Original Research Article

Prenatal Exposure to Organophosphate Pesticides in Vojvodina

Short running title: **Exposure to Organophosphate Pesticides**

Abstract

Organophosphate pesticides (OPs) are commonly used pesticides which are metabolized and excreted in urine. There are many studies reporting the levels of OP metabolites in urine of pregnant women from different countries, but there is no published data on this problem in Serbia. The aim of this study was to determine the level of OP pesticide exposure of pregnant women in Vojvodina region, Serbia. Sixty healthy pregnant women were recruited to participate in this study. A trained interviewer administered a questionnaire to each woman. The concentrations of five dialkylphosphate metabolites of the organophosphorus pesticides were determined in urine samples that were taken on a third postpartum day. Fifty-eight maternal urine samples were collected. Approximately two thirds (65,5%) of pregnant women reported living in urban area and rest living in rural area. Number of positive urine samples varied from 65,5% for DMP to 34,5% for DEDTP. Mean urine concentrations were highest for DMP (5,714 µg/L cre). Other metabolite concentrations averaged around 1 µg/L cre. This study confirms prenatal exposure to OP pesticides in Vojvodina region, Serbia. Levels of dialkylphosphate

metabolites in pregnant women from Serbia are comparable with those reported for Caribbean countries and Palestinian region.

Key words: organophosphate pesticides, exposure, pregnancy

Introduction

A pesticide is defined as "any substance or combination of substances used to prevent or eradicate unwanted insects, including vectors of diseases in human-beings and animals, weeds, fungi, or animals in order to enhance food production and help production, processing, storage, transport or marketing of the food and agricultural commodities" (1).

Organophosphate pesticides (OPs) are the most commonly used pesticides (2). OPs are absorbed by all routes, including ingestion, inhalation, and dermal absorption and humans are commonly exposed to OPs via ingested food and drink and inhalation of contaminated air, as well as being exposed through occupational contact and proximity to farms (3).

Once in the body, OP pesticides are rapidly metabolized and excreted in urine (4). Hydrolysis of ester linkages in the parent compounds yields dialkylphosphate metabolites, which are not considered toxic, such as dimethylphosphate (DMP), dimethylthiophosphate (DMTP), dimethyldithiophosphate (DETP), and diethylphosphate (DEP), diethylthiophosphate (DETP) and diethyldithiophosphate (DEDTP).

Several studies have examined the association between prenatal exposure to OPs and a child's health from the time of conception through later on in life. Although some studies did not establish link between prenatal OP pesticide exposure and postnatal health problems (5), prenatal exposure to these pesticides has been associated with decrease in gestational age and umbilical cord cholinesterase activity, as well as with decerase in intelectual and social abilites (6-8).

There are many studies reporting on the levels of OP metabolites in urine of pregnant women from different regions and countries (Jerusalem, Carribean, China). To the best of our knowledge there is no published data on pesticide exposure of pregnant women in Serbia. Therefore the aim of this study was to determine the level of OP pesticide exposure of pregnant women in Vojvodina, northern agricultural region of Serbia.

Materials and methods

Study Participants

From January 2015 to January 2016, 60 healthy pregnant women were recruited to participate in this study from the obstetric ward of The Clinic for Gyneacology and Obstetrics, Clinical Centre of Vojvodina. Eligible women were between 18 and 45 years of age, and reported no gestational or preexisting diabetes, hypertension, HIV infection or AIDS, and no use of illegal drugs in the preceding year. Women with multiple pregnancies were excluded from the study as well as women giving birth to babies with severe congenital anomalies. All women were fully informed about the study and they have signed a consent form.

The study protocol was approved by the Medical Ethics Committee of the Clinical Center of Vojvodina No. 00-01/306.

Questionnaire

A trained interviewer administered a brief questionnaire to each woman after delivery. Information was obtained on occupation, education, reproductive history, smoking habits, housing and diet. Women were specifically asked about presence of pests in their home/garden and use of household/garden pesticide products during pregnancy. In addition, women were asked about frequency of eating specific fruits, vegetables, and additional food items. Additional information on paternal effects such as OP exposure and occupation was also collected through personal interview.

Sample Collections

Urine samples were collected from each participant on a third postpartum day. Twenty milliliters of urine specimens were stored at -80°C. Analysis of dialkylphosphate metabolites were conducted at the Department for Pharmacology and Toxicology of Medical Faculty.

Chemicals and Materials

The concentrations of five dialkylphosphate metabolites of the organophosphorus pesticides were determined in urine samples. Analytical standards used were DMP (98% purity, Acros

Organics), DMTP (95% purity, Alfa Aesar), DEP (99.5% purity, Sigma Aldrich), DETP (98% purity, Sigma Aldrich), DEDTP (95% purity, Sigma Aldrich). Dibutylphosphate (DBP 97% purity, Acros Organics) was used as an internal standard. All of the solvents used were of liquid chromatography-mass spectrometry level of purity (JT Baker).

Sample Preparation

One milliliter of urine was pipetted into a 10ml screw-top glass test tube, 250µl of a DBP solution (4.0mg/l) were added. Subsequently, 4ml acetonitrile were added and the sample was mixed. After vigorous mechanical shaking for 5min, the test tube was centrifuged (1200×g, 5min, 25°C). The supernatant fluid containing DAP and DBP was transferred into a clean screw-top glass test tube. Sample volume was then reduced at 70°C to a volume of 0.5ml with a gentle nitrogen stream. Residues were re-extracted with 3ml of acetonitrile that contained 1g of Na_2SO_4, were shaken for 10min and then centrifuged. The resulting extract was repeatedly evaporated at 70°C to 0.1–0.2ml under a gentle stream of nitrogen. To the final extracts, 20mg of K_2CO_3, and 25µl of pentafluorobenzyl bromide (PFBBr) were added and heated at 50°C for 16h to covert the phosphate acids to their pentafluorobenzyl (PFB) esters. The PFB-DAP derivatives were dissolved in 100µl of toluene for injection into the GC–FPD.

GC-FPD and GC-MS conditions

A gas chromatograph – mass spectrophotometer (Agilent, USA) was used. The GC operating conditions were as follows: GC column- BP-10, 25mm x 0.33mm, 0,25µm film thickness;

column temperatures - 110°C (1min) – 8°C/min – 210°C (1min) – 20°C/min – 280°C (10min). Nitrogen gas (99.99% purity) was used as carrier gas at a head pressure of 150kPa. The detector gases used were air at 60kPa and hydrogen at 80kPa. The injection volume was 1.0µl.

A GC–MS was used for structural elucidation of PFB derivatives of DAP. Injector conditions and chromatographic conditions used were the same as for the GC-PPD. Operating conditions were as follows: carrier gas, helium at a flow rate of 1.0ml/min; ion-source temperature, 250°C; electron ionization, 70eV; interface temperature, 280°C. Chromatographic peaks were identified by target and qualify ions for each PFB-DAP.

Limit of Detection (LOD)

LOD ranged from 0.5ng/l for DEP, 0.8ng/l for DMDTP, 1ng/l for DETP and DEDTP, to 3ng/l for DMP.

Due to technical problems we were not able to provide DMTP standard. The content of this metabolite in urine was not analyzed.

Results

From January 2015 to January 2016, 58 maternal urine samples were collected from pregnant women. The mean age of enrolled women 29,66 years (range 18–41) and the mean duration of gestation at recruitment was 39,28 (range 36-42). High school education was reported by 35 women (60,34%), while 23 women reported higher (bachelor's or higher) degree. Approximately two thirds (65,5%) of pregnant women reported living in urban area, while remaining number

reported to be living in rural area (villages, near agricultural field). Most of the women reported to be employed and working during pregnancy. In 16 of interviewed pregnant women positive indirect exposure to pesticides was reported, while 2 women were using pesticides by themselves during pregnancy. Demographic characteristics of pregnant women enrolled in the study are presented in Table 1.

Urinary OP pesticide concentrations

Number of positive urine samples (OP metabolite concentrations above LOQ) varied from 65,5% for DMP to 34,5% for DEDTP.

Mean urine concentrations were highest for DMP (5,714 µg/L cre). Other metabolite concentrations averaged around 1 µg/L cre (Table 2).

The results of the current study show that pregnant women in Novi Sad, Vojvodina region are exposed to OP pesticides during pregnancy. When compared to exposure of Palestinian women median metabolite levels are similar or lower compared to concentrations measured in Palestinian women. However 95th percentile values and maximum values of metabolites are much lower in most of the metabolites in pregnant women from Vojvodina (Table 3).

Some other studies report lower exposure of pregnant women. In study by Forde et al, 2015 published data on OP metabolite concentration show lower median values for DMP, DEP, DETP compared to concentrations determined in urine of pregnant women in Vojvodina (Table 4, 5 and 6).

DMDTP was not detected in nine of the sampled Caribbean countries with this OP metabolite being detected in 60% of the samples from Bermuda with a geometric mean of 0.42 mg/L. All of the Caribbean samples tested below the LOD for the OP metabolite DEDTP.

Discussion

The results of our study suggest that women giving birth in Vojvodina are exposed to organophosphate pesticides. It should be of no surprise since Vojvodina is an agricultural region.

In this assessment, we assumed that 100% of absorbed maternal OP pesticides dose is expressed in urine as diethyl and dimethyl phosphate metabolites. Although the kinetics of elimination vary among the dimethyl and diethyl phosphate metabolites, toxicologic evidence suggest that the metabolites of many OP compounds are excreted primarily, but not exclusivelly in the urine (9,10). Further, total OP pesticides exposure may be underestimated because several OP pesticides, such as acephate, do not metabolize to any of the urinary dialkylphosphate metabolitesand are therefore not included in our exposure-dose estimate. These OP pesticides, which do not devolve into dialkylphosphate metabolites, represent approximately 20% of total OP pesticide use (11).

Once in the body, OP pesticides are rapidly metabolized and excreted in urine (12). Hydrolysis of ester linkages in the parent compounds yields dialkylphosphate metabolites, which are not considered toxic, such as dimethylphosphate (DMP), dimethylthiophosphate (DMTP), dimethyldithiophosphate (DMDTP), diethylphosphate (DEP), diethylthiophosphate (DETP), and diethyldithiophosphate (DEDTP). While direct exposure to dialkylphosphate metabolites is possible, they are primarily considered to be biomarkers of recent OP pesticide exposure (12). In an attempt to design a study in which we could assess the extent of fetal exposure to OP pesticides we considered a fact that a single urine sample may not adequately reflect overall extent of the exposure. There are authors who found a high degree of within person variability, high enough to present a challenge for designing well powered epidemiological studies (13). Since in the above mentioned study samples were taken at intervals of at least seven weeks apart, it is arguable if the seasonal variations in the level of pesticides are responsible for the change in the level of pesticides. Another important factor that must be taken into consideration is the fact that a single interview regarding use of pesticides increased awareness of the patients that also inevitabely affects the results (14).

There are studies showing that metabolite levels from a single urine sample taken from each subject reflect that subject's exposure over several months. Although nondifferential exposure misclassification is likely to occur to some extent, a single measure may adequately represent true average exposure over a longer period of time (15). Therefore we decided to use a single urine sample, and to compare our results with the results of the studies with the similar epidemiological design.

Urine samples of pregnant women in Novi Sad showed lower concentrations of DEP and DETP while the level of DMDTP was almost the same as in study conducted in Palestina. Interestingly, level of DEDTP was higher in urine samples of pregnant women in Serbia then in Palestina.Important difference in exposure is the fact that percent of samples positive to presence of OP metabolites was lower in Vojvodina compared to Palestine, although LOQ in our study was lower for all of the analyzed metabolites.

When comparing our results with the results of Caribbean islands geometric mean and median of the DMP were higher in Vojvodina then in all of the Caribbean islands, while DEP was less commonly detected in Serbia then in Caribbean islands. The level of DETP showed similar results in these two studies, but with the difference between different regions. Therefore, pregnant women in St. Vincent and the Grenadines showed lower median level of DETP then in Vojvodina, while higher median concentrations of DETP were detected in Jamaica comparing to Vojvodina.

We examined the registered OP pesticides in Vojvodina and we found that OP pesticides most commonly used are those containing following active substances: dimethoate, chlorpyrifos, malathion and pirimiphos-methyl. Although dialkylphosphate metabolites are not specific biomarkers, it is possible to assume the most probable source (parent pesticide) of a specific metabolite (16). Seven products containing dimethoate are currently registered in Vojvodina, and dimethoate is the most probable source of DMDTP. Chlorpyrifos, active ingredient in 16 of registered products, is most probable precursor of DEP. Malathion can be found in 4 registered pesticides, and is probable precursor of DMP (17) The most concerning conclusion is that DETP,

which was also determined in relatively high levels can be assigned as a metabolite of highly toxic and banned OPs such as Diazinon and Parathion (16).

This study on the concentrations of OP pesticide metabolites in maternal urine samples confirms prenatal exposure to these pesticides in Vojvodina region, Serbia. In general, levels of dialkylphosphate metabolites in pregnant women from Serbia are comparable with those reported for Caribbean countries and Palestinian region.

Since data on pesticide exposure in Serbia are scarce, results of this study are good starting point for future biomonitoring studies in this region. Use of pesticide in agricultural regions is inevitable, but data on pesticide use and exposure can be useful in improving the use of pesticides. By that manner potential health consequences can be prevented, especially in vulnerable population such are pregnant women.

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Table 1. Demographic characteristics of pregnant women enrolled in the study

Age (mean+SD; years)	29,66+5,44

BW (mean+SD; kg)	75,35+13,55
Smoking before pregnancy (%)	43,1
Smoking during pregnancy (%)	29,31

Table 2. OP metabolite concentrations

Metabolite	%>LOQ	Mean	SE	Min	Median	90th percentile	95th percentile	Max
DMP (µg/L)	65,5	7,509	0,971	0	5,640	16,613	19,679	32,57
DMP (µg/L cre)	65,5	5,714	0,810	0	4,640	13,278	17,866	30,625
DEP (µg/L)	53,4	0,496	0,072	0	0,535	1,362	1,655	1,760
DEP (µg/L cre)	53,4	0,415	0,063	0	0,342	1,105	1,289	2,066
DMDTP (µg/L)	56,9	0,566	0,073	0	0,680	1,255	1,353	2,070
DMDTP (µg/L cre)	56,9	0,469	0,071	0	0,476	1,176	1,751	2,393
DETP (µg/L)	43,1	0,569	0,091	0	0	1,478	1,893	2,360
DETP (µg/L cre)	43,1	0,401	0,065	0	0	1,034	1,353	1,562
DEDTP (µg/L)	34,5	0,439	0,082	0	0	1,426	1,571	1,810
DEDTP (µg/L cre)	34,5	0,284	0,053	0	0	0,867	0,983	1,204

Table 3. Comparison of results obtained in Vojvodina and Palestine

				Percentile		
Metabolite	Region	%>LOQ	Geometric mean	50th	95th	Maximum
DMP (µg/L)	NS	65,5	5,714*	4,640	17,866	30,625
	PA	89,7	3,7	4,9	41,5	203,3
DMTP (µg/L)	NS	-	-	-	-	-
	PA	97,2	8,6	8,6	84,9	1523,1
DMDTP (µg/L)	NS	56,9	0,469*	0,476	1,751	2,393
	PA	69	0,4	0,4	9,3	314,3
DEP (µg/L)	NS	53,4	0,415*	0,342	1,289	2,066
	PA	98,6	2,9	2,70	37,9	205,6
DETP (µg/L)	NS	43,1	0,401*	0	1,353	1,562
	PA	71,7	0,9	0,8	12,7	52,1
DEDTP (µg/L)	NS	34,5	0,284*	0	0,983	1,204
	PA	42,8	0,02	0,01	0,2	2,8

Table 4. Comparison of values of DEP obtained in Vojvodina and Caribbean Islands

Diethylphosphate (DEP) (µg/L)					
Region	Geometric mean	Median	90th	Minimum	Maximum
Serbia – Novi Sad	0,496*	0,535	1,362	0	1,760
Antigua and Barbuda	4,47	4,30	16,00	0,50	17,00
Belize		1,40	19,00	0,50	27,00
Bermuda	1,95	2,30	5,40	0,50	9,10
Dominica	1,31	1,10	4,50	0,50	5,40
Grenada	1,72	1,40	3,90	0,50	9,10
Jamaica	2,07	1,50	14,00	0,50	29,00
Montserrat			3,10	0,50	6,60
St Lucia		1,30	3,90	0,50	5,40
St Kitts and Nevis	2,52	2,00	6,40	0,50	41,00
St Vincent and the Grenadines			6,00	0,50	8,00
Caribbean islands	1,65	1,50	7,20	0,50	10,00

*arithmetic mean

Table 5. Comparison of values of DETP obtained in Vojvodina and Caribbean Islands

Diethylthiophosphate (DETP) (µg/L)					
Region	Geometric mean	Median	90th	Minimum	Maximum
Serbia – Novi Sad	0,569*	0	1,478	0	2,360
Antigua and Barbuda	0,43	0,48	2,00	0,15	2,50
Belize			3,90	0,15	23,00
Bermuda			0,53	0,15	0,70
Dominica		0,37	1,50	0,15	1,50
Grenada			0,61	0,15	1,70
Jamaica	0,71	0,45	3,10	0,15	16,00
Montserrat			1,10	0,15	4,00
St Lucia			0,70	0,15	1,00
St Kitts and Nevis		0,31	0,54	0,15	6,10
St Vincent and the Grenadines	0,35	0,37	1,00	0,15	2,00
Caribbean islands			1,50	0,15	23,00

*arithmetic mean

Table 6. Comparison of values of DMP obtained in Vojvodina and Caribbean Islands

Dimethylphosphate (DMP) (µg/L)					
Region	Geometric mean	Median	90th	Minimum	Maximum
Serbia – Novi Sad	7,509*	5,640	16,61	0	32,57
Antigua and Barbuda	3,30	3,00	14,00	0,50	20,00
Belize			2,00	0,50	2,60
Bermuda	3,84	4,60	11,00	0,50	11,00
Dominica		1,00	8,90	0,50	12,00
Grenada	1,29	1,20	4,50	0,50	13,00
Jamaica			2,60	0,50	7,50
Montserrat			14,00	0,50	34,00
St Lucia	1,28	1,00	5,90	0,50	12,00
St Kitts and Nevis	3,17	2,40	29,00	0,50	49,00
St Vincent and the Grenadines	1,51	1,40	7,70	0,50	11,00
Caribbean islands	1,60	1,40	11,00	0,50	49,00

*arithmetic mean