ORIGINAL ARTICLE

prenatal exposure timing

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Background: Exposure to organochlorine compounds (OCs) has been a subject of interest in recent years, given their potential neurotoxicity. Meconium is easily available and accumulates neurotoxicants and/or metabolites from the 12th week of gestation.
Aims: To determine whether neurotoxicants, specifically OCs, could be detected in serially collected meconium, and to compare the results with those obtained in cord blood samples.
Methods: A sample of cord blood and three serial stool samples were analysed in 10 newborns. Pentachlorobenzene (PeCB), hexachlorobenzene (HCB), polychlorinated biphenyls (PCBs), dichlorodiphenyl trichloroethane (p,p′-DDT) and its metabolite dichlorodiphenyl dichloroethylene (p,p′-DDE), and hexachlorocyclohexane isomers (α-, β-, γ-, and δ-HCH) were analysed by gas chromatography.
Results: From serial stool collection and analysis in newborns, there was an increase in the concentrations of HCB, p,p′-DDE, PCBs, and β-HCH between the first and last stools of the newborn. Levels of DDT diminished as pregnancy progressed. Concentrations in cord blood were positively associated with concentrations in meconium for p,p′-DDE and β-HCH.
Conclusions: Meconium is a very useful instrument for the investigation of fetal exposure to neurotoxicants; serial collection and analysis of meconium should estimate the timing and degree of in utero exposure of the fetus to neurotoxicants. Analysis and interpretation of neurotoxicants in meconium results is a complex process. Measurement in meconium of a wide range of neurotoxic substances should facilitate early identification of harmful exposures, and enable rehabilitation and instigation of preventive measures.

Exposure to organochlorine compounds (OCs) has been a subject of interest in recent years given their potential toxicity (carcinogenicity, inmunotoxicity, reproductive system illnesses, and neurotoxicity).[1] Contrary to what happens in adults, exposure to neurotoxicants during the windows of vulnerability in critical periods of brain development can produce permanent cerebral dysfunction from infancy, or it can delay its effect until adulthood.[2 3]

The ubiquity of neurotoxicants in the environment requires development of methods of measuring the scope of exposure. A useful approach consists of analysing biological samples that accumulate the neurotoxicants or their metabolites during the fetal period.[4]

Meconium, in addition to its diagnostic utility in certain paediatric pathologies (cystic fibrosis, meconium ileus, ileal stenosis or atresia, colon atresia, Hirschprung's disease), is very useful for investigation of fetal exposure to neurotoxicants during pregnancy. Meconium has multiple advantages: it is a discarded sample; there is a large amount of available material; it begins to accumulate from the 12th week of pregnancy (it may provide an indication of exposure timing); and it is easily obtained.[5]

Meconium constitutes the first stools of the newborn. Around 70% of full term newborns begin its expulsion within the first 12 hours of life; 93% within the first 24 hours; and 99.8% during the first 48 hours.[6] Some children in stress situations expel meconium into the amniotic liquid before being born, but even in those cases, the greatest amount of meconium remains in the intestine. Meconium has a viscous green-blackish aspect; it is very sticky. It is composed of water, lipids, proteins, esterols and precursors of cholesterol, products derived from swallowing the amniotic liquid (including free fatty acids), epithelial cells, bile acids/salts, and intestinal secretions.[7 8] It starts to accumulate from the 13th week of pregnancy. Physiological fetal defecation of small quantities of meconium occurs during the second three month period, up to the 34th week of pregnancy.[9]

The objective of this exploratory study was to determine whether neurotoxicants, specifically OCs, could be detected in serially collected meconium, and to compare the results with those obtained in cord blood samples. The present study was carried out within the framework of a larger project whose main objective is to measure the adverse effects of exposure, not only to low, but also to simultaneous doses of different neurotoxicants, and to study meconium as a measure of the intrauterine exposure to multiple environmental neurotoxicants.

MATERIALS AND METHODS

Between November and December 2004 we examined 10 mothers and their healthy full-term newborns in the maternity unit of our hospital. Information on the parents and newborns was obtained by means of a questionnaire. Data concerning weight at birth, gestational age, and history of meconium amniotic fluid were obtained from parental record charts. Written consent was obtained from all parents. This study has Institutional Review Board approval.

Cord blood and serial stool samples were collected and tested for organochlorine levels from 10 newborn infants in our maternity unit during the first 2–3 days of life before discharge. Midwives were instructed to collect 5 ml of cord blood using a glass syringe in the delivery room. Within 30 minutes blood samples were spun (4000 rpm) and fractionated in different tubes. Parents were trained by the

Abbreviations: α-, β-, γ-, δ-HCH, hexachlorocyclohexane isomers; HCB, hexachlorobenzene; OC, organochlorine compound; PCB, polychlorinated biphenyl; PeCB, pentachlorobenzene; p,p′-DDE, dichlorodiphenyl dichloroethylene; p,p′-DDT, dichlorodiphenyl trichloroethane; TBB, tetrabromobenzene; TOC, total organic carbon

Table 1 Limits of detection and quantification for cord blood and meconium samples

Compounds	Cord blood, limits (ng/ml)		Meconium, limits (ng/ml)	
	Detection	Quantification	Detection	Quantification
PeCB	0.018	0.024	0.018	0.027
HCB	2.53	3.87	0.373	0.490
β-HCH	0.07	0.11	0.065	0.12
PCB-28	0.064	0.088	0.117	0.143
PCB-52	0.516	0.789	0.147	0.187
PCB-101	0.069	0.083	0.166	0.239
4,4′-DDE	0.026	0.036	0.219	0.332
PCB-118	0.018	0.029	0.043	0.057
PCB-153	0.052	0.078	0.081	0.116
4,4′-DDT	0.026	0.039	0.032	0.047
PCB-138	0.028	0.035	0.114	0.159
PCB-180	0.047	0.076	0.025	0.034

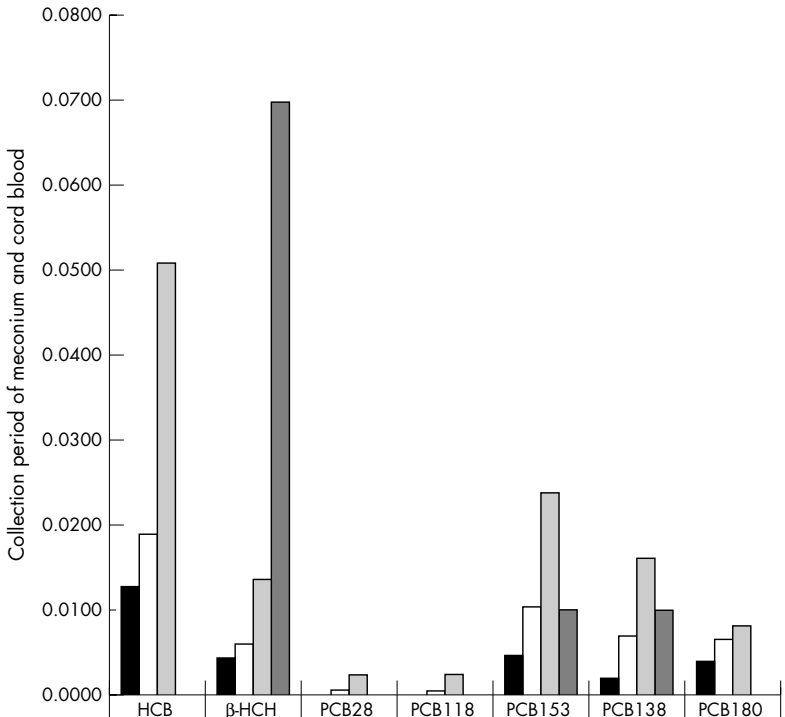
paediatrician to collect meconium in the ward, using a metal spatula and glass pots. Meconium obtained in the first 10 hours, between 11 and 20 hours, and afterwards until 48 hours of age were collected separately in three numbered pots. All samples were frozen at −20°C. Sample storage did not exceed 30 days. The metal spatula, glass pots, and tubes were tested for possible contamination with OCs prior to sample collection.

Materials

Standards of tetrabromobenzene (TBB), PeCB, HCB, α-, β-, γ-, and δ-HCH, PCBs, 4,4′-DDT, and 4,4′-DDE were purchased from Dr Ehrenstoffer (Augsburg, Germany). Analytical grade concentrated sulphuric acid, acetonitrile, iso-octane, and n-hexane were all purchased from Merck (Darmstadt, Germany). Neutral aluminium oxide type 507C was from Fluka AG (Switzerland).

Extraction

Cord blood (0.5 ml) and meconium (1–2 g) were introduced into 10 ml centrifuge tubes and the recovery standards TBB and PCB 209 were added. Concentrated sulphuric acid (2 ml) and n-hexane (3 ml) were added and the mixture mixed on a vortex mixer (ca. 1500 rpm, 30 seconds) and then centrifuged (ca. 1500 rpm, 10 minutes). The supernatant n-hexane layer was aspirated into a second centrifuge tube using a Pasteur pipette; a further 2 ml of n-hexane was added to the first tube containing the sulphuric acid/serum or sulphuric acid/meconium, the sample was mixed once more (vortex mixer, ca. 1500 rpm, 30 seconds), and then centrifuged (ca.



	HCB	β-HCH	PCB28	PCB118	PCB153	PCB138	PCB180
First 10 hours (ng/ml)	0.0130	0.0044	0.0000	0.0003	0.0047	0.0019	0.0040
11–20 hours (ng/ml)	0.0190	0.0061	0.0006	0.0004	0.0103	0.0070	0.0066
20–48 hours (ng/ml)	0.0508	0.0136	0.0023	0.0024	0.0238	0.0161	0.0081
Cord blood (ng/ml)	0.0000	0.0700	0.0000	0.0000	0.0100	0.0100	0.0000

Figure 1 Organochlorine concentrations in serial meconium/TOC (ng/ml) and cord blood serum samples (data expressed as means).

1500 rpm, 10 minutes). This last step was repeated, yielding a combined extract of 7 ml of n-hexane, to which 2 ml concentrated sulphuric acid was added, the sample mixed (vortex mixer, ca. 1500 rpm, 90 seconds), centrifuged as before, and the supernatant transferred to a conical, bottomed, graduated tube. In the case of meconium samples these extracts were cleaned up by adsorption chromatography with glass columns (Pasteur pipettes, with 2 g of aluminium oxide). The combined extracts were then reduced to near dryness under a gentle stream of nitrogen and 10 µl of an injection standard (PCB 142 in isooctane) was added. The sample was quantitatively transferred to GC vials using four 25 µl rinses of isooctane. If an emulsion was present at any stage of the extraction, 10–15 drops of MilliQ water were added before sample centrifugation.

Instrumental analysis

A gas chromatograph with electron capture detection (Hewlett Packard 6890N GC-ECD) was used to quantify PeCB and HCB, the ''ICES 7'' polychlorinated biphenyls (PCBs 28, 52, 101, 118, 138, 153, 180), p,p′-DDT, and p,p′-DDE, while a GC-MS (HP 5973 MSD) in negative chemical ionisation mode (NCI) was used to quantify α-, β-, γ-, and δ-HCH isomers. Samples were injected (2 µl) in splitless mode onto a 60 m, ''DB-5'' column with a retention gap (both from J&W/Agilent) in both instruments, with a flow of 1.5 ml/min helium for GC-ECD, and 1.0 ml/min ammonia for GC-MS-NCI. The temperature programme in both instruments was: 90°C, 2 minutes; 20°C/min to 140°C; 4°C/min to 200°C held for 13 minutes; 4°C/min to 310°C held for 10 minutes.

On both instruments, quantification was performed by external standards (50, 25, 15, 12, 10, 7, 5, 3, 2, and 1 ng/ml), using PCB 142 injection standard to correct for volume. Recoveries of TBB and PCB 209 were used to correct results, TBB recoveries were 74.5–99%, and PCB 209 82.6–98%. Limits of detection (LOD) and quantification (LOQ) were calculated from blanks (LOD = mean of all blanks plus three times the standard deviation, LOQ = mean plus five times the standard deviation), or if the compound was absent from the blanks, from instrument limits of detection using injections of dilutions of standards (table 1). This method performed satisfactorily in repeated international intercalibration exercises within the Arctic Monitoring and Assessment Programme (AMAP 2004).[10]

All the analyses were carried out in the Department of Environmental Chemistry (CID-CSIC). We present results for the most prevalent compounds found in meconium samples: HCB, p,p′-DDE, p,p′-DDT, β-HCH, and PCBs (congeners 28, 118, 138, 153, 180). Levels of OCs in meconium were adjusted for total organic carbon (TOC).

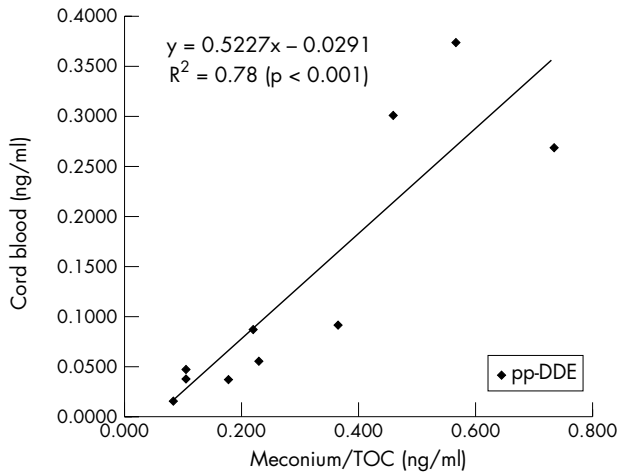


Figure 2 Cord blood and meconium/TOC regression analyses.

Statistical analysis

Initial descriptive statistical parameters were computed. Pearson's r, Kendall's tau, and Spearman's (r_s) correlation coefficient, and simple linear regression analysis were conducted to model the adjusted association between levels of OCs in cord blood and meconium. The criterion of statistical significance was $p < 0.01$.

RESULTS

We analysed 10 cord blood samples and 30 meconium samples. The mean age of the mothers was 29.6 years, gestational age 39.7 weeks, and weight of newborns 3352 g. All deliveries were spontaneous and with non-meconial amniotic fluid. Apgar of all newborns was 9/10 (score at 1 and 5 minutes). Levels of p,p′-DDE in cord blood were detected and quantifiable in 100% of the samples, and levels of β-HCH, PCBs, DDT, and HCB in 50%, 50%, 20%, and 0%, respectively. Levels of p,p′-DDE in meconium were detected and quantifiable in 100% of the samples, and levels of β-HCH, PCBs, DDT, and HCB in 90%, 90%, 80%, and 80%, respectively. Table 2 shows the detected levels of organochlorine in meconium and cord blood. We normalised them by dividing the results in meconium by the quantity of total organic carbon (TOC) (g/ml) present in meconium. Thus, the concentration differences show that different levels of pollutants have reached the fetus throughout gestation. Figure 1 shows, from serial stool sample collection in newborns, an increase of the concentrations of OCs from the first stool of the newborn to the last one. We observed an

Table 2 HCB, p,p′-DDE, p,p′-DDT, PCBs, and β-HCH levels in cord blood serum and meconium

	Cord blood serum (ng/ml) n = 10		Meconium (ng/g) n = 30		Meconium/TOC (ng/ml)
	Mean	SD (range)	Mean	SD (range)	Mean
HCB	0.00	0.000 (0.00–0.00)	1.39	1.30 (0.00–4.18)	0.028
β-HCH	0.07	0.083 (0.00–0.21)	0.40	0.36 (0.00–1.02)	0.008
PCB 28	0.00	0.000 (0.00–0.00)	0.06	0.08 (0.00–0.21)	0.001
pp′DDE	0.31	0.221 (0.08–0.73)	7.19	7.00 (0.61–38.66)	0.140
PCB 118	0.00	0.013 (0.00–0.04)	0.05	0.10 (0.00–0.51)	0.001
PCB 153	0.01	0.029 (0.00–0.092)	0.72	0.72 (0.00–1.48)	0.013
pp′DDT	0.01	0.027 (0.00–0.07)	0.18	0.49 (0.00–1.57)	0.003
PCB 138	0.01	0.024 (0.00–0.07)	0.44	0.47 (0.07–1.41)	0.008
PCB 180	0.00	0.000 (0.00–0.00)	0.38	0.38 (0.00–1.04)	0.006

increase of the concentrations of the HCB (Kendall's $\tau = 0.583$, $p < 0.04$), p,p'-DDE (Kendall's $\tau = 0.714$, $p < 0.03$), PCBs (Kendall's $\tau = 0.760$, $p < 0.03$), and β-HCH (Kendall's $\tau = 0.573$, $p < 0.05$) from the first stool of the newborn to the last one.

The associations between the organochlorinated compounds in cord blood and meconium were derived from simple linear regression analysis. The concentrations of p,p'-DDE and β-HCH in meconium were highly correlated with the concentrations in cord blood. Positive Pearson (r) and Spearman (r_s) correlation coefficients were observed in p,p'-DDE ($r = 0.883$, $p < 0.001$ and $r_s = 0.952$, $p < 0.001$) and β-HCH ($r = 0.858$, $p < 0.001$ and $r_s = 0.821$, $p < 0.01$) (figs 2 and 3). Total PCBs presented a moderate correlation ($r = 0.792$, $p < 0.01$ and $r_s = 0.623$, $p < 0.054$).

DISCUSSION

Traditionally, efforts to determine fetal exposure to neurotoxicants have focused on neonatal or maternal urine or cord blood analysis, but these samples reflect exposure to these agents only in the 2–3 days before delivery. Neonatal urine is difficult to collect, and cord blood is only available at the precise point of childbirth, making difficult and expensive to collect. We have observed that meconium analysis is useful for detecting fetal exposure to a number of neurotoxic agents that are deposited during pregnancy as early as the 12th week. Neurotoxicants and their metabolites may enter meconium via various pathways: (1) diffusion from blood borne chemicals; (2) swallowing those substances excreted by the skin and kidney, and fetal defecation into the amniotic liquid; and (3) excretion into the intestinal tract through the bile after hepatic metabolism or other intestinal secretions. Different studies suggest that once the toxic substances or metabolites reach the meconium, they remain "fossilised".[11–14] In the meconium there may be many chemical substances with potential neurotoxic properties: pesticides, metals, PCBs, and drugs.

The collection of meconium is easy, and cumulative samples may be collected over several days. From one sample multiple chemical substances can be analysed. Our preliminary study indicates that with a very small quantity of biological material, as little as 1 gram, we are able to detect exposure to several substances. It should be noted that with meconium, quantities weighing several grams can be obtained (up to 100 g in many cases), whereas with cord blood, as little as 1 ml is usually available. Meconium can therefore be very useful in detecting substances of prenatal exposure in comparison with other types of sample.[15–19]

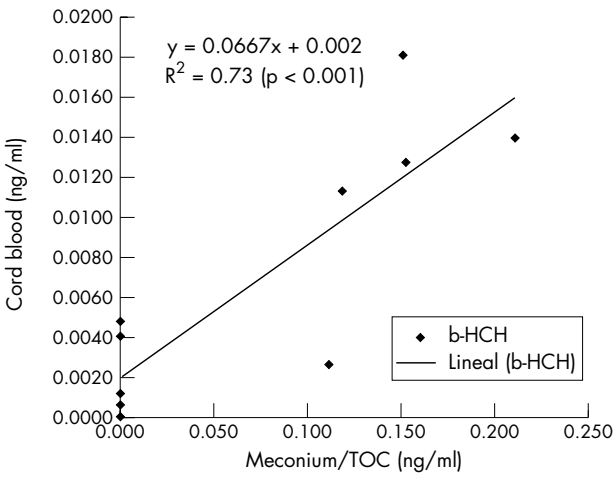


Figure 3 Cord blood and meconium/TOC regression analyses.

What is already known on this topic

- The fetal period probably constitutes the most important critical window of exposure for children's health
- The critical periods of brain development are especially vulnerable to numerous transplacental chemical neurotoxicants

What this study adds

- Due to its physiological characteristics, meconium could be a new and sensitive tool for studying the exposure timing to chemical neurotoxicants
- Knowledge of exposure timing could help to minimise and prevent short and long term adverse effects

Many questions remain to be answered about the deposition of neurotoxicants in meconium. Quantitative and qualitative differences in the OCs detected were observed in the different stools.

Could the presence of a particular analyte be a marker for neurotoxicant exposure at a certain gestational age? Meconium is not a homogeneous specimen, but a series of layers formed in the intestine during gestation. Most analytical methods advise a thorough mixing of the specimen before analysis because the distribution of neurotoxicants throughout a sample may not be uniform. Serial analyses of meconium could help to determine the degree and the timing of the exposure. We have used the suggestions of previous studies carried out by Ostrea *et al*, who proposed that meconium taken between 0 and 10 hours of life reflects the first 20 weeks of exposure, between 11 and 20 hours corresponds to 30 weeks of exposure, and between 21 and 36 hours corresponds to a gestational age greater than 30 weeks.[20] Although the sample size is small, an increase of the transplacental step is observed throughout pregnancy in most of the analysed substances. The increase in the fetal-placental blood circulation system could explain the increase in the concentration of metabolites throughout pregnancy. New data about the presence of DDT in meconium show how concentration levels decrease throughout pregnancy. These results could indicate a mechanism of fetal specific hydrolysis, characteristic only in the fetal period. Analysis and interpretation of results in meconium is a complex process, and over-interpretation of meconium data is a dangerous practice, especially at the current developmental stage of this new and useful technology.

There are some limitations in this study due to the small size of the population. An arbitrary decision was made to limit the analysis of meconium samples; this decision was taken in view of the fact that this study was an exploratory investigation.

We conclude that the analysis of meconium allows detection of prenatal exposure to environmental toxins, specifically neurotoxicants. For the accumulation of toxins it could be a good indicator of time of exposure and fetal internal dose or total fetal burden. We propose that this method constitutes an important tool for investigation of exposure to environmental pollutants of pregnant women and their potential adverse effects on infants in the study of the INMA cohort (Infancia y Medioambiente).[21] Such data will help to determine whether routine meconium

assessment will help clinical practice by showing harmful exposures and encouraging the use of appropriate preventive measures.

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