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Persistent environmental contaminants in human milk samples from the northeastern region of Bulgaria

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Organochlorine pesticides (OCPs) are persistent contaminants widely distributed in the environment and in the food chains. The aim of the present study was to determine the levels of 10 OCPs (DDT and its metabolites DDE and DDD, heptachlor, aldrin, heptachlor epoxide, endosulfan, endrin aldehyde, dieldrin and endrin) in human milk collected from northeastern Bulgaria. Breast milk samples from 45 first-time mothers were analyzed by capillary gas chromatography system with mass spectrometric detection (GC-MS). Important determinants such as mother's age, body mass, dietary habit and smoking were considered using a questionnaire. The inclusion criteria and the questionnaire were based on the World Health Organization's protocol. DDE *(p,p'*-dichlorodiphenyldichloroethylene), the main metabolite of DDT, was found in all breast milk samples at levels ranging from 16.89 to 94.48 ng/g lipids (mean 42.86 ng/g lipids). No concentrations of five of OCPs (heptachlor, aldrin, endosulfan, dieldrin, endrin) were detected in any of the milk samples. Heptachlor epoxide and endrin aldehyde (degradation product of endrin) were found in 18% of the milk samples only. The Sum DDTs (sum of DDE, DDD and DDT) in breast milk from Varna (54.62 ng/g lipids) was found higher than DDTs levels in milk samples from Dobrich (44.08 ng/g lipids). The levels of total DDTs in breast milk samples increased by age groups 31 – 35 and 36 – 40 years (53.47 ng/g and 69.16 ng/g lipids, respectively). Levels of OCPs in breast milk from northeastern region of Bulgaria were comparable to levels measured in other European countries.

Keywords*:* Organochlorine pesticides, OCPs, human breast milk, Bulgaria

INTRODUCTION

Organochlorine pesticides (OCPs) are classified as environmental organic pollutants due to their physical, chemical and toxicological properties, and their environmental and biological persistence [1]. OCPs such as DDT have been used successfully to control a number of diseases, such as malaria and typhus, and were banned or restricted in the 1970s in many parts of the world [2]. Pesticides were found as contaminants in soil, air, water and non-target organisms. Once there, they can harm plants and animals ranging from beneficial soil microorganisms and insects, non-target plants, fish, birds, and other wildlife [3]. Due to their persistence and potential immunotoxicity, carcinogenicity, neurotoxicity, reproductive toxicity and endocrine-disrupting effects, OCPs represent a significant public health problem [1]. These pollutants tend to bioaccumulate within the food chain [4]. The pesticides: DDT and its metabolites DDE and DDD, heptachlor, aldrin, heptachlor epoxide, endosulfan, endrin aldehyde, dieldrin and endrin were listed as persistent organic pollutants (POPs) in The Stockholm Convention [5].

Human exposure to OCPs can be assessed by investigation of biomarkers in body fluids such as blood, urine, saliva, breast milk and sweat [6]. Human milk is the best matrix for biomonitoring of OCPs, due to its non-invasive collection, and its high lipid content makes the extraction of lipophilic contaminants relatively easy [7]. The contamination of human milk with environmental pollutants is of special concern due of its importance as the first food for the newborn child.

In Bulgaria, a monitoring study for OCPs in breast milk was conducted as part of 2000 – 2003 WHO/UNEP global survey of POPs in human milk from a small group of donors [7, 8]. The aim of the present study was to determine the levels of 10 OCPs (DDT and its metabolites DDE and DDD, heptachlor, aldrin, heptachlor epoxide, endosulfan, endrin aldehyde, dieldrin and endrin) in human milk collected from Northeastern Bulgaria.

EXPERIMENTAL

Sample collection

The present study was based on the voluntary participation of donors, following the World Health Organization's (WHO) protocol [7]. Breast milk samples from lactating mothers living in two regions located in northeastern Bulgaria (Varna and Dobrich regions) were collected in October 2019 – July 2021.

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Inclusion criteria for the participant mothers were: 1) age 25–40 years, 2) First-time mothers (*primiparae*), 3) breastfeeding period 30 – 40 days after childbirth; 4) resident in Varna or Dobrich for ≥10 years; 5) informed consent signed. Data on age, pre-pregnancy weight, height, lactation period, smoking and dietary habits were obtained from a validated "face to face" questionnaire. The study design and questionnaires were approved by the Commission for Scientific Research Ethics at the Medical University – Varna, Bulgaria (protocol № 85/2019). The collected milk samples were stored at −18°C until the laboratory analysis.

Analytical method

Preparation of milk samples was performed by a previously described analytical method [9]. Briefly, individual milk samples were defrosted, then slowly warmed up to 36-37°C and carefully homogenized. Each individual milk sample (10 g) was three-step extracted with a mix of organic solvents: hexane / acetone in ratios 1:0 v/v (5 mL), 2:1 v/v (9 mL), 1:1 v/v (8 mL), respectively. The hexane layers were collected, and evaporated to near dryness in a rotary vacuum evaporator. The lipid content of the milk samples was determined gravimetrically. The cleaning-up procedure of the lipid extract was performed on a multilayer glass column filled with neutral silica and acid silica. The extract was dissolved in 2 mL of hexane and was placed in the clean-up column. The elution of OCPs was performed with 10 mL of n-hexane and 20 mL of a mix of n-hexane / dichloromethane in a ratio 9:1 (v/v). The concentrated and purified extracts from milk samples were analyzed using the gas chromatograph GC FOCUS using POLARIS Q Ion Trap mass spectrometer (IT-MS) (Thermo Electron Corporation, USA), equipped with an AI 3000 autosampler and splitless Injector. Experimental MS parameters were as follows: the Ion source and Transfer line temperatures were 220ºC and 250ºC, respectively. The splitless Injector temperature was 250ºC. The GC oven was programmed as follows: 50ºC (1 min), 30ºC/min to 180ºC, 5ºC/min to 260ºC, 30ºC/min to 290ºC with a final hold for 3.0 min. A TG-5ms capillary column with a length of 30 m, 0.25 mm ID and a film thickness of 0.25 µm was used. Helium was applied as carrier gas at a flow of 1 ml/min. Each individual sample was analyzed three times and the average of the results obtained was taken.

The detection and identification of target analytes were based on a selected precursor ion followed by application of an adequate excitation voltage for its subsequent fragmentation and the whole mass spectrum of its product ions (IT-MSn mode). GC/IT-MSn detection parameters of individual organochlorine compounds - retention times, precursor ions, voltage and product ions are present in Table 1.

Quality control

Quality control procedures included regular analysis of procedural blanks, analysis of replicate samples, and use of recovery surrogate. Pure reference standard solutions (EPA 625/CLP Pesticides Mix 2000 µg/ml - Supelco) were used for instrument calibration, recovery determination and quantification of compounds. All solvents (acetone, dichloromethane, hexane), reagents and chromatographic silica gel used for analysis were HPLC grade from Sigma-Aldrich (St. Louis, MO, USA).

The statistical analysis of the data was based on the comparison of mean values by a t-test at a statistical significance $p < 0.05$.

Table 1. GC/IT-MSn detection parameters of individual organochlorine compounds - retention times, precursor ions, voltage and product ions.

OCPs	RT (min)	Precursor ion (m/z)	Segment start (min)	Voltage (V)	Product ions (m/z)
Heptachlor	12.50	272	12.30	2.5	235+237+239
Aldrin	13.60	263	13.40	2.0	191+227+263
Heptachlor epoxide	14.91	353	14.70	3.0	253+261+263+282
p,p’-DDE	16.94	246	16.80	3.0	269+290+323+358
Dieldrin	17.20	263	17.00	2.0	205+221+249
Endrin	17.73	263	17.60	2.0	191+227+263
Endosulfan_I	18.04	195	17.95	4.0	123+159+161
p,p’-DDD	18.37	235	18.20	2.0	165+199
Endrin aldehyde	18.69	345	18.60	2.5	243+279+281+315
p,p’-DDT	19.66	235	19.60	2.2	165+199

RESULTS AND DISCUSSION

Population characteristics

The participating women were between 25 and 40 years old, with a mean age of 30.2 years. The mean pre-pregnancy body mass index (BMI) of 21.1 kg/m^2 indicated a normal BMI (BMI range 18.5–24.9). The consumption of meat and eggs from 2 to 4 times per week was reported by 60% and 51% of participants, respectively. Milk and milk products were part of the daily menu of almost half of the mothers (49%). In our study group, 93% of the mothers consumed fish much less than the World Health Organization recommendations (twice a week). Seafood consumption has been suggested as a major contributor to human exposure to POPs from a number of authors [7, 10]. Less than 30% of the participants in the present study defined themselves as smokers before pregnancy. Lipid content in milk samples was in the range of 0.8% to 5.7% (mean value 3.2%).

Levels of OCPs residues in breast milk

A total of 10 organochlorine pesticides were analyzed including: 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane (p,p'-DDT), 1,1-dichloro-2,2-bis(p-chlorophenyl)ethene (p,p'-DDE) and 1,1-dichloro-2,2-bis(p-chlorophenyl)ethane (p,p'-DDD), heptachlor, aldrin, heptachlor epoxide, endosulfan, endrin aldehyde, dieldrin and endrin. The residues of organochlorine pesticides were determined in 45 samples from mothers living in two regions of Northeast Bulgaria – Varna and Dobrich. The summarized results regarding levels of OCPs are reported in Table 2.

The OCPs contamination profile in breast milk was dominated by p,p'-DDE, detected in 100% of samples (both Varna and Dobrich) – Table 2. The p,p'-DDT positive milk samples from Varna were found lower than the positive samples from Dobrich (25.9% and 56.0%, respectively). p,p'-DDD was present in 22.2% and 20.0% of breast milk samples from Varna and Dobrich, respectively. p,p’-DDE, the main metabolite of DDT, was found in all breast milk samples at levels ranging from 16.89 to 94.48 ng/g lipids, mean 42.86 ng/g lipids and contributed 86.6% to the Sum DDTs (sum of DDE, DDD and DDT). Mean DDT concentration in human milk from Dobrich (typical rural area) was measured higher than in samples from Varna (8.44 ng/g and 3.18 lipids, respectively). The lowest levels were found for the metabolite p,p'-DDD, for which almost 80% of the results were below the LOQ (Table 2). These results showed that residues of DDT can still be found in the human body thirty years after its ban, due to its distribution in the environment.

Table 2. Organochlorine pesticides concentrations (mean, ng/g lipid weight) in the human milk samples collected from Varna and Dobrich regions, Bulgaria

Compound	Varna region (N = 27)				Dobrich region (N = 18)			
	% of positive samples ≥ LOQ[a]	ng/g lipid weight			% of positive samples ≥ LOQ [a]	ng/g lipid weight		
		Mean	95th percentile	Range [b]		Mean	95th percentile	Range [b]
p,p’-DDE	100	48.93	87.22	16.89 – 94.48	100	33.75	48.38	18.77 – 50.05
p,p’-DDD	22.2	2.50	12.85	< LOQ – 13.11	20.0	1.90	11.30	< LOQ – 12.56
p,p’-DDT	25.9	3.18	14.51	< LOQ – 16.30	56.0	8.44	15.53	< LOQ – 18.30
Heptachlor	0	nd	–	–	0	nd	–	–
Aldrin	0	nd	–	–	0	nd	–	–
Heptachlor epoxide	3.7	0.04	–	< LOQ – 1.07	0	nd	–	–
Endosulfan	0	nd	–	–	0	nd	–	–
Endrin aldehyde	22.2	1.50	–	< LOQ – 2.06	11.1	1.63	–	< LOQ - 1.80
Dieldrin	0	nd	–	–	0	nd	–	–
Endrin	0	nd	–	–	0	nd	–	–

[a] LOQ – Limit of quantification; [b] Concentration ranges (min – max), nd – not detected, N – number of individual milk samples

The residual amounts of DDE found in the breast milk samples from both studied regions are due to past use of the pesticide DDT and its metabolism. A similar distribution pattern of DDTs in human milk was reported by several authors in Belgium [10], Czech Republic [11], Croatia [12], Norway [13]. The Sum DDTs in breast milk from Varna (54.62 ng/g lipids) was found higher than DDTs levels in milk samples from Dobrich (44.08 ng/g lipids).

Heptachlor, aldrin, endosulfan, dieldrin, endrin were not detected in any of the milk samples. Similar results reported Dong *et al*., 2022 [14]. Heptachlor epoxide was found in one milk sample only. Endrin aldehyde was detected in 17.8% of all milk samples in the range from 1.25 to 10.51 ng/g lipids (from both Varna and Dobrich). The low levels of OCPs observed in breast milk samples correspond to the fact that these environmental pollutants were banned in Europe more than three decades ago.

Impact of maternal age and pre-pregnancy BMI

Because of the long biological half-lives of persistent pesticides and continuous human exposure to environmental pollutants, the concentrations of OCPs in the human body fat are expected to increase with age [13]. Maternal age has been reported as an important determinant affecting residual OCP levels in human milk [11–14]. The results in the present study were summarized by age groups and showed a positive relation between concentration of metabolites DDE and DDD and the age of mothers - Fig. 1.

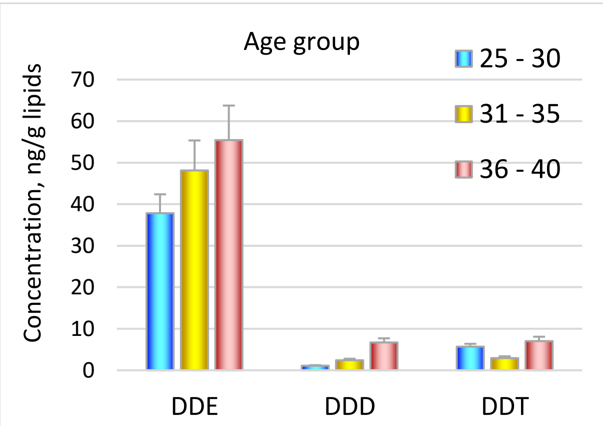

Figure 1. Distribution pattern of DDTs (ng/g lipids) in breast milk by age groups of the study population.

The mean value of the Sum DDTs in the breast milk samples was lowest in mothers aged 25-30 years (44.6 ng/g lipids), in general for both regions – Fig. 2. The levels of total DDTs in breast milk samples increased by age groups 31 – 35 and 36 – 40 years (53.47 ng/g and 69.16 ng/g lipids, respectively). The data obtained may be due to the longer exposure to DDTs of the 36 – 40-year mothers through their diet (as the main route of exposure). The comparison of DDTs residues detected in milk samples from different age groups showed that concentrations were found significantly higher ($p<0.05$) for mothers from Dobrich in 25-30 age than those in 31-35 age (39.33 ng/g and 61.54 ng/g lipids, respectively). Significant associations between OCPs levels in breast milk and age of *primiparae* mothers were described in a number of studies [10-13].

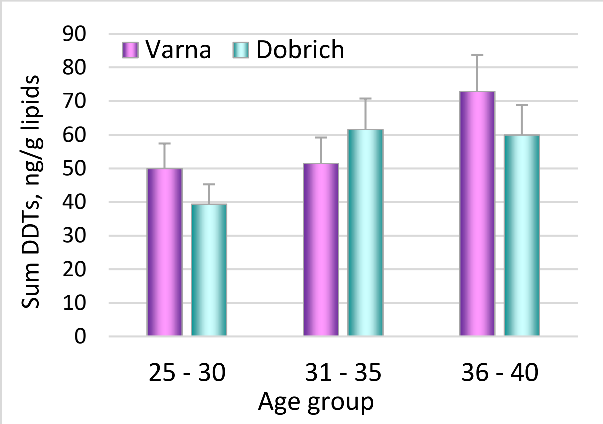

Figure 2. Comparison of Sum DDTs levels in breast milk samples from different age groups (Varna and Dobrich regions).

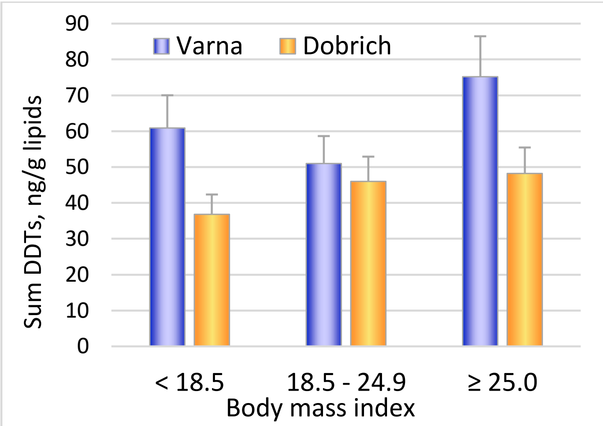

Figure 3. Comparison of Sum DDTs levels in breast milk by BMI groups (Varna and Dobrich regions).

A higher body mass index (BMI) may be associated with a higher body fat content in the human body, suggesting an accumulation of lipophilic pollutants in the adipose tissue of living organisms, including humans [4]. Several authors described a positive correlation between BMI and the serum levels of organochlorine compounds p,p′-DDE in pregnant women [4, 7]. Donors with normal BMI (18.5 – 24.9 kg/m^2) were the predominant group (73% of all participants studied) with the average value of Sum DDTs in breast milk samples – 49.02 ng/g lipids (Fig. 3). The comparison of DDTs levels in milk by BMI of participants showed statistically significant difference ($p<0.05$) between mothers from Varna with normal and high BMI (51.0 ng/g and 75.2 ng/g lipids, respectively). The milk samples from mothers with body mass index BMI ≥ 25 showed the highest Sum DDTs (mean value 66.2 ng/g lipids). Breast milk samples from Dobrich did not show a statistically significant difference in the levels of DDT and metabolites depending on the body mass index.

Comparison with other studies

In the present study, a total median OCPs concentration was 47.07 ng/g lipids. Similar OCPs levels were reported in Belgium by Aerts *et al*., 2019 [10] and in Croatia by Jovanović *et. al.,* 2019 [12]. Relatively higher concentrations of chlorinated pesticides were reported by Iszatt *et al.,* 2019 in Norway (mean, 88 ng/g lipids) [15] and Antignac *et al.*, 2016 in France (median, 106 ng/g lipids) [16]. The median levels of Sum DDTs (DDT, DDE and DDD) in breast milk samples from northeastern Bulgaria were comparable to the median levels reported by other studies - Table 3. Significantly higher were the levels of DDTs in India, one of the largest producers of pesticides in the world and first among the Asian countries [17].

Table 3. Comparison of median concentrations (ng/g lipid weight) of DDTs in human milk from European and Asian countries

Country	Period	N	Sum DDTs, ng/g lipids	Ref.
Bulgaria	2019 – 2021	45	46.8	Present study
Croatia	2014	79	16.8	[12]
Czech Republic	2019 – 2021	231	77	[11]
France	2011 – 2014	42	62.2	[16]
Belgium	2014	206	39.75	[10]
China	2016	60	57.4	[14]
India	2020	130	790.1	[17]

N – number of samples

CONCLUSIONS

Worldwide restrictive and preventive measures regarding persistent pollutants have resulted in a reduction in the residues of a large group of OCPs in human tissues. The pesticide DDT and its metabolites DDE and DDD are still present in traceable amounts in human milk. The residues of the main metabolite DDE indicate usage of DDT in the past. The mean level of DDTs in breast milk from Varna region was found higher than that in samples from Dobrich. Maternal age and pre-pregnancy BMI were found to be the major determinants for the pesticide residues in breast milk samples. Levels of OCPs in breast milk from the northeastern region of Bulgaria were comparable to the levels measured in other European countries. Future studies have to be focused to even further reduce human exposure to POPs and reduce *in utero* and lactation exposure of infants.

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