4,4′-tetrabromodiphenyl ether (BDE)-47, -99, -100, -153, -154 and 1,2,5,6,9,10-hexabromocyclododecane, HBCDD] and four phenolic [(pentachlorophenol (PCP), 4OH-CB-107 (4-hydroxy-2,3,3′,4′,5-pentachlorobiphenyl), -146 and -187)] OHCs were determined in 55 maternal serum samples taken at 35 weeks of pregnancy. Eight sex development-related hormones [testosterone, free testosterone, sex hormone-binding globulin (SHBG); LH, FSH, estradiol (E_2), free E_2 (FE_2) and inhibin B (InhB)] were determined in their sons at 3 months of age, and testes volume and penile length at 3 and 18 months of age. The following prenatal OHC levels correlated significantly with sex hormone levels: PCP with SHBG and InhB ($\rho = 0.30$ and -0.43, respectively), 4OH-CB-107 with testosterone ($\rho = 0.31$) and BDE-154 with FE_2, E_2 and InhB ($\rho = 0.49$, 0.54 and 0.34, respectively). BDE-154 levels correlated positively with testes volume at 18 months of age ($\rho = 0.34$).

CONCLUSIONS: Prenatal OHC exposure is correlated with aspects of sexual development outcome in boys up to 18 months of age.

Key words: organohalogen compounds / PCB / BFR / human sexual development / TDS

Introduction

Epidemiological studies in humans have reported falling sperm counts (Carlsen *et al.*, 1992; Irvine *et al.*, 1996) and an increased incidence of hypospadias, cryptorchidism and testicular cancer over the past decades (Toppari *et al.*, 1996). This combination is called testicular dysgenesis syndrome (TDS) (Skakkebaek *et al.*, 2001). The aetiology of TDS is most likely a disruption of fetal hormone homeostasis during reproductive development (Sharpe, 2003). Sharpe and Skakkebaek (1993) postulated that disruption of homeostasis could be due to over-exposure to estrogens or estrogenic chemicals during fetal life. Later, Sharpe (2003) partly

questioned his own earlier hypothesis, and postulated that decreased androgen production and/or action with subsequently decreased aromatization to estradiol (E_2), could be the key central change due to chemical compounds.

Chemicals which interfere with the endocrine system are called endocrine disruptors (EDs) (Zacharewski, 1998). Several organohalogen compounds (OHCs) appear to be EDs in humans, and may therefore be partly responsible for TDS. In adult males levels of pentachlorophenol (PCP), 2,2′-bis-(4-chlorophenyl)-1,1′-dichloroethene (4,4′-DDE) and 2,2′,4,4′,5,5′-hexachlorobiphenyl (CB-153) correlate negatively with sex hormone levels (Hagmar *et al.*, 2001; Richthoff *et al.*, 2003; Hsu *et al.*, 2005; Rylander *et al.*, 2006a).

Furthermore, 2,2′,4,4′,5,5′-hexabromodiphenyl ether (BDE-153) levels correlate with decreased sperm numbers and testes size, PCB levels with decreased sperm motility, and 4,4′-DDE levels with decreased sperm number, morphology and motility (Hauser *et al.*, 2005; Akutsu *et al.*, 2008; Messaros *et al.*, 2009).

Limited data are available on effects of these EDs and of the more recently introduced brominated flame retardants (BFRs) on hormone levels, testes volume and penile length in infant boys.

In 2001, the Groningen-infant-compare cohort (GIC) was founded to investigate the correlation between prenatal exposure to selected neutral and phenolic OHCs and infant growth and development. In this study, we describe the correlation between prenatal exposure levels of these neutral and phenolic OHCs on infant male sexual development, reflected by sex hormone levels in boys at 3 months of age, and testes volume and penile length at 3 and 18 months of age.

Materials and Methods

Cohort

The GIC cohort was founded between October 2001 and November 2002. The cohort consisted of 90 healthy pregnant women, living in the northern provinces of The Netherlands, who delivered a single, term, healthy infant. Fifty-six boys were born within the cohort. One boy was born after ICSI pregnancy, which may predispose to aberrations of sexual development (Wennerholm *et al.*, 2000), and was therefore excluded therefore 55 boys entered the study cohort. Before start of the study informed consent was obtained after fully explaining the protocol. The protocol was approved by the Medical Ethical Committee of the University Hospital Groningen, The Netherlands.

Body weight and height

Anthropometrics were determined at 3 and 18 months of age with a standardized balance and tapeline suitable for the infants' age. Measurements were performed by the same investigator. Owing to non-cooperative behaviour, interfering with reliable results, not all data are complete.

Testes volume

Testes volume was measured by ultrasound at 3 and 18 months of age $\pm$ one week, calculated from date of birth. The measurements were performed by three paediatric radiologists trained for the examination, on the same Antares ultrasound machine (Siemens, Erlangen, Germany). Testis volume was calculated using the formula for an ellipsoid ($1/6 \times \pi \times L \times B \times H$) in mm^3. Testes volume outcome was complete in 46 of 55 infants. At 3 months of age, seven examinations were cancelled due to interfering emergency consultations for the radiologist, one due to illness of the participant, and one due to a strike of medical personnel. Radiologist A performed 27 examinations, B 11 and C 8. At 18 months of age, four examinations were cancelled due to interfering emergency consultations for the radiologist, two due to loss to follow-up, two due to retractile testes (in both participants the left testis was not found) and one due to a strike of medical personnel. Radiologist A performed 36 examinations, B 9 and C 1. The outcome in testes volume was similar between the different radiologists, data can be obtained at request.

Penile length

Penile length was measured with a standardized tapeline by the same investigator throughout the entire study. Penile length was measured from the base (by performing pressure on the suprapubic fat) to the tip of the penis. Measurements were performed in supine position at stable room temperature. At 3 months of age, data were absent in four infants; three due to non-cooperative behaviour, and one due to illness of the participant. At 18 months of age, data were absent in eight infants; five due to non-cooperative behaviour, one due to cancelled testes volume measurement appointment and two due to loss to follow-up.

Analyses of prenatal neutral and phenolic OHC levels

Prenatal exposure to eight neutral and four phenolic OHCs was based on maternal serum levels taken at the 35th week of pregnancy. The eight neutral OHCs were: 4,4′-DDE, CB-153, 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99), 2,2′,4,4′,6-pentabromodiphenyl ether (BDE-100), BDE-153, 2,2′,4,4′,5,6′-hexabromodiphenyl ether (BDE-154) and 1,2,5,6,9,10-hexabromocyclododecane (HBCDD). The four phenolic OHCs were: 4-hydroxy-2,3,3′,4′,5-pentachlorobiphenyl (4OH-CB-107), 4-hydroxy-2,2′,3,4′,5,5′-hexachlorobiphenyl (4OH-CB-146), 4-hydroxy-2,2′,3,4′,5,5′,6-heptachlorobiphenyl (4OH-CB-187) and PCP.

Two neutral (4,4′-DDE and CB-153) and four phenolic (PCP, 4OH-CB-107, 4OH-CB-146 and 4OH-CB-187) OHCs were analysed at the Institute for Environmental Studies, Vrije Universiteit, Amsterdam, The Netherlands as described elsewhere (Meijer *et al.*, 2008). Analyses were performed in all 55 maternal samples obtained at 35 weeks of pregnancy. The limit of detection (LOD = three times the standard deviation of the blank values) was 10 pg/g serum for 4,4′-DDE, 21 pg/g serum for CB-153, 100 pg/g serum for PCP, 9 pg/g serum for 4OH-CB-107, 13 pg/g serum for 4OH-CB-146 and 6 pg/g serum for 4OH-CB-187. The limit of quantification (LOQ) was set as 3.3 times the LOD. 4OH-CB-107 levels were below LOD in 1/55 samples. Levels of all other compounds were above LOD.

Six neutral BFRs (BDE-47, BDE-99, BDE-100, BDE-153, BDE-154 and HBCDD) were analysed at the department of Environmental Chemistry, Stockholm University, Sweden, as described elsewhere (Meijer *et al.*, 2008). Owing to financial restraints, data on BFR levels were available in 44 randomly chosen matching maternal samples in the presently described sub-cohort. LOD (signal/noise = 5) was 0.08−0.16 pg/g for the PBDEs and 0.8 pg/g for HBCDD. LOQ was 1.2−30 pg/g serum for the PBDEs (caused by BDE-47 background levels) and 9 pg/g serum for HBCDD. Background levels were subtracted from reported results. BDE-99 levels were below LOQ in 3/44 samples, HBCDD levels were below LOD in 1/44 samples. All other BFR levels were above LOD and LOQ in all maternal samples.

Analyses of sex hormone levels

Levels of six gonadal function-related hormones were determined in serum of boys at 3 months of age: testosterone, sex hormone-binding globulin (SHBG), LH, FSH, E_2 and inhibin B (InhB). Blood (1 ml) was centrifuged at 3600 rpm for 10 min. Serum was stored at $-20°C$ until analysis. Sex hormone levels were determined at the Endocrine Laboratory, Department of Internal Medicine, Erasmus Medical Centre, Rotterdam, The Netherlands as described elsewhere (Laven *et al.*, 2004). Free testosterone (FT) and free E_2 (FE_2) were calculated as described elsewhere (de Ronde *et al.*, 2005). LOD values for these hormones were 0.1 nmol/l for testosterone, 3 nmol/l for SHBG, 0.1 IU/l for LH and FSH, 10 pmol/l for E_2 and 10 ng/l for InhB. Owing to insufficient amounts of serum in some infants, we decided to analyse sex hormones in the following order: InhB, SHBG, testosterone, LH, FSH and E_2. Sex hormone levels were above LOD in all analysed samples.

Serum lipid content

Total cholesterol was determined by the cholesterol oxidase-phenol $+$ aminophenazone method (Roche/Hitachi, Minneapolis MN, USA). Triglycerides were determined by the Trig/GB methods (Roche/Hitachi). The total lipid weight (LW) was calculated based on the strong correlation between cholesterol, Trig and phospholipids content in human serum [1.9 $+$ 1.3 (total cholesterol $+$ Trig)] (Rylander *et al.*, 2006b).

Statistical analyses

Results are presented as mean and standard error or median and range where appropriate. Testes volume is presented as mean volume (left $+$ right/2 from a total of three measurements). Levels of neutral OHCs are expressed on LW basis (ng/g lipid), and of phenolic OHCs on fresh weight basis (FW, pg/g serum). Bivariate correlations were calculated using Spearman's test. Differences were considered statistically significant at $P < 0.05$, with a trend towards significance at $P < 0.1$. All analyses were performed in SPSS 14.0 for Windows (Chicago, IL, USA).

Results

Infant characteristics, and levels of prenatal neutral and phenolic OHC and sex hormones

Characteristics of the participating infants are presented in *Table I*. All data are within Dutch reference values, except no reference values are available for testes volume and penile length. Prenatal neutral and phenolic OHC levels are presented in *Table II*. Sex hormone levels at 3 months of age are presented in *Table III*. Calculated FT and FE_2 levels were strongly positively correlated with total levels of testosterone and E_2 ($\rho = 0.89$ and $r = 0.88$, respectively, $P < 0.001$ for both). InhB levels were positively correlated with testosterone levels ($\rho = 0.39$, $P < 0.01$) and negatively with FSH levels ($\rho = -0.38$, $P < 0.05$). The correlations between levels of testosterone and LH showed a trend to significance ($\rho = 0.29$, $P < 0.07$), whereas levels of testosterone and SHBG were strongly correlated ($\rho = 0.45$, $P < 0.01$). Finally, E_2 levels were positively correlated with LH levels ($\rho = 0.44$, $P < 0.01$).

Correlations between sex hormone levels at 3 months of age and testes volume and penile length at 3 and 18 months of age

Several sex hormone levels showed a significant correlation with testes volume at 3 and 18 months of age. Serum levels of FT, and testosterone showed a significant positive correlation with testes volume at 3 months of age ($\rho = 0.33$ and $\rho = 0.36$, $P < 0.05$). InhB showed a significant positive correlation with testes volume at 18 months of age ($\rho = 0.36$, $P < 0.05$). In contrast, FSH levels were negatively correlated with testes volume at 3 months of age ($\rho = -0.41$, $P < 0.05$) and a trend towards a significant negative correlation with testes volume was found at 18 months of age ($\rho = 0.29$, $P < 0.1$). FE_2 showed nearly significant negative correlation with testes volume at

Table II Levels of neutral OHCs on LW basis (ng/g lipid) and phenolic OHCs on FW basis (pg/g serum) analysed in serum of women taken at 35th week of pregnancy.

	Median	Range	n.d./n.q.	n
Lipid weight basis (ng/g lipid)				
DDE	93	18–370		55
CB-153	64	19–230		55
BDE-47	0.9	0.0–6.1		44
BDE-99	0.3	0.0–2.1	−/3	44
BDE-100	0.3	0.0–1.4		44
BDE-153	1.6	0.3–20		44
BDE-154	0.5	0.2–3.5		44
HBCDD	0.7	n.d.–7.4	1/−	44
Fresh weight basis (pg/g serum)				
PCP	920	300–8500		55
4OH-CB-107	25	n.d.–100	1/−	55
4OH-CB-146	98	40–290		55
4OH-CB-187	76	36–180		55

n.d./n.q., not detected/not quantified; *n*, number of observations.

Table I Characteristics of weight, height, testes volume and penile length measured in boys at 3 and 18 months of age.

	Age (months)	Mean (SE)	n
Weight (g)	3	6371 (88)	55
	18	11851 (169)	52
Height (cm)	3	63.4 (0.3)	55
	18	84.3 (6.4)	52
Testes volume (mm³)	3	384 (19)	46
	18	421 (16)	46
Penile length (cm)	3	3.9 (0.1)	51
	18	5.0 (0.1)	47

n, number of participants in whom data are complete.

Table III Sex hormone levels in boys at 3 months of age.

Hormones	Unit	Median (range)	n
FT	(nmol/l)	0.03 (0.01–0.08)	45
Testosterone	(nmol/l)	2.7 (0.7–9.4)	45
SHBG	(nmol/l)	115 (40–219)	45
LH	(IU/ml)	0.9 (0.3–5.3)	44
FSH	(IU/ml)	0.8 (0.3–1.8)	41
FE_2	(pmol/l)	0.6 (0.1–1.2)	37
E_2	(pmol/l)	31 (6–60)	37
Inhibin B	(ng/ml)	323 (231–523)	47

n, number of observations.

Table IV Spearman rank correlation (ρ) between the concentrations individual OHCs in maternal blood in the 35th week of pregnancy and serum sex hormone levels in the baby boy at 3 months of age.

Organohalogen compoundss	Sex hormones	ρ	n
4,4′-DDE	LH	0.25[t]	44
PCP	SHBG	0.30*	45
	InhB	−0.43**	45
4OH-CB-107	FT	0.28[t]	45
	Testosterone	0.31*	45
4OH-CB-187	FSH	0.27[t]	41
BDE-154	FSH	−0.35[t]	32
	FE₂	0.49**	29
	E₂	0.54**	29
	InhB	0.34*	35
HBCDD	FT	−0.31[t]	34

[t]Trend to significance <0.10, *P < 0.05, **P < 0.01, n, number of subjects with data.

3 months of age ($\rho = 0.34$, $P < 0.1$). Except for a nearly significant positive correlation between FT and penile length at three months of age ($\rho = 0.29$, $P < 0.1$), none of the sex hormone levels correlated with penile length at 3 or 18 months of age.

Correlations between prenatal neutral and phenolic OHC levels and sex hormone levels at 3 months of age

Spearman correlations between levels of the OHCs and sex hormones are presented in Table IV. Significant correlations were observed between prenatal exposure to both older environmental contaminants like 4,4′-DDE, PCP and hydroxy-PCBs, as well as more recently introduced BFRs.

Correlations between prenatal neutral and phenolic OHC levels and testes volume and penile length at 3 and 18 months of age

Prenatal BDE-154 levels showed a significant positive correlation with testes volume at 18 months of age ($\rho = 0.34$, $P < 0.05$). No other significant correlations between prenatal exposure to OHCs and testes volume and penile length at 3 and 18 months of age were found. The two boys with retractile testes were not exposed to the highest levels of any of the OHCs.

Discussion

We investigated the correlations between prenatal OHC levels and infant male sexual development. Our results indicate that there are associations between prenatal exposure to both older OHCs (4,4′-DDE, PCP, 4OH-CB-107 and-187) and more recently produced BFRs (BDE-154 and HBCDD) and serum levels of several sex hormone levels in infant boys at 3 months of age. Prenatal levels of BDE-154 also correlated significantly with increased testes volume at

18 months of age. No other associations between prenatal OHC levels and testes volume or penile length could be observed.

As male sexual development is largely determined before birth, by the effect of sex hormones from the hypothalamus-pituitary-gonadal axis, it is possible that EDs can affect this development during this period. In male infants levels of testicular hormones peak at 3 months of age and decline thereafter. Therefore, the effects of EDs on infant male sexual development can probably be investigated best by correlating their levels to sex hormone levels, testes volume and penile length at infant age.

Associations between prenatal OHC levels on sex hormone levels at 3 months of age

Male sexual development is affected by a very complex interplay between a number of hormone levels which are linked by different feedback mechanisms. Both (anti-) androgenic and (anti-)estrogenic effects of OHCs can play a role. For example, anti-androgenic effects will block the effects of androgens at the level of the pituitary gland, leading to decreased negative feedback action and resulting in increased LH secretion and increased serum testosterone levels. The action of testosterone on peripheral target tissues however, will be inhibited by the anti-androgenic activity of the OHCs as well. The increased level of testosterone will result in increased levels of E₂ through peripheral aromatization, leading to an estrogen/androgen imbalance. Similarly, anti-estrogenic activity of OHCs will lead to increased LH levels, resulting in a reversal of the ratio of estrogenic and androgenic effects. The net effect of the imbalance in these hormones on the sexual development in the long-term is hard to predict.

Very few studies have investigated the potential effect of environmental pollutants on sex hormones in boys. A study conducted in Germany, where levels of metals and environmental pollutants are very comparable to those in The Netherlands, found a negative correlation between (polychlorinated dibenzo-dioxin and furan) PCDD/Fs in maternal blood with testosterone in cord blood in boys. A study in Denmark found a negative correlation between exposure to pesticides and serum concentrations of testosterone and InhB. In our study pregnant women were exposed to a larger number of environmental pollutants at background levels. For a number of the separate OHCs, significant effects on hormone levels were found. Functioning of Sertoli cells was affected, as indicated by positive and negative correlations with levels of InhB (BDE-154 and PCP, respectively), accompanied by a negative correlation with FSH for BDE-154. It has been shown that PCP negatively affects rat Sertoli cell viability *in vitro* (Xiong *et al.*, 2006). The effects on LH and testosterone might be explained on the basis of anti-androgenic effects of 4,4′-DDE (Kelce *et al.*, 1995) and 4OH-CB-107, whereas the reason for the negative correlation between HBCDD and FT is less clear. Finally, the positive correlation between BDE-154 and levels of free and total E₂, in the absence of a change in the concentration of E₂ direct precursor testosterone, is surprising.

Effects of prenatal OHC levels on testes volume and penile length

A study in Belgium found that higher levels of chlorinated compounds in serum are positively associated with early signs of pubertal staging (pubic hair and genital development). In the Danish study by

Andersen et al. (2008) a negative relationship between pesticide exposure and testicular volume and penile length was detected. In the present study, the only significant association with testis volume or penile length was the positive effect of BDE-154 on testis volume at 18 months of age. This observation is in accordance with the positive effect of BDE-154 on InhB levels at 3 months of age and may indicate a positive effect of BDE-154 on Sertoli cell development. The net result of exposure to chlorinated and brominated compounds might be result on our inability to detect effects on penile length.

Limitations of this study

We acknowledge the limitations of our study. We investigated only a limited group of healthy boys and measured a selected number of OHCs. In this cohort, we did not analyse compounds like phthalates that also might be related to sexual development (Hannas et al., 2011). On the other hand, we observed significant correlations between the levels of sex hormones in accordance with normal physiology. The correlations we observed between InhB, FSH and testis volume are also in accordance with the theoretical background. Levels of OHC are similar to those found in other studies in Europe (Meijer et al., 2008), but the levels of BFRs are lower than those reported in the USA (Meijer et al., 2008). The clinical implication of these associations has to be studied in further detail, probably in a larger cohort of infants and after a longer period of time.

Probably one of the best strategies to investigate the influence of prenatal OHC levels on sexual development is first to calculate a sum of endocrine potency (pro- or anti-estrogenic or -androgenic) for each of the compounds, similar to the sum of toxic equivalent quotient, designed for dioxin-like compounds. By in vitro determination of (anti-)estrogenic and/or (anti-) androgenic potencies per compound, calculation of summated potencies and correlation of these potencies to sexual outcome, a better understanding of the influence of these compounds on sexual development may be established.

In conclusion, we observed that prenatal OHC exposure is correlated with measures of early male sexual development. More and especially more long-term studies are needed to determine potential long-lasting effects and modes of action.

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Authors' roles

L.M., first author, designed the study and was primarily responsible for data collection and analysis. A.A.M., the primary consultant radiologist, who performed the majority of the ultrasounds of the testes. J.M. assisted with the execution of the study, and analyses of the results. A.B., project coordinator COMPARE project, theoretical background of the compounds and their toxicity. J.W. performed the analyses of the brominated flame retardants, and helped significantly with writing of the manuscript. F.H.J., professor of the endocrine laboratory in Rotterdam, was extremely helpful in getting my thoughts on paper and the rewriting stage. P.J.J.S., my PhD trainer, who together with the previous author was extremely helpful in writing this manuscript.

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Conflict of interest

None declared.

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