1. Introduction

Many environmental organochlorine pollutants including polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), coplanar polychlorinated biphenyls (cPCBs), *ortho*-substituted polychlorinated biphenyls (PCBs) and chlorinated pesticides have the potential to mimic or antagonize naturally occurring hormones and might affect wildlife and humans adversely (Kavlock et al., 1996; Wolff et al., 1993). PCDDs and PCDFs were unintended byproducts of several industries and processes: the herbicide industry, the chlorine and paper industry, melting processes and incineration of waste. More than 80% of total PCDDs and PCDFs released into the environment in Japan have been estimated to be derived from incinerators (Watanabe et al., 1999). PCBs and pesticides were used in industry and agriculture until the early 1970s. These organochlorines are resistant to metabolism and are lipid soluble; they bioaccumulate in the food chain, and are found in human adipose tissue, blood, and breast milk (Safe, 2000). Human exposure to organochlorines occurs almost exclusively through food consumption. Various kinds of fish from several supermarkets in Japan were reported to contain high levels of PCDDs, PCDFs and PCBs, and the mean daily intake of total toxic equivalency (TEQ) values of PCDDs, PCDFs and PCBs from fish and shellfish were higher than that from other foods (Tsutsumi et al., 2001). The main foods contributing to dietary intake of chlorinated pesticides including total hexachlorocyclohexane (HCH) and total bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT) have been also reported to be fish and meat among the Japanese population (Nakagawa et al., 1995). These findings suggest that people who often consume fish or meat would accumulate higher levels of PCDDs, PCDFs, PCBs and pesticides in their body.

In the present study, we measured serum levels of organochlorines including 8 PCDDs, 10 PCDFs, 4 cPCBs, 36 PCBs, and 13 chlorinated pesticides or their metabolites in 80 Japanese infertile women in a hospital-based cross-sectional study. The object of the present study was to evaluate background levels of exposure to organochlorines in Japanese women of reproductive age, and to estimate the effect of lifestyle factors, especially dietary factors, on serum organochlorine levels.

2. Subjects and methods

2.1. Subjects and sample collection

Eligible subjects were women aged 20 to 45 years who complained of infertility and consulted doctors in the Department of Obstetrics and Gynecology, the Jikei University School of Medicine, from 1999 to 2000. A total of 139 women were diagnosed laparoscopically according to the revised classification of the American Fertility Society (1985). Fifty-eight women with stage II or greater endometriosis were designated 'cases.' Eighty-one women who were laparoscopically confirmed not to have endometriosis (stage 0 or I) were designated 'controls.' Because accumulation of organochlorines in the body has been proposed as a risk factor for endometriosis (Rier et al., 1993), endometriosis cases might present higher serum organochlorine levels. Of eighty-one control subjects, we excluded one subject whose serum PCB levels could not be measured because of small blood sample volume. Consequently, eighty subjects, aged 26–43 (mean age, 32.9 years), were included in this study. All subjects gave their written informed consent. The study protocol was approved by the Institutional Review Board of the Jikei University School of Medicine, National Cancer Center, National Institute for Environmental Studies, and U.S. Centers for Disease Control and Prevention (CDC).

A fasting blood sample was obtained before the laparoscopic examination. Serum was immediately collected by centrifugation, transferred into a stock tube and stored at −80 °C until analyzed.

2.2. Questionnaire survey

Subjects were interviewed by a single trained interviewer using a structured questionnaire before the laparoscopic examination. The questionnaire included demographic and anthropometric information, occupation, and use of alcohol and tobacco. Regarding dietary habits, subjects were asked how often they consumed 6 food items (fish, meat, rice, vegetable, fruit and dairy products) over the previous year. The frequency of dietary intake was classified into nine categories, i.e., rare, 1–3 times/month, 1–2 times/week, 3–4 times/week, 5–6 times/week, 1 time/day, 2–3 times/day, 4–6 times/day, and more than 7 times/day.

Table 1
Lipid-adjusted serum median levels of organochlorines and total TEQs among Japanese women

	Subjects with detectable values[a]	Median level (25th, 75th)[b]	Mean LOD (SD/Maximum)
PCDDs/PCDFs/cPCBs (pg/g lipid)			
2,3,7,8-TetraCDD[c]	7/80	<LOD (<LOD,<LOD)	2.6 (1.8/12.2)
1,2,3,7,8-PentaCDD[c]	30/80	<LOD (<LOD, 5.5)	3.1 (2.2/15.9)
1,2,3,4,7,8-HexaCDD[c]	5/80	<LOD (<LOD, <LOD)	5.7 (4.2/25.1)
1,2,3,6,7,8-HexaCDD[c]	76/80	26.1 (20.7, 37.1)	5.0 (4.0/26.1)
1,2,3,7,8,9-HexaCDD[c]	34/80	<LOD (<LOD, 4.6)	5.2 (4.0/27.1)
1,2,3,4,6,7,8-HeptaCDD[c]	77/80	16.8 (13.2, 23.7)	5.6 (4.3/31.8)
1,2,3,4,6,7,8,9-OctaCDD[c]	80/80	265.5 (196.0, 389.0)	102.7 (87.2/637.0)
2,3,7,8-TetraCDF[c]	1/80	<LOD (<LOD, <LOD)	2.58 (1.6/12.7)
1,2,3,7,8-PentaCDF[c]	1/80	<LOD (<LOD, <LOD)	2.9 (2.0/14.3)
2,3,4,7,8-PentaCDF[c]	75/80	11.3 (8.7, 13.8)	3.0 (2.1/14.9)
1,2,3,4,7,8-HexaCDF[c]	78/80	6.2 (4.3, 8.4)	3.4 (2.5/16.4)
1,2,3,6,7,8-HexaCDF[c]	76/80	6.1 (5.0, 7.9)	3.3 (2.5/17.2)
1,2,3,7,8,9-HexaCDF[c]	1/80	<LOD (<LOD, <LOD)	3.5 (2.7/18.6)
2,3,4,6,7,8-HexaCDF[c]	47/80	2.0 (<LOD, 3.6)	3.5 (2.6/17.3)
1,2,3,4,6,7,8-HeptaCDF[c]	39/80	<LOD (<LOD, 5.4)	4.4 (3.4/24.3)
1,2,3,4,7,8,9-HeptaCDF[c]	1/80	<LOD (<LOD, <LOD)	4.8 (3.6/24.0)
3,4,4′,5-TetraCB[c]	79/80	8.2 (6.3, 11.6)	5.7 (3.8/26.8)
3,3′,4,4′,5-PentaCB[c]	77/80	47.6 (31.9, 70.2)	5.4 (3.7/22.7)
3,3′,4,4′,5,5′-HexaCB[c]	80/80	34.0 (26.5, 43.7)	6.4 (4.8/31.7)
PCBs (IUPAC nos.) (ng/g lipid)			
PCB44	76/80	4.4 (3.2, 5.7)	4.6 (4.8/18.2)
PCB49	63/80	3.0 (1.8, 4.0)	3.7 (3.0/11.9)
PCB52	71/80	5.5 (3.3, 7.7)	3.9 (3.5/13.8)
PCB66	79/80	1.8 (1.5, 2.6)	4.4 (2.5/15.4)
PCB74	80/80	6.4 (4.9, 9.2)	2.8 (1.6/9.9)
PCB87	79/80	1.1 (0.8, 1.9)	2.6 (1.5/9.3)
PCB99	80/80	6.8 (4.5, 9.5)	2.5 (1.5/8.5)
PCB101	79/80	2.6 (1.9, 4.6)	3.8 (2.2/14.8)
PCB105[c]	80/80	2.0 (1.4, 2.9)	4.0 (2.5/19.8)
PCB110	79/80	1.8 (1.1, 2.9)	3.6 (2.1/13.9)
PCB118[c]	79/79	10.5 (7.2, 15.0)	6.5 (3.6/25.2)
PCB128	67/80	0.3 (0.2, 0.5)	2.4 (1.5/8.3)
PCB138+158[d]	80/80	16.8 (11.6, 26.2)	2.9 (1.6/10.3)
PCB146	80/80	5.9 (3.3, 7.8)	2.5 (1.4/8.6)
PCB151	73/73	0.6 (0.4, 0.8)	2.5 (1.5/9.3)
PCB153	80/80	36.6 (23.3, 51.0)	2.7 (1.6/10.1)
PCB156[c]	79/79	3.4 (2.3, 4.9)	5.5 (3.3/22.9)
PCB157[c]	75/76	0.9 (0.6, 1.3)	6.4 (3.8/26.6)
PCB167[c]	71/72	1.6 (0.9, 2.2)	7.8 (5.1/36.0)
PCB170	80/80	8.2 (5.1, 11.4)	2.6 (1.4/9.29)
PCB172	80/80	1.4 (0.9, 2.0)	2.7 (1.6/11.0)
PCB178	78/80	2.0 (1.2, 2.7)	2.6 (1.5/9.7)
PCB180	80/80	21.6 (12.9, 28.9)	2.7 (1.6/11.0)
PCB183	80/80	2.4 (1.5, 3.2)	2.6 (1.5/9.7)
PCB187	80/80	8.7 (5.0, 12.5)	2.6 (1.4/9.2)
PCB189[c]	60/60	0.4 (0.2, 0.6)	8.4 (5.3/40.9)
PCB194	80/80	2.4 (1.5, 3.7)	3.9 (2.8/23.9)
PCB195	79/80	0.9 (0.6, 1.4)	7.9 (5.7/47.9)
PCB196+203[d]	80/80	1.9 (1.3, 2.9)	2.6 (1.6/9.7)
PCB201	79/80	2.4 (1.6, 3.7)	2.6 (1.6/9.7)
PCB206	78/80	0.8 (0.5, 1.1)	7.4 (4.8/40.3)
PCB209	80/80	0.7 (0.5, 0.9)	9.6 (6.5/56.6)

Table 1 (*continued*)

	Subjects with detectable values[a]	Median level (25th, 75th)[b]	Mean LOD (SD/Maximum)
Pesticides (ng/g lipid)			
HCB	2/80	<LOD (<LOD, <LOD)	16.1 (2.7/25.7)
b-HCCH	80/80	93.2 (60.8, 171.0)	8.1 (1.4/13.0)
g-HCCH	2/80	<LOD (<LOD, <LOD)	8.1 (1.4/13.0)
H.EPOX	8/80	<LOD (<LOD, <LOD)	8.1 (1.4/13.0)
Oxychlordane	54/80	9.0 (<LOD, 12.2)	8.1 (1.4/13.0)
trans-NONA	78/80	20.9 (16.0, 29.3)	8.1 (1.4/13.0)
pp-DDE	80/80	221.0 (146.0, 358.5)	8.1 (1.4/13.0)
Dieldrin	4/80	<LOD (<LOD, <LOD)	6.3 (1.1/10.1)
op-DDT	0/80	<LOD (<LOD, <LOD)	16.2 (2.8/25.9)
pp-DDT	7/80	<LOD (<LOD, <LOD)	16.2 (2.8/25.9)
Mirex	1/80	<LOD (<LOD, <LOD)	8.1 (1.4/13.0)
Total TEQ values (pg TEQ/g lipid)			
PCDDs	80/80	8.6 (6.4, 10.8)	
PCDFs	80/80	7.5 (6.3, 9.0)	
cPCBs	80/80	5.1 (3.5, 7.4)	
PCBs	80/80	3.6 (2.4, 5.0)	
PCDDs/PCDFs	80/80	16.1 (13.0, 19.6)	
PCDDs/PCDFs/cPCBs	80/80	21.6 (17.4, 26.9)	
Sum	80/80	25.1 (20.3, 31.8)	

Abbreviations: LOD—limit of detection; SD—standard deviation; CDD—chlorodibenzo-*p*-dioxin; CDF—chlorodibenzofuran; CB—chlorobiphenyl; HCB—hexachlorobenzene; HCCH—hexachlorocyclohexane; H.EPOC—heptachlor epoxide; NONA—nonachlor; DDE—bis(4-chlorophenyl)-1,1-dichloroethene; DDT—bis(4-chlorophenyl)-1,1,1-trichloroethene; TEQ—toxic equivalency.

[a] Number of subjects with values above LOD/number of measured subjects.

[b] 25th—25th percentile; 75th—75th percentile.

[c] WHO-TEF values were assigned.

[d] Combined levels for PCB138,158 and PCB196,203 were analyzed.

2.3. Analytical methods

Serum analyses for a total of 71 compounds, 8 PCDDs, 10 PCDFs, 4 cPCBs, 36 PCBs and 13 selected persistent chlorinated pesticides or their metabolites, were performed at the U.S. Centers for Disease Control and Prevention (CDC) by gas chromatography/high-resolution isotope dilution mass spectrometry. The analytical methods and quality control procedures have been described previously (DiPietro et al., 1997; Patterson et al., 1987; Turner et al., 1994). Because the PCDDs, PCDFs, cPCBs, PCBs and pesticides are lipophilic and concentrate in the body's lipid stores including the lipid in serum, the serum levels for these compounds were adjusted for serum lipid levels. Triglycerides and total cholesterol were used in calculating the total lipid level (2.27 × total cholesterol + triglycerides + 62.3). The mean volume of blood used for the analyses was 7.99 g (range: 0.89–13.2 g). Limits of detection (LOD) on a lipid-adjusted basis were calculated for each sample. Because we could not measure PCB138 and 158, or PCB196 and 203 separately, combined values for PCB138/158 and PCB196/203 were reported. The list of organochlorines measured and their mean LOD values are shown in Table 1. TEQ was assessed using the "toxic equivalency factor" (TEF) based upon the relative potency of each congener compared to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as the most potent of the compounds (Van den Berg et al., 2000). The World Health Organization (WHO)-TEF values were assigned 7 PCDDs, 10 PCDFs, 4 cPCBs and 6 PCBs to calculate TEQ values of PCDDs, PCDFs, cPCBs and PCBs in this study (Table 1) (Van den Berg et al., 1998). For values below LOD ('<LOD'), a value of one half the LOD was assigned (Hornung and Reed, 1990). The results were essentially similar when zeros were assigned to values <LOD.

2.4. Statistical analysis

Total levels of PCDDs/PCDFs/cPCBs, PCBs and pesticides were calculated for the sum of serum molar concentration of PCDDs/PCDFs/cPCBs, PCBs and

pesticides, respectively. Differences in log-transformed levels of total TEQ, PCDDs/PCDFs/cPCBs, PCBs and pesticides between subgroups were tested by the analysis of variance (PROC GLM, SAS, SAS Institute Inc., Cary, NC). Tests for trend were assessed by using serum organochlorine levels as continuous variables. P values less than 0.05 (two-tail) were considered to be statistically significant. All analyses were conducted using the SAS (version 8.2) program.

Table 2
Comparisons of serum organochlorine levels according to age, residence, occupation, BMI, smoking and alcohol habit among Japanese women

Variable	No.[a]	Total TEQ (pg TEQ/g lipid)	Total PCDDs/PCDFs/ cPCBs (pmol/g lipid)	Total PCBs (nmol/g lipid)	Total pesticides (nmol/g lipid)	p,p′-DDE (ng/g lipid)
		Median (25th, 75th)[b]	Median (25th, 75th)[b]	Median (25th, 75th)[b]	Median (25th, 75th)[b]	Median (25th, 75th)[b]
Total	80	25.1 (20.3, 31.8)	1.15 (0.84, 1.50)	0.46 (0.35, 0.66)	1.32 (0.92, 1.93)	221.0 (146.0, 358.5)
Age (years)						
24–29	15	23.7 (19.5, 25.7)	1.13 (0.78, 1.35)	0.41 (0.37, 0.54)	1.04 (0.83, 1.38)	191.0 (146.0, 271.0)
30–35	45	23.5 (19.6, 28.7)	1.13 (0.86, 1.41)	0.41 (0.31, 0.63)	1.18 (0.84, 1.70)	201.0 (119.0, 346.0)
36–43	20	31.9 (24.6, 39.0)	1.27 (0.91, 2.25)	0.61 (0.52, 0.83)	1.89 (1.42, 2.62)	297.5 (212.0, 537.0)
P for difference[c]		0.005	0.01	0.003	0.002	0.014
P for trend		0.003	0.01	0.005	0.01	0.02
Residence						
Residential area	61	24.6 (20.5, 32.0)	1.14 (0.84, 1.46)	0.45 (0.34, 0.63)	1.32 (0.89, 1.91)	221.0 (143.0, 323.0)
Shopping or office area	6	29.3 (28.6, 29.6)	1.15 (0.81, 1.27)	0.64 (0.45, 0.72)	1.64 (1.33, 1.70)	287.0 (215.0, 358.0)
Agricultural or fishing area	7	26.0 (17.8, 42.9)	1.41 (0.85, 1.86)	0.53 (0.38, 0.88)	1.26 (0.93, 3.24)	244.0 (146.0, 519.0)
Industrial area	2	26.7 (12.2, 41.2)	1.42 (0.62, 2.23)	0.42 (0.25, 0.60)	2.03 (0.95, 3.11)	441.5 (179.0, 704.0)
P for difference[c]		0.79	0.90	0.47	0.29	0.23
Occupation						
Office worker	43	24.6 (20.1, 32.0)	1.07 (0.83, 1.36)	0.47 (0.32, 0.63)	1.23 (0.85, 1.78)	215.0 (126.0, 318.0)
Specialist	18	25.3 (19.8, 32.2)	1.20 (0.86, 1.61)	0.48 (0.40, 0.71)	1.61 (1.01, 2.54)	250.0 (179.0, 592.0)
Others[d]	13	29.2 (20.7, 32.5)	1.35 (0.78, 1.86)	0.46 (0.38, 0.67)	1.70 (0.89, 1.97)	225.0 (179.0, 359.0)
P for difference[c]		0.86	0.09	0.54	0.29	0.44
BMI						
<19.4	25	27.4 (21.1, 32.5)	1.18 (0.83, 1.46)	0.54 (0.46, 0.67)	1.38 (0.93, 1.83)	221.0 (143.0, 346.0)
19.4–21.0	26	25.8 (20.7, 33.5)	1.18 (0.97, 1.53)	0.44 (0.32, 0.63)	1.32 (0.89, 1.95)	202.0 (150.0, 386.0)
>21.0	25	24.0 (17.8, 26.2)	1.00 (0.84, 1.29)	0.41 (0.31, 0.66)	1.33 (1.07, 2.33)	230.0 (146.0, 323.0)
P for difference[c]		0.22	0.27	0.02	0.82	0.66
P for trend		0.10	0.18	0.06	0.91	0.82
Smoking						
Never	50	25.7 (20.9, 32.2)	1.13 (0.84, 1.46)	0.48 (0.36, 0.66)	1.44 (1.01, 1.95)	228.5 (161.0, 359.0)
Past	10	24.4 (17.7, 31.7)	1.16 (0.83, 1.86)	0.45 (0.36, 0.60)	0.89 (0.62, 2.39)	157.5 (109.0, 453.0)
Current	16	24.6 (18.3, 31.2)	1.19 (0.82, 1.41)	0.41 (0.30, 0.67)	1.12 (0.84, 1.68)	195.5 (132.5, 282.5)
P for difference[c]		0.51	0.27	0.21	0.23	0.24
P for trend		0.36	0.24	0.24	0.89	0.54
Alcohol drinking						
≦3 times a month	37	23.3 (20.0, 26.2)	1.00 (0.83, 1.27)	0.41 (0.33, 0.60)	1.26 (0.92, 1.63)	203.0 (146.0, 281.0)
1–4 times a week	24	26.6 (22.3, 34.3)	1.26 (1.03, 1.94)	0.52 (0.36, 0.77)	1.65 (0.88, 2.55)	226.5 (134.0, 409.0)
≧5 times a week	15	30.1 (17.0, 34.0)	1.21 (0.99, 1.36)	0.60 (0.37, 0.72)	1.45 (0.68, 2.34)	256.0 (105.0, 618.0)
P for difference[c]		0.06	0.20	0.20	0.37	0.33
P for trend		0.06	0.40	0.20	0.11	0.16

[a] Total number of subjects for each item varied due to missing information.
[b] 25th—25th percentile; 75th—75th percentile.
[c] Differences in log-transformed levels between subgroups were tested by the analysis of variance.
[d] Others include merchant, housewife and so on.

3. Results

In this study, three PCDDs/PCDFs/cPCBs (1,2,3,4,6,7,9-heptachlorodibenzo-*p*-dioxin, octachlorodibenzofuran and 3,3′,4,4′-tetrachlorobiphenyl), four PCBs (International Union of Pure and Applied Chemistry nos. 18, 28, 149 and 177), and two pesticides (aldrin and endrin) could not be measured because of analytical conditions. Serum median levels of total TEQ of 7 PCDDs, 9 PCDFs, 3 cPCBs and 6 PCBs were 8.6, 7.5, 5.1 and 3.6 pg TEQ/g lipid, respectively (Table 1). The serum median level of the total TEQ of PCDDs/PCDFs/cPCBs/PCBs was 25.1 pg TEQ/g lipid. Serum median levels of total PCDDs/PCDFs/cPCBs, PCBs and pesticides were 11.5 pmol/g lipid, 0.46 nmol/g lipid and 1.32 nmol/g lipid, respectively (Table 2).

Table 2 shows the serum median levels of total TEQ, 19 PCDDs/PCDFs/cPCBs, 32 PCBs and 11

Table 3
Relationships between serum organochlorine levels and frequency of food intake among Japanese women

Frequency of food intake ($n=76$)	No.	Total TEQ (pg TEQ/g lipid)	Total PCDDs/PCDFs/cPCBs (pmol/g lipid)	Total PCBs (nmol/g lipid)	Total pesticides (nmol/g lipid)	p,p′-DDE (ng/g lipid)
		Median (25th, 75th)[a]	Median (25th, 75th)[a]	Median (25th, 75th)[a]	Median (25th, 75th)[a]	Median (25th, 75th)[a]
Fish						
≦3 times a month	7	17.9 (12.2, 27.7)	0.75 (0.62, 0.99)	0.36 (0.26, 0.61)	0.92 (0.63, 1.38)	146.0 (104.0, 215.0)
1–4 times a week	58	24.8 (20.5, 32.0)	1.16 (0.86, 1.46)	0.43 (0.34, 0.61)	1.25 (0.89, 1.83)	221.0 (143.0, 323.0)
≧5 times a week	11	30.7 (23.3, 42.9)	1.41 (0.81, 2.34)	0.73 (0.53, 0.88)	2.27 (1.72, 2.70)	367.0 (277.0, 578.0)
P for difference[b]		0.001	0.002	0.0002	0.001	0.001
P for trend		0.002	0.003	0.0003	0.006	0.002
Meat						
≦2 times a week	12	24.6 (21.0, 30.9)	1.05 (0.98, 1.29)	0.57 (0.35, 0.64)	1.42 (1.11, 1.84)	235.5 (206.0, 358.0)
3–4 times a week	42	25.7 (19.8, 32.0)	1.18 (0.84, 1.44)	0.44 (0.33, 0.61)	1.33 (0.85, 1.96)	205.0 (126.0, 323.0)
≧5 times a week	22	24.9 (21.5, 36.5)	1.16 (0.83, 2.10)	0.48 (0.37, 0.77)	1.32 (0.92, 2.70)	373.5 (146.0, 570.0)
P for difference[b]		0.80	0.39	0.69	0.57	0.73
P for trend		0.63	0.35	0.46	0.13	0.16
Rice						
≦6 times a week	9	29.2 (20.1, 31.7)	1.12 (0.71, 1.29)	0.47 (0.34, 0.66)	1.23 (0.93, 1.64)	221.0 (183.0, 256.0)
Once a day	24	25.8 (21.1, 36.7)	1.23 (0.91, 1.55)	0.46 (0.35, 0.73)	1.32 (0.82, 2.16)	214.0 (128.0, 444.0)
≧2 times a day	43	24.6 (19.8, 29.6)	1.13 (0.84, 1.46)	0.46 (0.36, 0.63)	1.42 (0.92, 1.95)	221.0 (146.0, 367.0)
P for difference[b]		0.38	0.81	0.94	0.87	0.86
P for trend		0.41	0.55	0.99	0.85	0.74
Vegetable						
≦6 times a week	9	25.9 (19.6, 29.6)	1.18 (1.07, 1.27)	0.44 (0.27, 0.63)	1.52 (1.07, 1.70)	203.0 (195.0, 359.0)
Once a day	18	23.6 (20.5, 32.3)	1.15 (0.83, 1.36)	0.46 (0.35, 0.66)	0.96 (0.78, 1.72)	149.0 (124.0, 225.0)
≧2 times a day	49	25.7 (20.7, 32.2)	1.13 (0.85, 1.46)	0.49 (0.37, 0.66)	1.38 (0.98, 2.27)	244.0 (179.0, 386.0)
P for difference[b]		0.56	0.59	0.35	0.73	0.57
P for trend		0.64	0.55	0.53	0.78	0.83
Fruit						
≦3 times a month	17	26.0 (20.5, 30.1)	1.04 (0.83, 1.44)	0.43 (0.35, 0.57)	1.07 (0.78, 1.64)	190.0 (124.0, 309.0)
1–4 times a week	34	23.9 (18.8, 32.2)	1.13 (0.84, 1.27)	0.47 (0.29, 0.66)	1.44 (0.93, 1.95)	222.0 (161.0, 359.0)
≧5 times a week	25	25.7 (23.3, 32.3)	1.24 (0.86, 1.61)	0.47 (0.41, 0.71)	1.38 (0.91, 2.27)	244.0 (150.0, 435.0)
P for difference[b]		0.31	0.31	0.18	0.37	0.35
P for trend		0.27	0.49	0.19	0.54	0.50
Dairy products						
≦6 times a week	23	26.2 (20.0, 31.7)	1.18 (0.99, 1.63)	0.61 (0.34, 0.72)	1.42 (0.93, 2.51)	227.0 (183.0, 555.0)
Once a day	40	25.8 (20.6, 32.8)	1.13 (0.84, 1.32)	0.45 (0.36, 0.64)	1.41 (0.87, 1.90)	211.0 (129.0, 363.0)
≧2 times a day	13	24.6 (20.6, 25.8)	1.06 (0.82, 1.46)	0.43 (0.33, 0.52)	1.18 (1.01, 1.61)	230.0 (179.0, 318.0)
P for difference[b]		0.76	0.31	0.09	0.31	0.34
P for trend		0.70	0.46	0.04	0.33	0.24

[a] 25th—25th percentile; 75th—75th percentile.
[b] Differences in log-transformed levels between subgroups were tested by the analysis of variance.

pesticides according to age, residence, occupation, body mass index (BMI), smoking and alcohol drinking habit among all subjects. Significantly higher levels of total TEQ, PCDDs/PCDFs/cPCBs, PCBs and pesticides were observed in older women (P for trend=0.003, 0.01, 0.005, 0.01, respectively). Serum total PCB levels were inversely related to BMI (P for difference=0.02). No significant differences in the levels of total TEQ, PCDDs/PCDFs/cPCBs, PCBs and pesticides were found with regard to residence, occupation, smoking or alcohol drinking habit.

Table 3 shows the association between serum organochlorine levels and frequency of food intake among all subjects. Levels of total TEQ, PCDDs/PCDFs/cPCBs, PCBs and pesticides were significantly increased with increasing frequency of fish intake (P for trend=0.002, 0.003, 0.0003 and 0.006, respectively). The median levels of total TEQ, PCDDs/PCDFs/cPCBs, PCBs and pesticides with subjects who consumed fish more than five times a week was about 1.7-, 1.9-, 2.0-, 2.5-fold significantly higher than in subjects who did so less than three times a month. Inverse association was observed between dairy product intakes and total PCBs levels (P for trend=0.04). Significant differences in levels of total TEQ, PCDDs/PCDFs/cPCBs, PCBs and pesticides were not found in terms of meat, rice, vegetable and fruit intakes. Furthermore, we analyzed the associations between the frequency of food intake and levels of PCDDs, PCDFs and cPCBs separately (data not shown). Statistically significant positive associations were found between the frequency of fish intake and TEQ levels of PCDFs and cPCBs, and the total levels of PCDDs, PCDFs and cPCBs, respectively. No significant differences in the TEQ levels of PCDDs, PCDFs and cPCBs, and the total levels of PCDDs, PCDFs and cPCBs were found with regard to frequency of meats, rice, vegetables, fruits and dairy products intakes.

4. Discussion

In the present study, we identified the serum levels of total TEQ, PCDDs/PCDFs/cPCBs, PCBs and pesticides among Japanese women of reproductive age, and the possible contributions of age and fish intake to such levels.

The mean or median total TEQ levels of PCDDs/PCDFs previously reported among Japanese with no occupational exposure were 9.8 to 24.9 pg TEQ/g lipid (Arisawa et al., 2003; Kumagai et al., 2002, 2000). The mean or median total TEQ levels of PCDDs/PCDFs/cPCB/PCBs previously reported among Japanese with no occupational exposure were 16 to 61 pg TEQ/g lipid (Arisawa et al., 2003; Tsuchiya et al., 2003). The median level of total TEQ of PCDDs/PCDFs and PCDDs/PCDFs/cPCBs/PCBs in the present study was 16.1 and 25.1 pg TEQ/g lipid. The serum TEQ levels in our study were consistent with those previously reported for other Japanese populations. The mean levels of PCBs (e.g., PCB105, 118, 156, 157, 167, 189) were also similar to the mean levels previously reported for Japanese populations (Arisawa et al., 2003; Tsuchiya et al., 2003). To our knowledge, the serum p,p′-DDE levels of Japanese have not been reported previously. The median level of serum p,p′-DDE was 221.0 ng/g lipid in the present study, and the serum p,p′-DDE level of the present subjects was lower than the serum p,p′-DDE levels of American or Swedish subjects (Laden et al., 2001; Weiderpass et al., 2000). In this study, the contribution of individual organochlorine compounds to the total TEQ was highest from 2,3,4,7,8-PentaCDF (21.8%), followed by 3,3′,4,4′,5-PentaCB (20.1%), 1,2,3,7,8-PentaCDD (13.7%) and 1,2,3,6,7,8-HexaCDD (11.4%) (data not shown).

Because organochlorines are lipophilic, slowly metabolized, and tend to bioaccumulate in the food chain, higher organochlorine levels should be found in the human body as people get older. In fact, some previous studies as well as the present investigation reported that total serum TEQ levels significantly increased with age (Arisawa et al., 2003; Chen et al., 2003; Kumagai et al., 2000; Wittsiepe et al., 2000a). In the present study, serum organochlorine levels tended to be lower as BMI was higher, and a inverse association was found between the BMI and level of total PCBs (P for difference=0.02). A few reports investigated the association between BMI and serum organochlorine levels, but no significant association was found in almost all of these investigations (Arisawa et al., 2003; Kumagai et al., 2002, 2000). One study revealed a positive association between BMI and serum DDE levels (Schildkraut et al., 1999). Further study will be needed to explore in

more detail the possible association between BMI and serum organochlorine levels in human.

Concerning the association between dietary intake and serum organochlorine levels in Japanese, higher serum TEQ levels in frequent fish and meat consumers are plausible, because the estimated mean daily intakes of total TEQ levels of PCDDs, PCDFs and PCBs contaminating foods were highest from fish and shellfish (76.9%), followed by meat and eggs (15.5%) in Japanese (Tsutsumi et al., 2001). In our study, we found that Japanese women of reproductive age who consumed fish frequently tended to accumulate TEQ levels of PCDFs, cPCBs, PCBs in their body. Similar to TEQ levels of PCDFs, cPCBs and PCBs, a positive association was found between fish intake and TEQ levels of PCDDs, although not significant. Because age might be a confounding factor of fish intake, we divided all subjects into three groups according to age (24–29, 30–35 and 36–43 years) and investigated the association between fish intake and serum levels of total TEQ, PCDDs/PCDFs/cPCBs, PCBs and pesticides. In each group, positive associations were also found between fish intake and serum levels of total TEQ, PCDDs/PCDFs/cPCBs, PCBs and pesticides (data not shown).

To our knowledge, five studies have reported on the associations between serum organochlorine levels and dietary intake in Japan (Table 4). Arisawa et al. (2003) measured serum total TEQ levels of 7 PCDDs, 10 PCDFs and 12 PCBs in relation to 11 food items consumed by randomly selected persons who resided in five prefectures of Japan and had no known occupational exposure to dioxins. They reported that frequent coastal fish intake was associated with higher serum TEQ levels of PCDFs ($P=0.03$), and the raw fish intake was positively related to TEQ levels of PCBs ($P=0.03$). Tsuchiya et al. (2003) measured serum total TEQ levels of 7 PCDDs, 10 PCDFs, 4 cPCBs and 8 PCBs among 10 fishermen, 10 farmers and 8 office workers. They reported that in frequent fish eaters, mean TEQ levels of PCDFs, cPCBs, PCBs and total sum TEQ levels were significantly higher than in the infrequent fish eaters. Kitamura et al. (2000) investigated the association between 9 factors of food intake and serum total TEQ levels of 7 TCDDs, 10 TCDFs and 3 PCBs among employees in waste incineration plants. Their study revealed that butter/cheese/lard intake was positively associated with TEQ levels of PCDDs, PCDFs and the total TEQ level, while ordinary daily food including fish, clam, egg, squid and vegetable was positively associated with serum TEQ levels of PCBs. They also analyzed the association between 5 preferable meals' intake and total serum TEQ levels. The observed higher fish intake was significantly associated with higher TEQ levels of cPCBs (77+126+169) in blood, but not with TEQ levels of PCDDs or PCDFs.

Contrary to these results, Kumagai et al. (2000) reported no association between the frequency of fish, meat and milk intake and serum TEQ levels of PCDDs and PCDFs among workers employed at waste-incineration plants. However, in their study, the total TEQ levels of PCDDs and PCDFs were compared between only two categories (<7 times/week and ≧7 times/week) of the frequency of fish, meat and milk intake. They indicated that more detailed information was necessary to clarify the relation between fish consumption and the serum PCDDs and PCDFs levels. Only one study investigated the association between dietary intake and serum levels of pesticides including β-HCH, hexachlorobenzene (HCB), pp′-dichlorodiphenyldichloroethane (pp′-DDD), bis(4-chlorophenyl)-1,1-dichloroethene (DDE) and DDT among Japanese farmers (Hanaoka et al., 2002). The authors reported that fish intake showed a positive but no significant relationship with HCB and DDT serum levels. In the present study, the serum pesticide level significantly increased with the increasing frequency of fish intake (P for trend=0.006), and this result was consistent with a previous report (Hanaoka et al., 2002).

Daily dietary intake of PCDDs, PCDFs and PCBs has been estimated using PCDDs, PCDFs and PCBs levels in foods. Because the PCDD, PCDF and PCB levels in foods and the consumption of foods vary from country to country, the kinds of foods contributing to daily dietary intake of PCDDs, PCDFs and PCBs obviously differ with the country. The main foods contributing to the daily dietary intake of total TEQ levels of PCDDs and PCDFs have been reported to be fish for Japanese (Tsutsumi et al., 2001), Spanish (Llobet et al., 2003b), and Belgian people (Focant et al., 2002), meat and meat products for Americans (Guo et al., 2001; Schecter et al., 2001), and British people (Harrison et al., 1998),

Table 4
Previous reports on the association between food and beverage intake and serum organochlorine level among Japanese populations

Subjects	Kinds of food and beverage	Analyzed organochlorines	Significant associations	Reference
Randomly selected people (n=253)	11 Items (beef, pork, milk, eggs, butter, cheese, grilled fish, boiled fish, raw fish, coastal fish, other fish)	TEQ of 7 PCDDs, 10 PCDFs, 12 PCBs	Positive association TEQ of PCDFs—coastal fish TEQ of PCBs—raw fish	Arisawa et al., 2003
Fishermen, farmers, office workers (n=28)	Fish	TEQ of 7 PCDDs, 10 PCDFs, 4cPCBs and 8PCBs	Positive association TEQ of PCDFf—fish TEQ of cPCBs—fish TEQ of PCBs—fish Total TEQ—fish	Tsuchiya et al., 2003
Incinerator workers (n=94)	9 Factors (ordinary daily food[a], clam/shrimp/bacon, fatty food, rice/egg, mushroom/ham, meat, butter/cheese/lard, dairy product, crab)	TEQ of 7 TCDDs, 10 TCDFs, 3 PCBs	Positive association TEQ of PCDDs—fatty food, mushroom/ham, butter/cheese/lard TEQ of PCDFs—butter/cheese/lard TEQ of PCBs—ordinary daily food, mushroom/ham Total TEQ—butter/cheese/lard	Kitamura et al., 2000
	5 Preferred meals (fatty meals, fish meals, noodles, broiled meat/tempura, grilled eel/fried dumpling)		Positive association TEQ of PCDDs—meat/tempra, eel/dumpling TEQ of PCDFs—eel/dumpling TEQ of PCBs—fish meals Total TEQ—eel/dumpling Inverse association TEQ of PCBs—fatty meals	
Incinerator workers (n=60)	3 Items (fish, meat, cow's milk)	TEQ of 5 PCDDs and 5 PCDFs 5 PCDDs, 5 PCDFs	Significant association was not found	Kumagai et al., 2000
Farmers (n=41)	7 Foods (meats, fish, vegetables, fruits, rice, green tea, milk)	5 Pesticides (b-hexachlorocyclohexane, b-hexachlorobenzene, pp′-DDE, pp′-DDT, pp′-dichlorodiphenyldichloroethane)	Significant association was not found	Hanaoka et al., 2002
Women of reproductive age (n=80)	6 Items (fish, meats, rice, vegetables, fruits, milk)	TEQ of 7 PCDDs, 9 PCDFs, 3 cPCBs, 6 PCBs 7 PCDDs, 9 PCDFs, 3 cPCBs, 32 PCBs, 11Pesticides	Positive association TEQ of PCDFs—fish TEQ of cPCBs—fish TEQ of PCBs—fish Total TEQ—fish PCDDs—fish PCDFs—fish cPCBs—fish PCBs—fish Pesticides—fish Inverse association PCBs—dairy products	This study

[a] Ordinary daily food contains fish, clam, eggs, squid, vegetables etc.

and milk and dairy products for Germans (Malisch, 1998). The main food contributing to daily dietary intake of TEQ levels of PCBs is fish in various countries including Japan (Tsutsumi et al., 2001), Spain (Llobet et al., 2003a), the USA (Schecter et al., 2001), England (Harrison et al., 1998), meat and meat product in England (Harrison et al., 1998), and the dairy product in Canada (Wittsiepe et al., 2000b). The present study revealed that the frequency of fish consumption was the most significant contributor to serum total TEQ levels of PCDDs, PCDFs, cPCBs and PCBs among Japanese women of reproductive age, and these results were consistent with the results of the above studies estimating daily dietary intake for Japanese.

The human health effects associated with low exposure to organochlorine compounds which levels we found in the present study have not yet been fully characterized. However, cancer mortality has been reported to be unaffected by such low levels of exposure to organochlorines (Bertazzi et al., 2001). We will examine the effects on endometriosis of exposure to organochlorine compounds, and the results will be reported in a separate paper in the near future.

In conclusion, we found that among various lifestyle factors, fish consumption was positively associated with serum levels of total TEQ, PCDDs/PCDFs/cPCBs, PCBs and pesticides in Japanese women of reproductive age.

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Decreased Serum Free Testosterone in Workers Exposed to High Levels of Di-*n*-butyl Phthalate (DBP) and Di-2-ethylhexyl Phthalate (DEHP): A Cross-Sectional Study in China

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BACKGROUND: Observations of adverse developmental and reproductive effects in laboratory animals and wildlife have fueled increasing public concern regarding the potential for various chemicals to impair human fertility.

OBJECTIVE: Our objective in this study was to assess the effect of occupational exposure to high levels of phthalate esters on the balance of gonadotropin and gonadal hormones including luteinizing hormone, follicle-stimulating hormone, free testosterone (fT), and estradiol.

METHODS: We examined urine and blood samples of 74 male workers at a factory producing unfoamed polyvinyl chloride flooring exposed to di-*n*-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) and compared them with samples from 63 male workers from a construction company, group matched for age and smoking status.

RESULTS: Compared to the unexposed workers, the exposed workers had substantially and significantly elevated concentrations of mono-*n*-butyl phthalate (MBP; 644.3 vs. 129.6 μg/g creatinine, $p < 0.001$) and mono-2-ethylhexyl phthalate (MEHP; 565.7 vs. 5.7 μg/g creatinine, $p < 0.001$). fT was significantly lower (8.4 vs. 9.7 μg/g creatinine, $p = 0.019$) in exposed workers than in unexposed workers. fT was negatively correlated to MBP ($r = -0.25$, $p = 0.03$) and MEHP ($r = -0.19$, $p = 0.095$) in the exposed worker group. Regression analyses revealed that fT decreases significantly with increasing total phthalate ester score (the sum of quartiles of MBP and MEHP; $r = -0.26$, $p = 0.002$).

CONCLUSION: We observed a modest and significant reduction of serum fT in workers with higher levels of urinary MBP and MEHP compared with unexposed workers.

KEY WORDS: di-*n*-butyl phthalate (DBP), di-2-ethylhexyl phthalate (DEHP), free testosterone (fT), mono-*n*-butyl phthalate (MBP), mono-2-ethylhexyl phthalate (MEHP), occupational exposure. *Environ Health Perspect* 114:1643–1648 (2006). doi:10.1289/ehp.9016 available via *http://dx.doi.org/* [Online 27 July 2006]

Observations of adverse developmental and reproductive effects in laboratory animals and wildlife have fueled increasing public concern regarding the potential for various chemicals to impair human fertility. Included among the list of chemicals suspected of impairing human fertility are the phthalate esters (PEs), which are used extensively as plasticizers in household and consumer goods and in certain medical products. Each year 2–8 million tons of PEs are produced and consumed worldwide [Colborn et al. 1993; Toppari et al. 1996; World Health Organization (WHO) 1992]. Several studies have demonstrated the extent of exposure to PEs in the general population (Blount et al. 2000; Koch et al. 2003b; Silva et al. 2004b).

Phthalate monoesters, including mono-2-ethylhexyl phthalate (MEHP) and mono-*n*-butyl phthalate (MBP), are known testicular toxicants in rodents. The Leydig cells (LCs) and Sertoli cells (SCs) that play crucial roles in spermatogenesis and testosterone production are considered the primary targets of phthalate monoester toxicity (Akingbemi et al. 2004; Barlow et al. 2003; Foster et al. 2001; Gray et al. 2000; Jones et al. 1993; Mahood et al. 2005; Wang et al. 2005). Although adverse effects on male reproduction are suggested, research findings remain inconsistent (Kumar 2004). There is a large gap between results from studies investigating exposure to relatively high levels of PEs in a laboratory setting and the relatively low levels found in the general environment (Mylchreest et al. 2002). Both fetal and adult exposure to PE are suspected to contribute to impaired human fertility (Mahood et al. 2005; Parks et al. 2000; Skakkebaek et al. 2001).

Although many toxicologic studies on PEs have been conducted in the past decade, few epidemiologic studies have assessed the relationship between PE exposure and the effect on human reproduction. Obstacles that have hindered human studies include the long latency period from fetal exposure, low exposure levels, difficulties in sampling sperm, and the involvement of complex cell–cell interactions between the cells and hormones associated with the hypothalamo–pituitary–testis (HPT) system. Although several studies have demonstrated high levels of PEs in patients under dialysis or extracorporeal membrane oxygenation and in workers having occupational exposures, very few studies have evaluated the effects of PE exposure on reproductive function (Cooper and Kavlock 1997; Duty et al. 2005; Hoppin 2003; Kavlock 1999; Main et al. 2006; Takahashi et al. 2004). Thus findings from epidemiologic studies are not conclusive as to whether exposure to environmental levels of PEs can cause sperm damage and/or disrupt the gonadal hormone balances in adult males.

Because occupational exposure to PEs is generally higher than environmental exposure, the workplace provides an appropriate setting to study the effects of PEs on reproductive function (Kumar 2004). The present study was designed to assess the effect of occupational exposure to high levels of di-*n*-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) on the circulating concentration and/or balance of free testosterone (fT), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and estradiol (E_2).

Methods

Subjects and sample collection. The study was conducted in a factory producing unfoamed polyvinyl chloride (PVC) flooring in Liaoning Province, China. DBP and/or DEHP were used as plasticizers in four similar production lines; the workers were exposed to DBP and/or DEHP by dermal contact and through

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dust inhalation. For our exposed workers group, we selected all 74 male workers currently working in the four production lines. The exposed workers were involved in raw material preparation and mixing (n = 14), filtering (n = 13), refining (n = 23), rolling and pressing (n = 14), packaging (n = 4), and other duties (n = 6). For a comparison group without occupational exposure to DBP and/or DEHP, we randomly selected 63 male workers from 89 employees of a construction company, group matched for age and smoking status. They were woodworkers (n = 24), bricklayers (n = 16), workers in material preparation and loading (n = 13), scaffolders (n = 4), reinforcing steel bar workers (n = 4), and electricians (n = 2).

Urine and blood samples were collected from each subject between 800 and 1100 hours on the same day, but not on the first day of the subject's work week or the day after a night work shift. Peripheral blood was collected in an EDTA-2Na tube, and plasma was collected by centrifugation. A simple questionnaire was used to obtain lifestyle information, including smoking and alcohol consumption habits, personal plastic material usage, and consumption of soybean products. We developed a summary index for plastic material contact (SIP) to ascertain if the subject came into contact with plastic tableware (0/1), water or tea in polyethylene terephthalate bottles (0/1) and food packaging (0/1). The frequency of soybean product consumption (FSPC) was defined as the frequency per week of consuming soybean products.

This study was conducted in accordance with the Declaration of Helsinki (World Medical Assoiation 2004). All subjects volunteered to participate in the study and gave written informed consent.

Chemicals, instruments, and analytical conditions for MBP and MEHP. Stock solutions of the standard chemicals were prepared in acetonitrile at a concentration of 100 mg/mL. We purchased MBP, MEHP, and d_4-labeled internal standards from Hayashi Pure Chemical Industries (Osaka, Japan). Organic solvents for PE analysis and sample stock preparation were obtained from Kanto Chemical Co. (Tokyo, Japan). Ammonium acetate, acetic acid, and β-glucuronidase solution from *Escherichia coli* (85 U/mL) were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Liquid chromatography-electrospray-ionization (ESI) tandem mass spectrometry (MS/MS) was performed using a Waters Alliance HT2795/Micromass Quattro micro system (Waters Co., Milford, MA, USA). The injection volume was 20 μL; the precolumn was a Mightysil RP-18 GP 5-2.0 (5 μm; Kanto Chemical Co.); and the analytical column was an Inertsil ODS-3 (2.1 × 50 mm, 5 μm; GL Sciences Inc., Tokyo, Japan). The column temperature was maintained at 40°C. The linear gradient program was as follows: 96%A/1%B/3%C (0 min), 4%A/1%B/95%C (3–7 min), and 96%A/1%B/3%C (7.2 min). We used a flow rate of 0.2 mL/min. For operation of the ESI-MS/MS, the flow rates of dry nitrogen for desolvation and cone gas were 350 and 50 L/hr, respectively, and the temperatures for desolvation and source were 350 and 100°C, respectively. The MS/MS data for MBP, MEHP, and the d_4-internal standards were collected in negative ion mode by multiple reaction monitoring of the transition m/z 221 → 77 (MBP), m/z 277 → 134 (MEHP), m/z 225 → 81 (MBP-d_4), and m/z 281 → 138 (MEHP-d_4). The optimized parameters for ESI with monitoring ions of MBP and MEHP were a cone potential of –22 and –28 V and a collision energy of 17 and 16 eV, respectively. Other working conditions for the MS/MS in multiple reaction monitoring mode included a cone gas of nitrogen, a collision gas of argon, an interchannel delay of 0.1 sec, and repeats of one time span of 0.1 sec.

For cleanup and preconcentration determination, we modified a solid phase extraction (SPE) method for measuring urinary mono-phthalate ethers previously described by Blount et al. (2000) and Silva et al. (2004b). Briefly, a 3-mL aliquot from a human urine sample (spiked internal standard, 30 μL) was buffered with 100 mM ammonium acetate/acetic acid (1 mL, pH 6.2); after the addition of β-glucuronidase solution (50 mL, 4.25 U), the sample was sealed in a glass tube and gently mixed. This solution was then incubated at 37°C for 60 min to deconjugate the glucronidated phthalate metabolites. This reaction time was established in preliminary experiments, which showed maximal reaction at 60 min. We used OASIS MAX (6 cc/150 mg; Waters Co.) with *N*-vinylpyrrolidone/divinylbenzene hydrophilic–lipophilic balanced copolymer mixed anion-exchange phase. Because MBP and MEHP have carboxyl group and alkyl groups, this anion exchange/reverse phase column was suitable for extracting these metabolites. The SPE cartridge was preconditioned with 15 mL acetonitrile and 5 mL 100 mM ammonium acetate/ammonia solution and then loaded with deconjugated sample in 3 mL 100 mM ammonium acetate/ammonia solution. The SPE cartridge was then rinsed with 5 mL water and 5 mL acetonitrile, after which it was dried under vacuum for 3 min. The sample was eluted with 5 mL acetonitrile

Table 1. Demographic characteristics [no. (%)] of exposed (n = 74), unexposed (n = 63), and total workers (n = 137).

Characteristic	Exposed	Unexposed	All
Age at interview (years)[a]	33.5 ± 9.4	34.3 ± 9.9	33.9 ± 9.6
Age (years)			
< 20	5 (7)	4 (6)	9 (7)
20–29	25 (34)	18 (29)	43 (31)
30–39	21 (28)	20 (32)	41 (30)
40–49	20 (27)	17 (27)	37 (27)
≥ 50	3 (4)	4 (6)	7 (5)
Years working in current job[a]	1.0 ± 0.8*	2.6 ± 5.5	1.7 ± 3.8
Marriage status			
Single	21 (28)	15 (24)	36 (26)
Married	50 (68)	48 (76)	98 (72)
Divorced	3 (4)	0	3 (2)
Smoker	44 (60)	39 (62)	83 (61)
Drinker	40 (54)	40 (64)	80 (58)
SIP score			
0	33 (45)	24 (38)	57 (42)
1	27 (36)	29 (46)	56 (41)
2	10 (14)	8 (13)	18 (13)
3	4 (5)	2 (3)	6 (4)

[a]Mean ± SD. *p < 0.01.

Table 2. Selected percentiles and GMs of urinary MBP and MEHP concentrations (μg/g creatinine) among exposed workers, unexposed workers, and the American male population.

		Percentiles							
Marker/subjects	No. (%)[a]	10th	25th	50th	75th	90th	95th	GM	p-Value
MBP									
Exposed	74 (100)	156.7	252.1	548.4	1492.6	2455.5	8781.2	644.3	
Unexposed	63 (100)	58.2	74.7	113.5	206.8	338.2	434.5	129.6	< 0.001
NHANES[b]	1,215 (99)	6.5	10.2	17.0	28.6	49.1	63.6	17.3	
MEHP									
Exposed	74 (100)	78.0	209.6	562.3	1884.4	3303.7	5379.7	565.7	
Unexposed	63 (98)	2.0	3.7	5.4	9.9	15.4	23.2	5.7	< 0.001
NHANES[b]	1,215 (81)	< LOD	1.3	2.8	5.6	10.3	21.6	2.9	

[a]Sample size and percentage of detection. [b]Data from Silva et al. (2004a).

containing 1% formic acid. The elution sample solution was dried under a stream of nitrogen at 40°C before resuspension in 300 µL acetonitrile/water (50/50, vol/vol). The final sample solution was analyzed by liquid chromatography-MS/MS.

Analytical validation by liquid chromatography-MS/MS. Using the liquid chromatography-MS/MS conditions described above, the retention times for MBP and MEHP were 5.6 and 6.4 min, respectively, with a relative SD (RSD) of 0.5–0.7% on 3 different days (n = 10). The calibration graphs (peak area ratios of the internal standard versus sample concentration) obtained for MBP and MEHP (slopes 0.014 and 0.017 and intercepts 0.0541 and 0.034, respectively; r > 0.999) were linear over the calibration range from 5 ng/mL to 50 µg/mL. The limit of detection (LOD) was 0.5 and 0.6 ng/mL (signal/noise ratio = 3) for MBP and MEHP, respectively. If the observed level was below the lowest calibration standard in the HPLC-MS analysis, we repeated the measurement using a sample volume that was double (or more) that used in the original analysis. The limit of quantitation was 5 ng/mL (signal/noise ratio > 10). The average recovery for MBP and MEHP (10 and 100 ng/mL) in urine samples ranged from 97.8% to 100.8% (RSD < 7.5%, n = 6).

Determination of plasma hormones. Plasma levels of LH, FSH, fT, and E_2 were measured by radioimmunoassay in a commercial laboratory (SRL Inc., Tokyo, Japan). The reference values for the determinations provided by the laboratory were 1.8–5.2 IU/mL, 2.9–8.2 IU/mL, 14–40 pg/mL, and 20–60 pg/mL, respectively. For LH, FSH, fT, and E_2, respectively, the LODs were 0.10 mIU/mL, 0.05 mIU/mL, 0.6 pg/mL, and 1.0 pg/mL, and the coefficients of variance (interday variation) were 4%, 3%, 1.6–3.6, and 3–4%. The reference range provided by the commercial laboratory were as follows: LH, 1.8–5.2 mIU/mL; FSH, 2.9–8.2 mIU/mL; fT, 3.3–21.3 pg/mL; and E_2, 20–59 pg/mL.

Table 3. Concentrations (mean ± SD) of $\log_{10}$-transformed serum FSH, LH, fT, and E_2 among exposed (n = 74) and unexposed (n = 63) workers.

Hormone	Exposed	Unexposed	p-Value
FSH	5.0 ± 1.5	5.4 ± 1.7	0.360
LH	4.3 ± 1.5	4.9 ± 1.7	0.102
T	8.4 ± 1.5	9.7 ± 1.4	0.019
E_2	22.4 ± 1.6	20 ± 1.7	0.187

Statistical analyses. We calculated medians, geometric means (GMs), and distribution percentiles of creatinine-adjusted concentrations for urinary levels of MBP and MEHP. Urinary levels of MEHP and MBP and serum levels of FSH, LH, fT, and E_2 were transformed to $\log_{10}$ for statistical analysis. The ratio of LH to fT was calculated by simple division. Values for MEHP and MBP were specified as 0.5 ng/mL and 0.6 ng/mL, respectively, when levels fell below the LOD. GMs were compared between subgroups by the two-sample t-test. We estimated daily intake of DEHP for each subject according to the method of Koch et al. (2003a). The standardized partial correlation coefficient was calculated to assess bivariate relationships adjusting for potential confounding variables. We performed a stepwise multiple regression analysis to determine the independent variables [among MEHP, MBP, age, alcohol consumption (yes/no), tobacco smoking (yes/no), body mass index (BMI), SIP, and FSPC] important in predicting the serum concentrations of FSH, LH, fT, and E_2. The significance levels for entry and inclusion in the model were p < 0.05 and p < 0.10, respectively. Age was forced into the final model when we assessed the relationship between hormones and the statistically significant variables. Because MBP and MEHP are highly correlated and because the limited sample size in the present study prohibited us from examining the effects of the individual exposure and coexposure, we calculated the total phthalate esters score (TPES) according to the method of Swan et al. (2005). MBP and MEHP were divided into quartiles and assigned values of 0, 1, 2, and 3, respectively. TPES equals the sum of scores for MBP and MEHP. Differences in proportions were tested by the chi-square method or, when the expected values in cells were small, by Fisher's exact test. A p-value < 0.05 (two-tailed) was considered significant.

Results

The baseline characteristics of all subjects are shown in Table 1. The unexposed workers were comparable in terms of age, marital status, and smoking and alcohol consumption habits, as well as the plastic material exposure index. Subjects in the exposed worker group had worked < 1 year on average, which was significantly lower than the corresponding time for the unexposed workers (p < 0.01).

Table 2 shows the GMs and selected percentiles of urinary MBP and MEHP for exposed and unexposed workers. As a reference, the corresponding values of American males from the National Health and Nutrition Examination Survey (NHANES) are also presented (Silva et al. 2004a). MBP and MEHP were detected in all workers, apart from one subject in the unexposed group for which MEHP could not be detected. Exposed workers had significantly higher levels of MBP and MEHP than the unexposed workers. Table 3 shows the concentrations of FSH, LH, fT, and E_2 for the exposed and unexposed groups. We found significantly lower fT levels in exposed workers than in unexposed workers, but no significant difference between the two groups for FSH, LH, or E_2.

Table 4 shows the standardized partial correlation coefficients between urinary levels of MBP and MEHP and plasma levels of FSH, LH, fT, E_2, and LH/fT in exposed, unexposed, and total workers. MBP and MEHP correlated positively in both the exposed and unexposed groups (Table 4). fT was negatively correlated with MBP and MEHP in the exposed worker group and in all subjects but not in the unexposed group. We found a nonsignificant negative correlation between FSH and MBP and MEHP in the exposed group (Table 4). There was a positive correlation between FSH and LH only in the unexposed group but not in the exposed worker group. fT levels correlated positively with LH and E_2 in

Table 4. Standardized partial correlation coefficients between levels of urinary metabolites and plasma hormones in all subjects.

	Exposed[a]						Unexposed[a]						All[a]					
	MEHP	FSH	LH	fT	E_2	LH/fT	MEHP	FSH	LH	fT	E_2	LH/fT	MEHP	FSH	LH	fT	E_2	LH/fT
MBP[b]	0.716	−0.180	0.087	−0.253	−0.029	0.216	0.549	0.002	0.078	0.095	−0.061	−0.032	0.799	−0.103	−0.042	−0.237	0.032	0.073
p-Value	0.000	0.129	0.466	0.032	0.808	0.034	0.000	0.998	0.550	0.467	0.639	0.402	0.000	0.107	0.632	0.006	0.712	0.199
MEHP[b]		−0.191	0.035	−0.198	0.007	0.146		0.092	0.127	−0.045	−0.128	0.109		−0.103	−0.109	−0.242	0.077	0.035
p-Value		0.109	0.768	0.095	0.955	0.110		0.480	0.330	0.728	0.325	0.202		0.235	0.207	0.005	0.376	0.345
FSH[b]			0.156	0.084	0.051	0.093			0.319	−0.081	−0.229	0.328			0.262	0.032	−0.075	0.224
p-Value			0.192	0.486	0.669	0.218			0.012	0.533	0.076	0.005			0.002	0.713	0.385	0.004
LH[b]				0.315	0.229	0.715				0.225	0.167	0.855				0.294	0.177	0.784
p-Value				0.007	0.053	0.000				0.082	0.199	0.000				0.001	0.040	0.000
fT[b]					0.465	−0.402					0.418	−0.255					0.402	−0.318
p-Value					0.000	0.000					0.001	0.023					0.000	0.000
E_2[b]						−0.101						−0.022						−0.058
p-Value						0.200						0.432						0.251

[a]Standardized partial correlation coefficients were adjusted for age and alcohol consumption status (yes/no). [b]$\log_{10}$-transformed MBP, LH, FSH, LH, fT, and E_2.

both the exposed and unexposed groups, and a negative correlation was observed between LH/fT and MBP in the exposed group. We found no associations between MBP, MEHP, LH, and E_2. A regression analysis showed a significant decrease in fT with increasing TPES ($r = -0.26$, $p = 0.002$).

Table 5 shows the results of multiple regression analyses. A positive correlation was found between both FSH and LH and age, whereas a negative correlation was found between fT and age ($p < 0.10$), MEHP ($p < 0.01$), and alcohol consumption ($p < 0.10$) for all subjects. MBP levels were the only significant predictor of fT levels in the exposed workers. When TPES was included in the model replacing MEHP and MBP, TPES remained as a significant predictor of fT levels for all subjects and exposed workers (data not shown). BMI was the only significant predictor of fT in the unexposed workers. We found a positive correlation between FSPC and E_2 in the exposed group.

Discussion

In this cross-sectional study we evaluated the relationship between occupational exposure to DBP and/or DEHP and serum sex hormones in male Chinese workers. Urinary MBP and MEHP were detected in all 137 subjects, except 1 subject in the unexposed group whose urine contained undetectable levels of MEHP. The detection rates of MEHP we found in the present study—100% in exposed workers and 98% in unexposed workers—are higher than the 81% reported for American males by Silva et al. (2004a). By contrast, the detection rate for MBP found here in Chinese workers was similar to that found in American males (Silva et al. 2004a). These findings indicate that exposure to DBP and DEHP is ubiquitous in China and other parts of the world.

The levels of urinary MBP and MEHP in the exposed group (GMs of 644.3 and 565.7 μg/g creatinine, respectively; Table 2) was 5–100 times that of the unexposed group. Of the 74 exposed workers, 3 had urinary MBP levels > 21,000 μg/g creatinine, which is greater than the highest MBP level ever identified in a male patient with ulcerative colitis [16,868 μg/g creatinine (Hauser et al. 2004a)]. The levels for MBP and MEHP were 7.5 and 2.0 times higher in unexposed workers than in American males (Silva et al. 2004a). Because the factory described in the present study used waste plastic materials as raw materials, it is possible that more DBP and/or DEHP were used than in other PVC flooring factories. Poor environmental control of dust and vapor and the insufficient use of air masks are likely contributors to high DBP and DEHP exposure. In the present study, workers handled raw and mixed materials containing DBP and/or DEHP on production lines, with dermal contact occurring frequently and directly via hands, arms, and other parts of the body, or indirectly through contaminated work clothes. The high temperature attained in the workshops may have increased the levels of DEHP and DBP in the air as well as the dermal absorption rate. It is also possible that workers ingested DBP and/or DEHP by drinking or eating contaminated water and food. We suspect that the high levels of urinary MBP and MEHP in the exposed worker group are caused primarily by air pollution and heavy dermal contamination.

The levels of urinary MBP and MEHP among the unexposed workers (median 113.5 and 5.4 μg/g creatinine, respectively; Table 2) were similar to those found among German males [111.0 and 8.2 μg/g creatinine, respectively (Koch et al. 2003b)], and 7 to 2 times those found among American males (Silva et al. 2004a). A number of studies have demonstrated widespread pollution of DBP and DEHP in environmental and biological samples in China (Hu et al. 2003; Zhang et al. 2003). Before the present study, there were no reports of urinary MBP or MEHP levels in China. The significant correlation between urinary MBP and MEHP in the exposed ($r = 0.72$, $p < 0.001$) and unexposed workers ($r = 0.51$, $p < 0.001$) suggests that exposure to DBP and DEHP in working environments and in the general environment is simultaneous. A similar correlation between MBP and MEHP ($r = 0.37$, $p < 0.0001$) was reported among American males by Silva et al. (2004a).

The median of estimated daily intake (DI) of DEHP in the exposed group was 48.2 μg/kg body weight/day, which was significantly higher than that of the unexposed group (0.5 μg/kg body weight/day). Of 74 exposed workers, 30 (32.1%) had a DI above the tolerable daily intake (TDI; 37.0 μg/kg body weight/day) (Committee for Toxicity, Ecotoxicity and the Environment 1998).

In the present study we demonstrate for the first time a significant negative correlation between serum fT and urinary MBP ($r = -0.24$, $p = 0.006$) and MEHP ($r = -0.24$, $p = 0.005$) (Table 4). Furthermore, the exposed workers had significantly lower fT levels than the unexposed workers (8.4 vs. 9.7 μ/g creatinine, $p = 0.019$; Table 3). Multiple regression analyses showed that age, MEHP, and alcohol consumption were significantly related to reduced levels of fT. MBP was the only significant predictor of fT in the exposed workers. When TPES, MBP, and MEHP were assessed in the same model, only TPES was retained in the model for total subjects and exposed workers, indicating that coexposure to both MBP and MEHP was responsible for the reduction in fT. The negative relationship we observed between fT, age, and alcohol consumption is consistent with findings from other studies (Emanuele and Emanuele 1998; Ma and Zheng 2004).

Toxicologic studies have invariably shown that MEHP and MBP are toxicants of LCs and SCs in the testis. The MEHP-induced inhibition of testosterone production in LCs is thought to be associated with decreased pituitary LH secretion and reduced steroidogenic enzyme activity (Akingbemi et al. 2001). Maternal exposure to DBP leads to a decrease in testosterone biosynthesis by reducing cholesterol synthesis, transport, and storage in fetal LCs (Barlow et al. 2003). The relationship between testosterone and PE exposure has been explored in a few epidemiologic studies in recent years. Yin et al. (1998) reported a nonsignificant decrease of serum fT levels in 85 male Chinese workers with occupational exposure to di-octyl phthalate compared with 72 controls (30.7± 2.0 nmol/L vs. 36.0 ± 3.3 nmol/L). Zhang et al. (2003) reported a nonsignificant negative correlation between serum fT and serum DEP ($r = -0.36$, $p = 0.398$) and DBP ($r = -0.23$, $p = 0.588$) in 8 healthy Chinese males. Duty et al. (2005) observed a negative correlation between

Table 5. Multiple regression analyses predicting levels of FSH, LH, fT, and LH in exposed, unexposed, and all workers.

	FSH[a]			LH[a]			fT[a]			E_2[a]		
	Exposed	Unexposed	All	Exposed	Unexposed	All	Exposed	Unexposed	All	Exposed	Unexposed	All
Age[b]	0.257*	0.585#	0.474#	0.305#	0.450#	0.383#	−0.097	−0.129	−0.152*	−0.159	−0.069	−0.138
BMI	—	—	—	—	—	—		0.287**	—	—	—	—
MBP	—	—	—	—	—	—	−0.257**	—	—	—	—	—
MEHP	—	—	—	—	—	—		—	−0.235#	—	—	—
Drinking alcohol	—	—	—	—	—	—	—	—	−0.230*	—	—	—
FSPC	—	—	—	—	—	—	—	—	—	0.330#	—	—
Adjusted R^2	0.159	0.331	0.219	0.081	0.19	0.14	0.058	0.084	0.098	0.012	0.09	0.019
p-Value	0.001	< 0.001	< 0.001	0.008	< 0.001	< 0.001	0.045	0.043	0.001	0.177	0.022	0.109

—, Excluded from model.
[a]Values are beta coefficients except for R^2 and *p*-value. [b]Age was forced in each model. *$p < 0.10$. **$p < 0.05$. #$p < 0.01$.

MEHP and testosterone ($r = -0.17$, $p < 0.05$) in 295 American males. Main et al. (2006) reported that MBP was negatively correlated with fT ($r = -0.22$, $p = 0.033$) in 96 3-month-old boys. Jonsson et al. (2005) examined the relationships between various urinary PEs and gonadal hormones among Swedish males, but they did not observe any significant effects of PEs on serum fT. In a study of 19 adolescents exposed to DEHP as neonates under extracorporeal membrane oxygenation support, levels of FSH, LH, fT, and E_2 were within the normal range (Rais-Bahrami et al. 2004). The first evidence that prenatal PE exposure can adversely affect human male reproductive development was provided by Swan et al. (2005). Although reduced testosterone production in LCs was put forward as a likely cause of adverse male reproductive development, the authors did not measure either testosterone or other gonadal hormones. It should be noted that testicular atrophy and decreased sperm production observed in rats occurs at PE exposure levels substantially higher than human exposure levels in the general environment. To clarify the effects of PE exposure on human sex hormones, more studies are warranted in populations with various exposure levels, especially populations with high exposure (Mantovani et al. 1999).

The low level of fT observed in workers exposed to PEs is mostly due to the exposure to DBP and DEHP in the working environment. In the present study, MBP and MEHP levels in the exposed workers were 5–100 times the levels observed in the unexposed workers and may be in the range required to induce human testicular toxicity. The results from the present study support the notion from animal studies that PEs can suppress testosterone biosynthesis in humans.

The mechanisms underlying testicular toxicity that lead to adverse reproductive effects are complex. Normally, when androgen biosynthesis is sufficiently depressed, the lowered serum fT concentrations act via negative feedback mechanisms to induce increased LH output from the pituitary gland. LH then stimulates LCs to secrete more testosterone, which in the course of time, and acting via the same pathway, restores pituitary LH secretion to normal levels. In this manner, the negative feedback mechanism serves as a homeostatic control for the HPT axis (Kumar 2004). Compared with the unexposed workers, exposed workers had nonsignificant reductions of FSH and LH levels. The decreased LH concentration was not consistent with our hypothesis that LH should increase in response to reduced fT levels. The positive correlation between LH/fT and MBP ($r = 0.216$, $p = 0.034$) and MEHP ($r = 0.146$, $p = 0.110$) in the exposed workers was caused by a decrease in fT, not by an increase in LH. The most likely explanation for the simultaneous occurrence of significantly decreased fT and nonsignificant decreases of LH and FSH levels in the exposed worker group is that the combined exposure to high levels of MBP and MEHP may have caused dysfunction of both testosterone biosynthesis in the testis and the normal feedback regulation of the HPT axis. The negative relationship between fT and MBP/MEHP in the exposed group was not present in the unexposed group, a finding consistent with the results of a study of Swedish males (Jonsson et al. 2005). This implies that the relatively low level of environmental PE exposure may not cause significant serum fT reduction or that the reduction was subtle and compensated by the feedback regulation of the HPT axis. Other raw materials used in the production line of this factory, including PVC resin, azodicarbonamide as a forming agent, and calcium carbonate as a filler, would not have contributed to the decreased fT. FSH, LH, and testosterone play crucial roles in the initiation, maintenance, and restoration of spermatogenesis. Toxicants that damage the LCs can lead to a reduction in the secretion of testosterone, which in turn can affect SC function and spermatogenesis. Although spermatogenesis may be maintained by intratesticular testosterone produced in response to LH stimulation of the LCs, it is generally recognized that combined stimulation with FSH and LH leads to maximal sperm production. The circulating concentration of FSH is thought to provide the signal that sets the level of sperm production above the basal rate induced by intratesticular testosterone (Plant and Marshall 2001). The simultaneously decreased levels of FSH, LH, and fT among exposed workers may have an adverse effect on spermatogenesis. Although the normal range of serum fT in Chinese males is 5.6–10.2 ng/dL (Liao and Cao 2001), in the present study we found that 9.5% (7/74) of exposed workers had serum fT levels < 5.6 ng/dL, which was not significantly higher than the 4.8% (3/63) we observed in the unexposed group. Understanding the clinical relevance of the decreased serum fT levels requires assessment of sperm levels. In the present study, we found no obvious effects of daily plastic material usage on either PE levels or serum hormones.

The results of the present study must be interpreted with caution because the phthalate and hormone levels were determined from single spot urine and blood samples. It is well known that there is significant minute-to-minute variation in endogenous serum LH and FSH concentrations. For this reason, it is possible that spot sampling may cause a bias by not reflecting average hormone levels. Phthalates have short half-lives, and urinary samples reflect only recent exposure. In support of the methodology we used in the present study, Hauser et al. (2004b) reported that a single urine sample was moderately predictive of each subject's exposure over a 3-month period. Because the subjects in the exposed group had worked in the factory an average of < 1 year, the low levels of fT would have been caused by the current exposure to high levels of PEs. To further evaluate the effect of PEs on reproductive function, other potentially important biomarkers such as other PEs (e.g., monoethyl phthalate), inhibin B, and gonadotropin-releasing hormone could be assessed in addition to measuring sperm levels.

Conclusions

In the present study we observed a modest and significant reduction of serum fT in workers with higher levels of urinary MBP and MEHP compared with unexposed workers. fT was significantly and negatively correlated with urinary levels of DBP and DEHP. In future studies, analysis of the effects of PE exposure on gonadotropin and steroid hormone levels should form part of an overall risk assessment for PEs.

Correction

In the the description of the MS/MS procedure for MEHP and MEHP-d_4 in "Materials and Methods" of the article published online, the molecular transitions used in multiple reaction monitoring were incorrect; they have been corrected here.

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Soy Product and Isoflavone Consumption in Relation to Prostate Cancer in Japanese Men

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Abstract

The incidence of prostate cancer is much lower in Asian than Western populations. Environmental factors, such as dietary habits, may play a major role in the causation of prostate cancer. Although isoflavones have been suggested to show a preventive effect against prostate cancer in animal experiments, the results of epidemiologic studies are inconsistent. Here, we conducted a population-based prospective study in 43,509 Japanese men ages 45 to 74 years who generally have a high intake of isoflavones and low incidence of prostate cancer. Participants responded to a validated questionnaire, which included 147 food items. During follow-up from 1995 through 2004, 307 men were newly diagnosed with prostate cancer, of which 74 cases were advanced, 220 cases were organ localized, and 13 cases were of an undetermined stage. Intakes of genistein, daidzein, miso soup, and soy food were not associated with total prostate cancer. However, these four items decreased the risk of localized prostate cancer. In contrast, positive associations were seen between isoflavones and advanced prostate cancer. These results were strengthened when analysis was confined to men ages >60 years, in whom isoflavones and soy food were associated with a dose-dependent decrease in the risk of localized cancer, with relative risks for men in the highest quartile of genistein, daidzein, and soy food consumption compared with the lowest of 0.52 [95% confidence interval (95% CI), 0.30-0.90], 0.50 (95% CI, 0.28-0.88), and 0.52 (95% CI, 0.29-0.90), respectively. In conclusion, we found that isoflavone intake was associated with a decreased risk of localized prostate cancer. (Cancer Epidemiol Biomarkers Prev 2007;16(3):538–45)

Introduction

The incidence of prostate cancer is much lower in Asian than in Western populations (1). However, Japanese migrants to the United States and Brazil have an increased incidence (2, 3), and the incidence of latent or clinically insignificant prostate cancer in autopsy studies among men from Asian countries and the United States is similar (4, 5). It has therefore been suggested that environmental factors may play an important role in the progression of prostate cancer. Asian populations consume large quantities of soy food that contained isoflavones such as genistein and daidzein (6). Mean serum or plasma concentrations of isoflavones in Japanese men are 10 to 100 times higher than those in men from the United Kingdom (7) and Finland (8). Moreover, Morton et al. (9) reported a higher concentration of daidzein in the prostatic fluid of Asian men than in Western men. Genistein and daidzein exhibit anticarcinogenic properties and estrogenic activity *in vitro* and have shown a protective effect against prostate cancer development in some animal studies (6). On these bases, isoflavones have been recognized as key substances that may decrease the incidence of prostate cancer in Asia. However, previous findings from epidemiologic studies regarding isoflavone or soy food intake and prostate cancer are equivocal (10-17).

This inconsistency may be due to errors in exposure measurement and limited variation in soy intake. Some of the previous epidemiologic studies investigated association between prostate cancer and a single soy food only, such as tofu or soy milk, and most were conducted in Western countries, in which physiologically meaningful amounts of soy are not consumed (10-12). Here, we investigated the association between isoflavone intake and risk of prostate cancer in a prospective study in Japanese who consume large amounts of soy.

Materials and Methods

Study Population. The Japan Public Health Center–Based Prospective Study was initiated in 1990 for cohort I and in 1993 for cohort II. The study design has been described in detail previously (18). Cohort I included those residents ages 40 to 59 years who had registered their addresses in five public health center areas (Iwate, Akita, Nagano, Okinawa, and Tokyo). Cohort II included those residents ages 40 to 69 years who had registered in six public health center areas (Ibaraki, Niigata, Kochi, Nagasaki, Okinawa, and Osaka). The Tokyo subjects were not included in the data analysis because incidence data were not available. This study was approved by the institutional review board of the National Cancer Center, Tokyo, Japan. The initial cohort consisted of 68,557 men.

Food Frequency Questionnaire. At baseline, participants completed a self-administered questionnaire that assessed information on lifestyle factors, medical, and smoking histories. The food frequency questionnaire (FFQ) in the baseline survey had 44 food items for cohort I and 52 food items for cohort II with four (cohort I) or five (cohort II) frequency categories but without standard portions/units. In contrast, the 5-year follow-up survey included a self-administered FFQ, which included lifestyle factors, medical history, and 147 food

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Study Group members are listed in Appendix A.

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and beverage items with standard portions/units and nine frequency categories. Owing to this greater detail, the present study therefore used the 5-year follow-up survey as baseline and followed the subjects from 1995 for cohort I and from 1998 for cohort II until 2004. After the 5-year follow-up survey, 128 subjects were found to be ineligible and were excluded because of non-Japanese nationality (n = 28), late report of emigration occurring before the start of the follow-up period (n = 97), incorrect birth data (n = 3), and subjects with self-reported prostate cancer (n = 21), leaving 58,427 men eligible for participation. Among eligible subjects, 46,001 men (79%) returned valid responses to the 5-year follow-up FFQ.

We dealt with two item groups: consumption of miso soup and soy food. Soy food referred to the consumption of "Tofu, Yushidofu (pre-drained tofu), Koyadofu (freeze-dried tofu), Aburaage (deep-fried tofu), Natto (fermented soybean), and soymilk," for which the major ingredient is soybeans. The questionnaire asked about the usual consumption of 147 foods and beverages during the previous year. The frequency of miso soup consumption was divided into six categories (almost never, 1-3 days/mo, 1-2 days/wk, 3-4 days/wk, 5-6 days/wk, and daily). Portion sizes were specified, and the amounts provided in three categories (less than half, same, and more than 1.5 times). One bowl of miso soup was calculated as 150 mL. Nine frequency categories were used for soy foods (almost never, 1-3 times per month, 1-2 times per week, 3-4 times per week, 5-6 times per week, once a day, 2-3 times per day, 4-6 times per day, and ≥7 times per day). Portion sizes were specified, and the amounts were determined in three categories (less than half, same, and more than 1.5 times). Ten frequency categories were used for soy milk (almost never, 1-3 times per month, 1-2 times per week, 3-4 times per week, 5-6 times per week, 1 glass per day, 2-3 glasses per day, 4-6 glasses per day, 7-9 glasses per day, and >9 glasses per day). The total consumption of miso soup (mL/d) and soy food (g/d) was calculated from these responses, whereas that of isoflavones (daidzein and genistein) was calculated using values in a specially developed food composition table for isoflavones in Japanese foods (19, 20).

Validity was assessed among subsamples using 14- or 28-day dietary records. Spearman's correlation coefficients between the energy-adjusted intake of miso soup and soy food consumption from the questionnaire and from dietary records were 0.54 and 0.53 for cohort I and 0.48 and 0.52 for cohort II, respectively, whereas those for energy-adjusted intake of daidzein and genistein were 0.65 and 0.65 for cohort I and 0.49 and 0.48 for cohort II, respectively. Moreover, Spearman's correlation coefficients for daidzein and genistein between energy-adjusted intakes from FFQ and those from serum concentration were 0.26 and 0.40, respectively, and with those from creatinine-adjusted urinary excretion were 0.22 and 0.33, respectively (21). These correlation coefficients are considered acceptable (22). With regard to the reproducibility of estimations between two questionnaires administered 1 year apart, respective correlation coefficients for the energy-adjusted intake of miso soup, soy food, daidzein, and genistein were 0.80, 0.64, 0.75, and 0.75 for cohort I and 0.75, 0.57, 0.53, and 0.51 for cohort II (23-25).

Among the 46,001 men who responded to the questionnaire, 2,492 who reported extreme total energy intake (<800 or >4,000 kcal) were excluded, leaving 43,509 men for analysis.

Follow-up. Subjects were followed from the 5-year follow-up survey until December 31, 2004. Changes in residence status, including survival, were identified annually through the residential registry in each area or, for those who had moved out of the study area, through the municipal office of the area to which they had moved. Generally, mortality data for residents included in the residential registry are forwarded to the Ministry of Health, Labour, and Welfare and coded for inclusion in the national Vital Statistics. Residency and death registration are required by the Basic Residential Register Law and Family Registry Law, respectively, and the registries are believed to be complete. Here, information on the cause of death was based on death certificates from the respective public health center for those who had not moved out of the original area. Among questionnaire respondents to the 5-year follow-up FFQ, 3,855 men (8.4%) died, 1,492 men (3.2%) moved out of the study area, and 66 men (0.1%) were lost to follow-up during the study period.

The occurrence of cancer was identified by active patient notification from major local hospitals in the study area and data linkage with population-based cancer registries, with permission from the local governments responsible for the cancer registries. Cases were coded using the International Classification of Diseases for Oncology, Third Edition (26). Death certificate information was used as a supplementary information source. The proportion of cases of prostate cancer first notified by death certificate was 0.9%. The ratio of incidence to mortality was 7.7. The registration rate as introduced by Parkin et al. (27) was 94.3%. The proportion of case patients with prostate cancer ascertained by death certificate only was 0.6%. These ratios were considered satisfactory for the present study. A total of 307 newly diagnosed prostate cancer cases were identified by December 31, 2004.

Finally, a population-based cohort of 43,509 men (18,105 in cohort I and 25,404 in cohort II) was established for analysis. During the 325,371 person-years of follow-up (167,611 in cohort I and 157,760 in cohort II), 307 cases of prostate cancer were newly diagnosed (156 in cohort I and 151 in cohort II).

Statistical Analysis. Person-years of follow-up were calculated for each man from the date of completion of the 5-year follow-up FFQ to the date of prostate cancer diagnosis, the date of emigration from the study area, or the date of death, whichever came first; or if none of these occurred, follow-up was through to the end of the study period (December 31, 2004). Men who were lost to follow-up were censored at the last confirmed date of presence in the study area. The crude incidence rate for prostate cancer was calculated by dividing the number of prostate cancer cases by the number of person-years. The relative risks (RR) of prostate cancer were calculated in quartile for the categories of miso soup consumption, soy food consumption, and isoflavone intake, with the lowest consumption category as the reference. RRs and 95% confidence intervals (95% CI) were calculated by the Cox proportional hazards model, adjusting for age at 5-year follow-up survey and study area (10 public health center areas) according to the SAS PHREG procedure (version 9.1; SAS Institute, Inc., Cary, NC). For further adjustment, additional possible confounders were incorporated into the model: smoking status (never, former, and current); alcohol intake (almost never, <3-4 days/wk, and >5 days/wk); marital status (yes/no); body mass index; and consumption of dairy foods, vegetables, fruit, and total fatty acids.

We conducted additional analyses according to the stage of prostate cancer. Advanced cases were defined by a diagnosis of extraprostatic or metastatic cancer involving lymph nodes or other organs. If this information was not available, advanced cases were defined as those with a high Gleason score (8-10) or poor differentiation. These criteria were selected to allow the identification of advanced cases with a high likelihood of poor prognosis. The remaining cases were organ localized. In this study, there were 74 advanced cases, 220 localized cases, and 13 (4% of total) cases of undetermined stage.

The trend was assessed by assigning ordinal values for categorical variables. All Ps were two sided, and statistical significance was determined at the $P < 0.05$ level.

Results

Distribution of subject characteristics at the 5-year follow-up survey according to quartile of energy-adjusted isoflavone consumption is shown in Table 1, in which the results for genistein were used as a surrogate for isoflavones owing to the high correlation among results for genistein, daidzein, miso soup, and soy food. Men with high genistein consumption were slightly older. Body mass index was higher in the highest category than in the other categories. The proportion of current smokers increased as genistein intake increased, whereas alcohol intake decreased. The proportion of men who live with their wife was lower in the lowest category than in the other categories. Screening-detected prostate cancer accounted for >40% of cases in all categories. Dairy food, fruit, vegetable, and total fatty acid consumption were positively correlated with genistein intake. As expected, soy food and miso soup increased as genistein intake increased and were highly correlated with it ($P < 0.0001$).

Table 2 shows age- and area-adjusted and multivariable RRs and 95% CIs for total prostate cancer by each quartile of genistein, daidzein, miso soup, and soy food consumption. Genistein, daidzein, and soy food consumption slightly decreased the risk of total prostate cancer. Multivariable RRs for the highest versus lowest quartile of genistein, daidzein, and soy food consumption were 0.71 (95% CI, 0.48-1.03), 0.77 (95% CI, 0.52-1.13), and 0.82 (95% CI, 0.57-1.19), respectively. Tests for linear trends were not statistically significant. No statistically significant association was seen between miso soup consumption and total prostate cancer risk (highest versus lowest: RR, 1.04; 95% CI, 0.72-1.50).

We next classified the data according to prostate cancer stage (Table 3). Consumption of genistein decreased the risk of localized prostate cancer in a statistically significant manner (RR, 0.59; 95% CI, 0.38-0.93), although P_{trend} was not statistically significant. Men in the highest quartile of daidzein also had a decreased the risk of localized prostate cancer, although with only marginal significance (RR, 0.66; 95% CI, 0.42-1.04). Miso soup and soy foods tended to decrease the risk of localized prostate cancer, with respective multivariable RRs for the highest versus lowest quartile of 0.78 (95% CI, 0.51-1.20) and 0.77 (95% CI, 0.50-1.19). In contrast, genistein and daidzein increased the risk of advanced prostate cancer, with respective RRs for men with the highest versus lowest consumption of genistein and daidzein of 1.26 (95% CI, 0.56-2.83) and 1.43 (95% CI, 0.63-3.28). Miso soup was dose-dependently increased the risk of advanced prostate cancer, with multivariable RR for the highest versus lowest quartile of 2.79 (95% CI, 1.19-6.55; $P_{trend} = 0.02$). Consumption of soy food was not associated with advanced cancer.

Table 4 shows multivariable RRs and 95% CIs for prostate cancer by stage categorized according to age. We found that the negative association with genistein, daidzein, and soy food and localized prostate cancer became clear when we analysis was restricted to men ages >60 years. Genistein, daidzein, and soy food were dose-dependently associated with a decreased risk of localized prostate cancer, with multivariable RR for the highest versus lowest quartile of 0.52 for genistein (95% CI, 0.30-0.90; $P_{trend} = 0.03$), 0.50 for daidzein (95% CI, 0.28-0.88; $P_{trend} = 0.04$), and 0.52 for soy food (95% CI, 0.29-0.90; $P_{trend} = 0.01$). Miso soup also decreased the risk of localized prostate cancer, although without statistical significance. In contrast, RRs of daidzein and miso soup showed an increased risk of advanced prostate cancer in men ages >60 years old. In particular, miso soup was positively associated with advanced cancer, with a multivariable RR for the highest versus lowest quartile of 2.86 (95% CI, 1.01-8.11). Daidzein tended to increase the risk of advanced cancer (highest versus lowest: RR, 1.49; 95% CI, 0.55-4.03). Genistein was not associated with advanced cancer. Soy food tended to be negatively associated with advanced cancer in men ages >60 years. In men ages ≤60 years, genistein, daidzein, and soy food increased the risk of both localized and advanced prostate cancer. Multivariable RR for the highest versus lowest quartile was 1.18 for genistein, 1.38 for daidzein, and 1.38 for soy food in localized prostate cancer and 2.00 for genistein, 2.46 for daidzein, and 1.48 for soy food in advanced prostate cancer. Miso soup was not associated with localized prostate cancer (RR, 0.82) but was associated with an increased risk of advanced prostate cancer (RR, 2.65). However, none of these values was statistically significant.

To weaken the influence of localized prostate cancer detected by prostate-specific antigen screening, we also analyzed the association between prostate cancer and the four items after excluding screening-detected tumors by stage in men ages >60 years, notwithstanding that screening information was available for only 70% of subjects (Table 5). Results in both localized and advanced prostate cancer were similar to those in Table 4 when screening-detected prostate cancer was included, although the statistical significance in these results was lost. Genistein, daidzein, and miso soup tended to decrease the risk of localized prostate cancer (highest versus lowest: RRs of genistein, daidzein, miso soup, and soy food of 0.52, 0.49, 0.73, and 0.51, respectively) but without statistical significance. In advanced prostate cancer, multivariable RR for the highest versus lowest quartile was 0.85 for genistein,

Table 1. Characteristics of study subjects according to genistein consumption

	Genistein consumption				$P_{difference}$*
	Lowest	Second	Third	Highest	
Age ± SD (y)	56.2 ± 8.2	56.5 ± 7.9	56.7 ± 7.7	57.7 ± 7.6	<0.0001
Body mass index ± SD (kg/m^2)	23.6 ± 3.0	23.6 ± 2.9	23.6 ± 2.8	23.7 ± 2.9	0.009
Current smoker (%)	49.8	52.3	54.5	58.8	<0.0001
Alcohol intake, ≧5 d/wk (%)	49.1	49.7	49.5	46.2	<0.0001
Men who live with their wife (%)	81.1	83.9	83.8	83.2	<0.0001
Screening-detected tumors (%)	40.4	43.9	47.4	42.6	0.97
Dairy food (g/d)	161.2 ± 260.1	159.5 ± 215.1	164.7 ± 200.4	170.1 ± 188.5	0.002
Fruits (g/d)	152.3 ± 169.5	175.7 ± 168.2	193.5 ± 177.6	201.4 ± 177.2	<0.0001
Vegetables (g/d)	163.9 ± 141.6	192.0 ± 143.2	209.5 ± 146.1	225.2 ± 159.7	<0.0001
Total fatty acids (g/d)	48.7 ± 26.9	48.9 ± 23.8	51.0 ± 24.2	51.6 ± 23.0	<0.0001
Soy food (g/d)	33.0 ± 18.8	62.4 ± 26.4	92.3 ± 39.6	164.5 ± 144.6	<0.0001
Miso soup (mL/d)	51.3 ± 39.6	167.4 ± 57.3	301.8 ± 74.2	449.6 ± 139.0	<0.0001
Genistein (mg/d)	8.5 ± 4.3	17.0 ± 5.7	26.8 ± 9.1	49.1 ± 30.9	<0.0001
Daidzein (mg/d)	5.4 ± 2.8	10.8 ± 3.5	16.8 ± 5.5	29.9 ± 17.8	<0.0001

NOTE: Results for genistein are reported as isoflavones because the intake estimates for genistein and daidzein were highly correlated ($P < 0.0001$).
*$P_{difference}$ values of characteristics between categories of genistein consumption were calculated by ANOVA and the χ^2 test for homogeneity.

Table 2. RRs and 95% CIs for total prostate cancer according to quartile of energy-adjusted intake of genistein, daidzein, miso soup, and soy food

	Intake by quartile				P_{trend}
	Lowest (<13.2 mg/d)	Second (13.2-21.2 mg/d)	Third (21.3-32.7 mg/d)	Highest (≧ 32.8 mg/d)	
Genistein					
No. cases	75	76	91	65	
Person-years of follow-up	78,439	81,443	83,208	82,282	
Age/area–adjusted RR (95% CI)	1.00	0.92 (0.67-1.27)	1.16 (0.84-1.59)	0.80 (0.56-1.14)	0.48
Multivariate RR (95% CI)	1.00	0.81 (0.62-1.23)	1.13 (0.81-1.57)	0.71 (0.48-1.03)	0.22
	Intake by quartile				P_{trend}
	Lowest (<8.5 mg/d)	Second (8.5-13.4 mg/d)	Third (13.5-20.3 mg/d)	Highest (≧ 20.4 mg/d)	
Daidzein					
No. cases	70	79	93	65	
Person-years of follow-up	78,260	81,548	83,193	82,370	
Age/area–adjusted RR (95% CI)	1.00	1.01 (0.73-1.40)	1.25 (0.91-1.72)	0.87 (0.61-1.25)	0.77
Multivariate RR (95% CI)	1.00	0.95 (0.67-1.33)	1.21 (0.87-1.70)	0.77 (0.52-1.13)	0.43
	Intake by quartile				P_{trend}
	Lowest (<110.0 mL/d)	Second (110.0-225.9 mL/d)	Third (226.0-355.9 mL/d)	Highest (≧ 356.0 mL/d)	
Miso soup					
No. cases	58	79	85	85	
Person-years of follow-up	75,651	79,621	84,403	85,696	
Age/area–adjusted RR (95% CI)	1.00	1.11 (0.79-1.57)	1.10 (0.78-1.56)	1.06 (0.75-1.51)	0.82
Multivariate RR (95% CI)	1.00	1.10 (0.77-1.58)	1.08 (0.75-1.55)	1.04 (0.72-1.50)	0.94
	Intake by quartile				P_{trend}
	Lowest (<46.6g/d)	Second (46.6-71.8 g/d)	Third (71.9-107.3 g/d)	Highest (≧ 107.4 g/d)	
Soy food					
No. cases	66	88	79	74	
Person-years of follow-up	77,756	81,557	83,116	82,941	
Age/area–adjusted RR (95% CI)	1.00	1.18 (0.85-1.63)	1.05 (0.75-1.47)	0.91 (0.65-1.29)	0.43
Multivariate RR (95% CI)	1.00	1.10 (0.78-1.54)	0.95 (0.67-1.36)	0.82 (0.57-1.19)	0.18

NOTE: Multivariate RRs were adjusted for age, area, smoking status, drinking frequency, marital status, body mass index, and intake of total fatty acids, dairy, vegetables, and fruits.

1.10 for daidzein, 1.97 for miso soup, and 0.73 for soy food; however, these results were not statistically significant. Results in subjects ages ≤60 years were similar to those which included screening-detected cancers. Multivariable RR for the highest versus lowest quartile was 1.28 (95% CI, 0.33-4.97) for genistein, 1.55 (95% CI, 0.42-5.78) for daidzein, and 1.94 (95% CI, 0.58-6.52) for soy food in localized prostate cancer and 2.22 (95% CI, 0.50-9.91) for genistein, 2.93 (95% CI, 0.53-16.29) for daidzein, and 1.82 (95% CI, 0.33-9.91) for soy food in advanced prostate cancer. However, these values were not statistically significant (data not shown).

Discussion

In the present study, we observed a dose-dependent decrease in the risk of localized prostate cancer with isoflavone consumption. Men with the highest intake of isoflavones (as genistein, ≧32.8 mg/d) had a decreased risk of prostate cancer compared with those with the lowest intake of isoflavones (as genistein, <13.2 mg/d). To our knowledge, this is the first prospective study to report an inverse association between isoflavone and localized prostate cancer in Japanese, whose intake of soy food is high.

Our results support previous studies, which reported that soy food is protective for prostate cancer. Among case-control studies, Sonoda et al. (17) reported that *natto* (fermented soy) consumption showed a significantly decreasing linear trend for risk of prostate cancer in Japanese; Lee et al. (13) found that the highest intake of tofu and genistein had a statistically significant association with a decreased risk of prostate cancer in Chinese compared with the lowest intake; and Strom et al. (11) reported an inverse association between daidzein intake and prostate cancer risk in American men. Soy foods were also inversely related to prostate cancer in a large multicenter case-control study (12). In prospective studies, Jacobson et al. (10) reported that frequent consumption of soy milk was associated with a decreased risk of prostate cancer in Californian Adventist men. However, no association was seen between tofu consumption and a decreased risk of prostate cancer in Japanese men living in Hawaii (16), nor was tofu or miso soup significantly associated with prostate cancer risk in native Japanese (14). The reason these studies did not show a protective effect of soy foods on prostate cancer may have been due to their evaluation of a single soy food only or their failure to assess specific nutrients such as genistein or daidzein.

Studies *in vivo* and *in vitro* experiments have also shown a protective effect of isoflavones against prostate cancer development. Isoflavones possess weak estrogen activity, inhibit tyrosine protein kinases and angiogenesis, and reduce serum testosterone levels (6, 28, 29). Isoflavones also inhibit 5α-reductase, an enzyme that metabolizes testosterone to dihydrotestosterone (30). Any or all of these mechanisms may explain the inverse association between isoflavones and localized prostate cancer seen here. Moreover, our results are plausible because the incidence of prostate cancer in Japanese is much lower than in Western men (1).

However, when the data were analyzed by stage, we found that the results differed between advanced and localized