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Dialkyl phosphates in amniotic fluid as a biomarker of fetal exposure to organophosphates in Crete, Greece; association with fetal growth

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ABSTRACT

The aim of this study was to evaluate fetal exposure to organophosphate pesticides (OPs) by measuring their non-specific dialkyl-phosphate metabolites (DAPs) in amniotic fluid (AF), and to examine the potential association between prenatal exposure and fetal growth. AF samples were collected from 415 women during the second gestational trimester. The determined OPs metabolites were DMP, DMTP, DEP, DETP, and DEDTP. DAPs were extracted by liquid–solid extraction, derivatized and analyzed by gas chromatography–mass spectrometry. 97.8% of AF samples were positive for at least one DAP. DAPs levels did not differ between urban and rural areas. Macrosomic neonates have significantly higher sum levels of DMPs ($p = 0.043$), which exerted a linear positive association with birth-weight centile ($b = 4.43$, $p = 0.016$). Conclusively, as DAPs are detectable in AF they may be used as a potential biomarker of fetal exposure to OPs. Sum levels of DMPs appear to be associated with birth weight independently of other covariates.

1. Introduction

The residents of the island of Crete, Greece are a mixture of rural and urban population whose most important source of income is agriculture, mainly based on the production of olive oil, vegetables and fruits. In these types of crops, organophosphate pesticides (OPs) consist of a class of widely used neurotoxic insecticides and it is well established that environmental and/or dietary exposure to OPs results in the bioaccumulation of these chemicals in the human body, especially in adipose tissue, urine and breast milk [1–6]. During pregnancy, OPs which are lipophilic chemicals stored in maternal adipose tissue can be mobilized to the blood stream reaching the fetus through the placenta [7]. The next step is their rapid metabolism in the human body by hydrolysis or oxidative desulfuration, giving the non-specific metabolites dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), dimethyl dithiophosphate (DMDTP), diethyl phosphate (DEP), diethyl thiophosphate (DETP), and diethyl dithiophosphate (DEDTP), referred as dialkyl phosphate metabolites (DAPs) [7–9]. These metabolites are polar water-soluble compounds and are used as biomarkers of OPs exposure on human population in various biological samples such as blood, urine, post-mortem tissue, hair, AF and meconium [2–4,10–12].

Maternal exposure to pesticides during pregnancy is a topic of major public concern as there are human and animal studies that associate the prenatal exposure to these compounds with aberrations in neuronal proliferation, migration, differentiation, synaptogenesis, myelination, and apoptosis. Prenatal exposure to organochlorine pesticides has been associated with adverse effects in neurodevelopment and growth in infancy and childhood [13–15]. Similarly, significant associations have been reported after prenatal exposure to OPs with structural changes in the developing human brain in terms of abnormal reflexes [16], reduced cognitive abilities [17–19], and attention problems [20,21]. Recent studies associate the prenatal exposure to OPs with pregnancy-associated complications such as shorter length of gestation and lower birthweight [22]. In addition, there is increasing evidence in support of a

Abbreviations: AF, amniotic fluid; CV, coefficient of variability; DAPs, dialkyl phosphate metabolites; DEDTP, diethyl dithiophosphate; DEP, diethyl phosphate; DETP, diethyl thiophosphate; DMP, dimethyl phosphate; DMTP, dimethyl thiophosphate; OPs, organophosphate pesticides; GC–MS, gas chromatography–mass spectrometry; sumDMPs, sum levels of DMP and DMTP; sumDEPs, sum levels of DEP, DETP and DEDTP; sumDAPs, sum levels of DMP, DMTP, DEP, DETP and DEDTP.

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potential link between increased risk for childhood leukemia, brain cancer, neuroblastoma, non-Hodgkin's lymphoma, Wilms' tumor, and Ewing's sarcoma with pesticide exposure [23,24]. Although there is an increasing number of studies that determine pesticide metabolites levels in maternal urine or serum samples, there is no extending literature for measuring pesticides or metabolites in AF samples [25–27], and there is only one study that evaluates OP metabolite levels in AF [28]. The concentration of OP metabolites in AF collected during amniocentesis from pregnant women who are exposed to pesticides through a number of sources, including residential and agricultural applications, is likely to be a useful biomarker of direct fetal exposure to these chemicals between 16th and 20th weeks of gestation which is a critical period for the developing embryo.

The aim of this study was first, to determine the presence of OP non-specific metabolites (DAPs) in AF as an index for in utero exposure to these contaminants among a cohort of pregnant women in the island of Crete and second, to evaluate any potential link between prenatal exposure to OPs and birth weight. This is in continuation and advancement of the authors' studies hitherto focused on the determination of the parent compounds in fetal and maternal compartments [1–6,9].

2. Materials and methods

2.1. Study's design

The study was carried out between August 2006 and May 2008. 415 women with singleton pregnancies, who were permanent residents of Crete for at least two years, were enrolled. Women were recruited at the time of referral for amniocentesis to the Fetal-Maternal Unit, Department of Obstetrics and Gynecology, University Hospital of Heraklion, Crete, Greece. The indications for referral included increased risk for chromosomal abnormalities (based on advanced maternal age or the results of the first trimester combined test for fetal aneuploidies, or sonographically detected markers or abnormalities between 18 and 22 weeks of gestation) suspicion of genetic syndromes or increased risk for single gene disorders (based on history or sonographic findings), abnormal fetal growth, and suspicion of congenital infection. Written informed consent was obtained from each woman that agreed to participate. The Ethics Committee of the University Hospital of Crete approved the study's protocol.

2.2. Data collection

82.5% of the amniocenteses were carried out between 16th and 20th weeks of gestation and an additional volume of 8–10 ml of AF was obtained during each procedure. Each AF sample was frozen at −20 °C immediately after collection. The samples were analyzed to the laboratory of Toxicology, Medical School, University of Crete. The participants were asked to complete a detailed questionnaire about their medical history, demographic data, socioeconomic status, occupational and residential status and exposure to other potentially embryotoxic factors.

2.3. Materials and assays

Diethyl ether (95.5%), toluene (99.5%), hydrochloric acid (37%), natriumdisulfit (98%) and potassium carbonate were obtained from Merck (Darmstadt, Germany). Dimethyl phosphate (DMP, 98%) and dimethyl chlorothiophosphate (DMClTP, 97%) were purchased from Acros Organics (Geel, Belgium). Diethyl phosphate (DEP, 98.9%) was obtained from Chem Service (West Chester, USA), *O*,*O*-diethyl thiophosphate potassium salt (DETP, 98%) and diethyl dithiophosphate salt (DEDTP, 95%) from Sigma–Aldrich (Steinheim, Germany). Methanol and acetonitrile, both HPLC-grade, were purchased from Roth (Karlsruhe, Germany). Sodium chloride (NaCl) was from Riedel-de Haen (Seelze, Germany). The derivatization agent 2,3,4,5,6-pentafluoro benzylbromide (PFBBr, 99%) was purchased from Sigma–Aldrich (Steinheim, Germany) and water (LC–MS grade) from Sigma–Aldrich (Buchs, Switzerland). The synthesis of dimethyl thiophosphate (DMTP) was achieved by hydrolysis of 5 g of DMClTP in a solution of 40 ml of HPLC grade water–acetonitrile (10:30, v/v) and triethylamine (3 ml). After hydrolysis, acetonitrile was added in order to achieve a final volume of 200 ml [9,29].

2.4. Stock solutions

Stock solutions (1 mg/ml) of each individual DAP were prepared in methanol and stored at −20 °C. Mixed working solutions of DMP, DEP, DMTP, DETP and DEDTP were prepared monthly and stored at 0 °C, in the dark, covering concentration range from 0 to 500 ng/ml.

2.5. Sample treatment

2.5.1. Liquid–liquid extraction

AF samples were treated according to previously reported procedure by Ueyama et al. [30] with slight modifications. Briefly, 5 ml of AF were transferred to a clean 15 ml screw-top glass vial. Four grams of NaCl, 1 mL of HCl (6 M), 50 mg of $Na_2S_2O_5$ were added. Liquid–liquid extraction was performed by adding 4 ml of diethyl ether–acetonitrile (1:1, v/v) followed by mechanical shaking for 5 min. After the extraction, the samples were centrifuged at 2000 × *g* (5 min) at 4 °C. The supernatant was collected in another vial containing 15 mg of K_2CO_3 and the liquid–liquid extraction step was repeated. The two extracts were combined and evaporated to dryness under a stream of nitrogen at 30 °C.

2.5.2. Derivatization procedure

Fifteen milligrams of K_2CO_3 was added to the residue, which was reconstituted in 1 ml of acetonitrile and 0.1 ml of PFBBr in acetonitrile (1:3, v/v) and incubated in a water bath at 80 °C for 30 min with occasional shaking [30]. After incubation, the mixture was brought to room temperature and acetonitrile was evaporated to dryness under a stream of nitrogen at 35 °C. The residue was dissolved in 50 μl of toluene and 2 μl was injected to GC–MS.

2.6. Chromatography and mass spectrometry conditions

Electron ionization mass spectrometric analysis was performed on a GC-2010 Shimadzu system equipped with a BPX5 (30 m × 0.25 mm × 0.25 μm) capillary column (SGE, Argent Place, Ringwood, Victoria, Australia). Pure helium (99.999%) was used as a carrier gas. The column temperature was initially held at 60 °C for 1 min, raised to 180 °C at 20 °C/min, held for 1 min, raised to 250 °C at 4 °C/min, held for 1 min and finally raised to 300 °C, at 25 °C/min. The injector, interface and ion source temperatures were 270 °C, 310 °C and 230 °C, respectively.

Quantitative analysis was performed in selected ion monitoring (SIM) mode with a total run time 32.2 min per sample, using one target ion for quantification and one fragment ion for confirmation for each DAP. Specifically, m/z = 110, 306 for DMP; 258, 334 for DEP; 322, 211 for DMTP; 350, 274 for DETP; 366, 185 for DEDTP; and 335 for DBP (IS) [4,9].

2.7. Data management and statistical analysis

Exploratory data analysis was carried out to investigate the distributions of the examined continuous variables. Normality of