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Low Levels of Organochlorine Pesticides in Subjects with Metabolic Disturbances: A Survey Taken in Rome in 2001–2002

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Pesticides are used in agriculture to protect crops from a number of pests. In particular organochlorine (OC) pesticides are man-made organic chemicals that have been used to control many pests, from fungi to grasshoppers. DDT was the first to be used on a large scale; it was heavily applied in agricultural regions. Because of their high chemical stability and toxicity, the use has been restricted and then banned, in North America and Europe, in the 1970s. Actually, in some developing countries, DDT and other OC pesticides are still used for malaria control. Some other compounds are still used in industrialized countries; however their use is decreasing due to regulatory limitations. Most OC compounds or their metabolites break down slowly and are stored in fatty tissues of animals because of their liposolubility and persistence, entering the food chain (Stefanelli et al. 2002). They show a high degree of bioaccumulation and biomagnification along the chain itself (Borga et al. 2001). Moreover man is at the top of the chain, so it is possible to detect the presence of these contaminants in human tissues or blood, even years after they have been banned (Nagakawa et al. 1999; Najam et al. 1999; Waliszewski et al. 1999). Recent evidence has suggested that OC pesticides, even at low concentrations, may disrupt the endocrine system, which is responsible for proper hormone balance (Mantovani 2002; Figà-Talamanca et al. 2001).
An Endocrine Disrupter is an exogenous substance or a mixture, that alters functions of the endocrine system, causing adverse health effects in an intact organism or its progeny or subpopulations (*European Commission. Endocrine Disrupter Website*). In the framework of a pilot project of the Italian National Health Institute aimed at the evaluation of risks for reproduction and development, we have analysed biomarkers of 26 OC pesticides in blood samples from 32 subjects with metabolic disturbances, collected in Italy between July 2001 and December 2002. One of the objectives of the project was to investigate a possible correlation between OC residue levels and metabolic disturbances.

MATERIALS AND METHODS

In July 2001-December 2002, blood samples were collected from 32 obese patients with endocrine problems attending the endocrinological day hospital of the Rome's hospital "Policlinico Umberto I"; informed consent was obtained by each subject. Samples, an amount of about eight grams, were delivered in plastic

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Table 1. Details of analyzed samples.

code	sex	gross weight (tube+blood+ acid solution) [g]	volume of acid solution [mL]
1	F	18.16	0.2
2	M	17.84	0.4
3	F	17.76	0.5
4	F	17.33	0.5
5	F	18.12	0.5
6	F	17.85	1.0
7	F	17.58	0.5
8	M	17.38	1.0
9	F	18.14	0.5
10	F	17.42	1.0
11	F	17.51	0.5
12	F	17.61	0.5
13	M	17.69	0.5
14	F	18.29	0.5
15	F	17.48	0.5
16	F	16.39	1.0
17	F	18.35	0.5
18	F	16.88	0.5
19	M	17.40	0.5
20	F	17.23	0.5
21	F	15.18	1.0
22	M	17.29	1.0
23	F	17.80	1.0
24	F	17.42	0.5
25	F	17.88	0.5
26	M	17.38	1.0
27	M	17.11	1.0
28	F	15.76	1.0
29	F	17.85	0.5
30	M	17.96	0.5
31	F	15.61	1.0
32	M	17.07	1.0

tubes weighing 10.1 g. A known volume of acid saline solution (pH 1) had been added in each tube for safety reasons, immediately after sampling. Tubes were stored in a fridge (4°C) until analyses. In table 1 details of 32 samples analysed are reported.

The exact amount of blood contained in each tube was calculated by subtracting the weight of the added acid and a fixed weight of 10.1 g to allow for the weight of the plastic tube. Pesticide-grade reagents and solvents were used throughout.

The blood contained in the tube was poured into a becker and mixed with 15 g of a mixture of Extrelut powder (Extrelut NT, Merck, cod. 1.15092.1000) + magnesium sulphate (anhydrous, reagent grade, Fluka, cod. 63135) (3 + 1, w + w), washing the tube with bidistilled water and collecting the washings into the becker. The mixture was transferred into a glass column, 300 x 25 mm i.d., with PTFE stopcock and slightly compressed by a glass rod to an height of 14 cm. The column was eluted with 5 x 20 mL dichloromethane, collecting into a 150 mL Erlenmeyler flask (weighed at ± 0.01 g). The eluted solvent was concentrated to a small volume (ca. 1-2 mL) by rotary evaporator (bath temperature 40°C; reduced pressure) and then to dryness by manually rotating the flask. The residue was weighed and was in the range 6 - 21 mg. The residue was redissolved in 1 mL n-hexane and cleaned up and fractionated by adsorption chromatography over activated Florisil (2.5 g; 60-100 mesh, Supelco, Bellefonte, PA, USA, cat. N.2-0280, activated at 130°C overnight).

The OC pesticides analysed are listed in table 2: in particular heptachlor, aldrin, mirex, were eluted from the column by 30 mL n-hexane (1st fraction). Most OC pesticides were eluted by a fraction of 25 mL n-hexane + toluene (80 + 20, v + v) (2nd fraction). Other pesticides, such as heptachlor epoxide (HEPO), α-endosulfan, endrin, dieldrin and methoxychlor were eluted by 30 mL n-hexane + toluene + ethyl acetate (180 + 19 + 1, v + v + v) (3rd fraction). Other pesticides were split between 1st and 2nd fraction [trans-nonachlor, hexachlorobenzene (or HCB), α-chlordene, γ-chlordene and p,p'-DDE] or between 2nd and 3rd fraction (β-HCH and δ-HCH). In this case the recoveries were calculated as sum of both fractions. The fractions were separately collected and analysed. 1 mL isooctane (as "keeper") was added to the 1st fraction only. The fractions were concentrated to a small volume (ca. 1 mL) by rotary evaporator (bath temperature 40°C for 1st fraction and 50°C for the others; reduced pressure). The internal standard solution (PCB 209) was added before analysing by gas chromatography (GC) with electron capture detector (ECD).

OC pesticides were determined using a capillary gas chromatograph equipped with twin splitless injectors and twin electron capture detectors. The GC used was an HP 5890 Series II equipped with two fused silica capillary columns, DB-XLB and SP-1701, both 30 m x 0.25 mm i.d. x 0.25 μm film thickness operated under the following conditions: Helium carrier gas, flow rate 1.5 mL/min supplied through Electronic Pressure Control (EPC) in constant flow mode; oven temperature programming: 60°C, 2 min hold, ramp to 160°C at a rate of 10°C/min, 160-250°C at a rate of 2°C/min, with a final hold of 10 min: injection 1 μL into split/splitless injectors used in splitless mode (with vent time 1 min) equipped with a dual-tapered deactivated glass liner using HP-7673 autosamplers. Samples were subjected to a qualitative confirmation by gas chromatography/mass spectrometry (GC/MS). The GC/MS used was an Agilent mod. 5973 Network, equipped with a fused silica capillary column HP-5MS, 30 m x 0.25 mm i.d. x 0.25 μm film thickness, operated under the following conditions: Helium carrier gas, flow rate 1.5 mL/min in constant flow mode; oven

Table 2. Recovery values of the twenty-six OC pesticides investigated.

OC pesticides	Florisil fractions	Added amount [ng]	Spiking level for 8 g of blood sample [ng/g = ppb]	Mean recovery % [n = 3]	Reporting limit [ng/g = ppb]
heptachlor	1	20	2.50	76	0.20
aldrin	1	20	2.50	85	0.15
mirex	1	55	6.88	92	0.18
trans-nonachlor	1-2	19	2.38	90	0.15
HCB	1-2	19	2.38	88	0.10
α-chlordene	1-2	40	5.00	106	0.19
γ-chlordene	1-2	40	5.00	103	0.21
p,p'-DDE	1-2	39	4.88	90	0.17
α-chlordane	2	37	4.63	85	0.22
γ-chlordane	2	49	6.13	76	0.20
quintozene	2	20	2.50	63	0.14
α-HCH	2	19	2.38	76	0.24
γ-HCH	2	20	2.50	70	0.23
oxychlordane	2	45	5.63	87	0.27
o,p'-DDD	2	59	7.38	100	0.37
p,p'-DDD	2	40	5.00	95	0.44
o,p'-DDT	2	60	7.50	73	0.32
p,p'-DDT	2	57	7.13	86	0.51
o,p'-DDE	2	58	7.25	87	0.27
β-HCH	2-3	39	4.88	84	0.25
δ-HCH	2-3	20	2.50	56	0.14
HEPO	3	19	2.38	79	0.17
α-endosulfan	3	21	2.63	57	0.17
endrin	3	39	4.88	75	0.28
dieldrin	3	39	4.88	78	0.20
methoxychlor	3	100	12.50	54	0.72

temperature programming: 60°C, 2 min hold, ramp to 160°C at a rate of 10°C/min, 160-260°C at a rate of 3°C/min, with a final hold of 20 min; injection 1 μL into split/splitless injector used in a splitless mode (with vent time 1 min) equipped with a dual-tapered deactivated glass liner using a HP-7673 autosampler; quadrupolar mass filter, transfer line temperature 280°C, Electron Impact (EI) ion source with ionisation energy 70 eV. Finally, statistical comparison of analytical results between male and female subjects was performed (Miller & Miller, Third Edition).

RESULTS AND DISCUSSION

In classical methods to analyse blood samples, compounds are extracted using a mixture of solvents. For example a first extraction of blood is carried out with a

Table 3. Blood concentrations of HCB and p,p'-DDE.

Sample and Sex	HCB [ng/g = ppb]	p,p'-DDE [ng/g = ppb]
2M	–	–
8M	0.22	0.58
13M	–	–
19M	–	–
22M	0.17	–
26M	0.14	1.90
27M	–	–
30M	–	0.56
32M	–	–
$\bar{x}$ (positive value)	0.18	1.01
S.D.	0.04	0.77
n	3	3
1F	–	–
3F	0.16	0.36
5F	–	0.27
6F	–	–
7F	–	–
9F	0.21	0.45
10F	0.11	–
11F	0.12	–
12F	0.28	0.18
14F	0.10	–
15F	0.12	0.21
16F	–	–
17F	–	0.22
18F	–	–
20F	1.06	1.69
21F	–	–
23F	0.28	0.56
24F	–	0.59
25F	–	0.26
28F	–	0.30
29F	–	–
31F	–	0.23
$\bar{x}$ (positive value)	0.27	0.44
S.D.	0.30	0.42
n	9	12
F	56.59	3.41
F_{cr}	38.80	7.96
Pooled s	–	0.49
t	–	1.81
t_{cr}	–	2.16

mixture of methanol and (1+1) diethyl ether + hexane and a following re-extraction is done with diethyl ether + hexane (Rathore et al. 2002). Using an other procedure, compounds are extracted from plasma with a mixture of saturated aqueous ammonium sulphate, ethanol and hexane. The extraction with hexane is repeated (Butler Walker et al. 2003). In any case, a clean-up is carried out on the extracts. On the contrary, we used a direct extraction followed by a clean-up with Florisil. An aliquot of some samples found free of the investigated OC pesticides was spiked and analysed for recovery tests. In table 2 the mean of recovery values obtained is reported; for the most part results are in the range 70-106%, except for quintozene, α-endosulfan, δ-HCH and methoxychlor. The reporting limit for most of that were in the range 0.10 - 0.30 ng/g, except for o,p'-DDD, p,p'-DDD, p,p'-DDT and methoxychlor. The reporting limit was established by assuming a minimum area counts of 5000 and converting into the equivalent level (ng/g) according to the pertinent response factor. The method used was suitable also for polychlorobiphenyls (PCB), that share analogous properties as OC pesticides and are still widely diffused contaminants, following their past use in electrical transformers and other industrial products. However, concerning the analysis of OC pesticides in our study, there was not interference from PCB because of the different retention times.

In table 3 results of the analysed samples are reported; only two OC pesticides (HCB and p,p'-DDE) show amounts higher than reporting limit in some of the samples. For each compound a statistical comparison has been carried out between male and female population. At first two-tailed F-test has been accomplished to compare the standard deviations. F-values obtained ($F = 56.59$ for HCB and $F = 3.41$ for p,p'-DDE) compared with F critical values ($F_{cr} = 38.80$ for HCB and $F_{cr} = 7.96$ for p,p'-DDE) allowed to carry out a t-test to compare the means between male and female populations only for p,p'-DDE. The null hypothesis adopted is that there is not difference between the populations. The t-value obtained ($t = 1.81$) is lower than the corresponding critical value ($t_{cr} = 2.16$), therefore the null hypothesis is retained.

To our best knowledge, there are not recent data available on the blood concentrations of OC in Italy. Generally analysed samples showed low contamination by OC pesticides, proving the positive, favourable long term effect of regulatory measures. In particular, DDT was represented only by the presence of p,p'-DDE, its main animal metabolite; this seems to indicate that it is not a recent pollution but the consequence of DDT previous use in public health programs or agricultural practice. HCB was the only other contaminant detected, possibly due not only to its high persistence, but also to its importance as by-product of industrial chlorination processes. Other OC were not detected, indicating a lower exposure and/or persistence. OC pesticides are highly liposoluble, so the contamination levels in human blood show the mobility within adipose tissue due to lipoproteins. Obviously analyses carried out directly on adipose tissue reveal higher amounts (Smeds and Saukko, 2001). Comparing the obtained results with analogous monitoring studies on human blood in other

countries, two different situations can be noticed. In the countries where OC pesticides are still used to fight vector borne diseases, monitored samples reflect a higher extent of exposure for all the population (Waliszewski et al. 1999, Rathore et al. 2002). On the other hand, higher amounts of OC pesticides were observed in some other European surveys; however, such studies were performed on population groups with additional occupational exposure, either past or still occurring, i.e. farmers and pesticide applicators (Hernàndez et al. 2002). It is noticeable that, although our sample was composed by people with obesity and related endocrine and metabolic disturbances, their levels were not significantly higher than those expected in the general populations, according to data obtained in other western countries (Covaci and Schepens, 2001). Nevertheless, a full evaluation of exposure data will require the setting of reference values and their possible variation according to such factors as age, sex, etc. It is noticeable that sex was not a significant confounding factor in our data series.
In conclusion all the available studies show the importance of frequent monitorings to control and to prevent potential risks to human health.

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