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Endocrine disrupting chemicals in urine of Japanese male partners of subfertile couples: A pilot study on exposure and semen quality

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ABSTRACT

The concentrations of chemicals with suspected endocrine disrupting effect were measured in urine samples collected from 42 Japanese male partners of couples who had infertility consultation at a gynecology clinic in Tokyo. The urinary analytes included metabolites of 5 phthalate diesters, pyrethroid insecticide (3-phenoxybenzoic acid, 3-PBA) and soy isoflavones (daidzein and equol), and cadmium. The semen parameters (semen volume, concentration and motility) of the male subjects were examined at the clinic as a diagnostic screening. Multiple regression analysis using one of the semen parameters examined as dependent variable and urinary biomarkers with age, body mass index, abstinent period, alcohol drinking, smoking and consumption frequency of selected foods as independent variables. For sperm concentration, urinary mono-n-butyl phthalate was selected as a significant independent variable with positive beta, while urinary daidzein was with negative beta. Consumption frequency of coffee (negative) and fruits (positive) were also significant. For sperm motility, urinary 3-PBA was selected as significant with negative beta as well as detectability of equol and frequency of coffee consumption with negative beta while smoking was with positive beta. This pilot study suggested the pyrethroid exposure level and dietary habit (coffee and soy products) as a significant contributor to poorer semen quality.

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Introduction

Increased concern over the reported decline in sperm concentration in the past 50 years (Carlsen et al., 1992) has been noted. This secular trend has often been discussed in relation to increased exposure to chemicals, particularly those with endocrine disrupting actions, among general populations. Although it is still controversial as to whether the secular decline in sperm concentration has actually taken place and whether the hypothesis on the involvement of chemical exposure is valid, it is certain that chemicals with male reproductive toxicity, as revealed by *in vitro* and *in vivo* studies, are present in our general environment.

To date, exposure to persistent organochlorine compounds (e.g., PCB and DDE), pyrethroid insecticides, phthalates, heavy metals and others in non-occupationally exposed population was studied in relation to human semen parameters. Statistically significant deterioration of semen parameters with increasing exposure to these chemicals were found in some studies (e.g., Telišman et al.,

2000; Richthoff et al., 2003; Duty et al., 2003; Toft et al., 2006; Hauser et al., 2006; Xia et al., 2008; Meeker et al., 2008) but not in others (e.g., Jurasović et al., 2004; Jönsson et al., 2005; Herr et al., 2009). It is necessary to accumulate more human data in different settings (ethnicity, dietary habit, etc.) to elucidate if the effect really takes place and the extent of the effect if any. In these studies, exposure to chemicals was assessed by biomarkers of exposure which is indispensable in more accurate exposure assessment. Moreover, there are many factors other than chemical exposure that are thought to be involved in semen quality, such as dietary habit and lifestyle (Jurewicz et al., 2009; Li et al., 2011).

The present pilot study was designed to measure the concentrations of several metabolites of chemicals in urine of Japanese male partners of couples who had infertility consultation, and to preliminarily relate those biomarkers of exposure to semen parameters of the subjects taking dietary and other factors into consideration. The analytes of this study include metabolites of chemicals with suspected endocrine disrupting effect, i.e., phthalate esters and pyrethroid insecticide, and cadmium, as well as soy-derived phytoestrogen metabolites (daidzein and equol) as potential contributor (Chavarro et al., 2008) because of its abundance in Japanese diet.

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Materials and methods

Subjects

The subject of the present study was the male partner of couple who visited a gynecology clinic in Tokyo for infertility consultation during January to June 2010. We asked them if they voluntarily participate in our study after being explained the purpose and procedure of the study from a gynecologist. Written consent was obtained from 42 male subjects.

At the time of clinic visit, the semen sampling kit was distributed to the subject for routine examination of diagnostic screening purpose. The subject was instructed to sample semen by masturbation into a wide-mouthed sterilized plastic cup after at least 2-days abstinence. Semen sampling was done at home in most of the cases: the subject was instructed to take semen sample to the clinic within 3 h after sampling while paying utmost attention not to expose the sample to heat or low temperature. Note that the present method of semen transportation to laboratory did not strictly follow WHO guideline (e.g., semen has to be transported at 37 °C within 1 h of sampling, Working Group for Standardized Guideline for Semen Examination, 2003) because of practical limitations under clinical situation.

Urine cup, polypropylene (PP) bottle and self-administer questionnaire on smoking and dietary habit was also distributed to the subject for this study. The subject was asked to sample urine in the cup and transfer approximately 20 mL of it to PP bottle by themselves just before semen sampling. According to the record, the urine samples were taken in the morning (7–11 a.m.). Urine sample was taken to the clinic at the time of submission of semen sample for examination. Urine sample was stored in a freezer at –20 °C until analysis at University of Tokyo.

Methods

The semen sample was examined for volume by gravimetric method (assuming density=1) and concentration and motility of sperm was on Makler counting chamber. Counting the number of sperm and evaluation of motility was based on the Guideline of Urology Society of Japan (Working Group for Standardized Guideline for Semen Examination, 2003), which was based on the 1999 Guideline of WHO, as much as possible. Semen examination was carried out at the clinic by one person who was not aware of urinary biomarker results.

Determination of 7 metabolites of 5 phthalate diesters in urine was carried out by enzymatic deconjugation and solid-phase extraction (OASIS MAX 150 mg/6 cm³, Nihon Waters Co. Ltd., Tokyo, Japan) followed by liquid chromatography tandem mass spectrometry (LCMSMS) (LC: 1100 Series, Agilent Technologies, Inc., CA, USA; MS: Micromass Quattro Ultima, Manchester, UK) according to Suzuki et al. (2009). The metabolites analyzed were mono methyl phthalate (MMP), mono ethyl phthalate (MEP), mono-n-butyl phthalate (MnBP), mono benzyl phthalate (MBzP), mono-2-ethylhexyl phthalate (MEHP), mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-oxohexyl phthalate (MEOHP). Determination of a metabolite of pyrethroids, 3-phenoxybenzoic acid (3-PBA), was carried out by LCMSMS according to Olsson et al. (2004) and Baker et al. (2004) except that acidic deconjugation was used in the present analysis. Cartridge used for solid phase extraction of 3-PBA was OASIS HLB 150 mg/6 cm³ (Nihon Waters Co. Ltd., Tokyo, Japan). Urinary metabolites of isoflavones (daidzein and equol) were analyzed by gas chromatography mass spectrometric (GCMS) method of Grace et al. (2003) using 6850 GC with 5975 MS detector from Agilent Technologies, Inc. (CA, USA). ¹³C labeled internal standard was used for phthalate metabolites and daidzein analyses, and

deuterium labeled internal standard for 3-PBA. ¹³C labeled bisphenol A was used as internal standard for equol. Urinary cadmium concentration was determined by electrothermal atomic absorption spectrometry (ETAAS) (Contra AA, Analytik Jena Japan, Kanagawa, Japan) after 5-fold dilution of urine sample. Palladium nitrate matrix modifier and standard addition calibration was employed.

These analyses were validated by extensive internal quality assurance practices including periodic blank measurements, recovery test, and X-R chart based on in-house control urine samples. External quality control was carried out by joining in international proficiency test (phthalate metabolites) or by the analysis of reference material (cadmium, Seronorm[®] Urine Blank, Norway).

Specific gravity of urine sample was measured by a refractometer to correct for dilution effect. Urinary concentrations of chemicals were expressed as specific gravity-corrected value by the following equation:

$$C_c = C_m \times \frac{1.020 - 1}{SG - 1}$$

where C_c and C_m is the corrected and measured concentration, respectively, and SG is the measured specific gravity.

From the self-administered questionnaire, information on smoking status, consumption frequency of some food items (vegetable, fruit, soy products, alcohol, coffee and tea), which were considered the potential modifier of semen quality, was obtained.

Statistical analysis

The specific gravity-corrected concentrations of chemicals had skewed distribution: log-transformation was applied for statistical analyses. Semen parameters were used as crude value. The association between semen parameters and urinary biomarkers were examined in two different ways: one was comparison of urinary biomarker concentrations between the subjects with semen parameter above or below the former WHO reference value of 2010 (semen volume 1.5 mL, sperm concentration 15×10^6 count/mL and motility 40%). Student's *t*-test was used for the comparison of log-transformed urinary chemical/metabolite concentrations between the groups. The other was by using semen parameters as continuous variables and analyzed the association with urinary biomarkers by correlation analysis and multiple regression analysis. Urinary equol concentration was not used as continuous variable because of distorted distribution. Equol is a metabolic product of intestinal microflora, of which composition varies from person to person. Therefore, some person can make equol in response to soy isoflavone intake (called "equol producer") but others cannot (Setchell and Clerici, 2010). In the statistical analyses of the present study, dichotomized variable was used for urinary equol by the detectability. These statistical analyses were performed by using SPSS ver 12.0 J.

Ethical consideration

Only the subjects who voluntarily agreed to participate and those who gave written informed consent were the subjects of the present study. The Ethical Committee of the University of Tokyo approved this study.

Results

The characteristics of the present subjects and the results of semen examination are shown in Table 1. Mean age of the subjects was 36.8 and 11 of the 41 was current smoker (data for one subject was missing). Abstinent period before semen sampling of one of the subjects was <2 days, so this subject was excluded in the following analyses on semen parameters. There were no subjects whose

Table 1

Characteristics and semen parameters of the male subjects.

	Unit	Mean (SD)	Min	Max
Age	years	36.8 (5.4)	29	58
Length	m	1.73 (0.05)	1.63	1.86
Weight	kg	70.2 (7.8)	56	93
BMI	kg/m ²	23.4 (2.6)	18.7	31.1
Semen volume	mL	3.6 (1.3)	1.1	7.1
Sperm concentration	× 10 ⁶ /mL	80.6 (47.8)	0.8	236
Motility (A + B)	%	40.9 (20.7)	6.8	85.0
Abstinence period	days	4.8 (2.0)	1	7
Current smoker	%		26.8 (11/41)	
Drinking >1 bottle of beer/day	%		26.2 (11/42)	
Drinking >1 cup of coffee/day	%		63.4 (26/41)	
Drinking >1 cup of tea/day	%		71.4 (30/42)	
Eating vegetable everyday	%		58.5 (24/41)	
Eating fruits >1 time/week	%		48.8 (20/41)	
Eating soy products >1 time/week	%		76.2 (32/42)	

abstinent period exceeded 7 days. Two of the 41 had <1.5 mL semen volume, 1 of 41 had sperm concentration of <15 × 10⁶ count/mL and 23 of 41 had <40% motility.

The results of internal quality assurance of urinary biomarkers analysis (phthalates, 3-PBA, daidzein, equol, and cadmium) were as follows: reproducibility of analysis for the analytes other than MEHHP was 2.7–10.4%. That of MEHHP was 26%. Recovery of added native standard was 90–112%. No procedural blank was detected for any analytes.

In Table 2, urinary concentrations of 7 phthalate metabolites, 3-PBA, daidzein, equol and cadmium of the present subjects were shown. In this table, data of the subject who donated semen sample with <2 days abstinence period was included. Urinary concentration was shown as specific gravity corrected values. All of the analytes were detectable in the subjects' urine samples except for equol. Equol was detected in 21 of 42 subjects.

When the subjects were divided into two groups by the reference value for each semen parameter (1.5 mL for volume, 15 × 10⁶ count/mL for concentration and 40% for motility) and urinary metabolite concentrations were compared between the two groups, higher MnBP ($p < 0.05$) and MBzP ($p < 0.01$) concentration was found in the subjects with ≥ 1.5 mL semen volume ($n = 39$) than in those <1.5 mL ($n = 2$). Equol concentration was marginally higher ($p = 0.051$) in the subjects with ≥ 40% motility. No other biomarker concentrations were significantly different between the two groups for sperm concentrations and motility. Since there was only one subject whose semen volume was <1.5 mL, comparison was not possible for this parameter.

Pearson correlation analyses between semen parameters and urinary biomarkers (except for equol) found only a weak positive correlation between sperm concentration and urinary cadmium as significant ($r = 0.316$, $p = 0.044$). Negative correlation between sperm concentration and urinary daidzein concentration was marginally insignificant ($r = -0.292$, $p = 0.064$). None of the variables were significantly correlated with motility or semen volume. Semen quality was compared between two groups of subjects, i.e., equol detected ($n = 21$) and non-detected subjects ($n = 20$), to find that sperm motility was significantly different between the two groups (48.2 ± 21.0% in non-detectable vs 32.5 ± 16.8% in detectable, $p = 0.013$).

Association was examined between semen parameters and smoking, alcohol drinking, and dietary habit of the subjects. Sperm concentration was significantly higher in the subjects who consume vegetable daily (95.4 ± 51.5) × 10⁶ count/mL, $n = 23$) than in those who did not (64.7 ± 36.9) × 10⁶, $n = 17$). It was higher in those who consume fruits more than once a week (108.1 ± 44.0) × 10⁶, $n = 19$) than in those who did not (59.0 ± 39.0) × 10⁶, $n = 21$). Motility was significantly lower in the subjects who drink more than

1 cup of coffee a day (35.5 ± 19.5%, $n = 26$) than in those who did not ($n = 50.7 \pm 18.2$ %, $n = 14$). Semen parameters were not statistically significantly related to smoking, alcohol drinking, tea drinking and consumption of soy products in the present subjects except for marginally insignificant ($p = 0.064$) difference in motility between current smokers (48.4%) and non-smokers (35.6%).

The association between semen parameters and urinary biomarkers of chemical exposure was further assessed by multiple regression analysis. Dependent variable was either one of semen volume, sperm concentration or motility. Independent variables included urinary biomarker concentration (specific gravity corrected), equol detectability, and the following variables as potentially influencing co-variables as deduced from literature or the aforementioned analyses: age, BMI, abstinence period, smoking status, consumption frequency of vegetable, fruits, and coffee. For smoking status and consumption frequency of selected items, as well as equol detectability, dichotomized variable was used. Table 3 shows the summary of the result. Frequency of fruits and coffee consumption, urinary concentration of daidzein and MnBP were selected as significant for sperm concentration. Significant correlation found between urinary cadmium and sperm concentration diminished when other co-variables were controlled. Frequency of coffee consumption, equol detectability, smoking status and urinary 3-PBA concentration were selected for motility, though univariate correlation analysis did not find any significant correlations. No independent variable was selected for semen volume.

Although data are not shown, the results of multiple regression analyses were essentially similar to Table 3 when creatinine-corrected urinary biomarker concentration was used.

Discussion

The subjects of the present study were male partners of couples who had not conceived for a certain period of time and had infertility consultation at a gynecology clinic in Tokyo. Therefore, the present subjects could include both fertile and infertile male: in fact, screening semen examination revealed 19 out of 42 subjects who had all of the semen parameters above the reference values of WHO of 2010 (one subject with less than 2 days abstinence period was one of the 19 subjects). However, we should be cautious in comparing the data of semen parameters in this study with WHO reference values because semen examination of the present study was for diagnostic screening purpose and it was not performed by strictly following the WHO guideline.

In this study, urinary concentrations of chemicals that have been considered to have endocrine disrupting actions were measured (Table 2). Except for equol, all other metabolites and cadmium were detected in all of the subjects confirming that the target

Table 2
Specific gravity corrected urinary concentrations of chemicals.^a

	Unit	Detection limit	Geometric mean (SD)	Min	Median	Max
MMP	ng/mL	0.02	7.98 (2.33)	1.22	7.22	139
MEP	ng/mL	0.03	12.8 (4.14)	0.252	10.7	201
MnBP	ng/mL	0.2	62.4 (1.82)	18.3	65.7	183
MBzP	ng/mL	0.03	9.73 (3.12)	1.44	9.18	164
MEHP	ng/mL	0.02	6.23 (2.13)	1.21	5.94	55.7
MEHHP	ng/mL	0.006	11.2 (1.98)	2.62	11.5	50.9
MEOHP	ng/mL	0.02	8.09 (1.89)	2.16	7.93	31.4
3-PBA	ng/mL	0.04	0.547 (2.76)	0.160	0.458	7.72
Daizidin	μg/mL	0.008	0.943 (3.38)	0.0454	1.14	7.13
Equol	μg/mL	0.005	0.0470 (24.0) ^b	<0.005	0.0391	14.4
Cadmium	ng/mL	0.03	0.558 (1.71)	0.148	0.561	2.32

^a Adjusted to the specific gravity of 1.020.^b One half of detection limit value was substituted when undetectable for the calculation of the mean.

chemicals of suspected endocrine disrupting effect are ubiquitous in our general environment. With regard to equol, this metabolite is detectable in only the person who has equol-producing intestinal microflora (Setchell and Clerici, 2010). It is known that equol has capability to bind estrogen receptor and that the estrogenic activity is than its precursor in *in vitro* system (Miyahara et al., 2003). Thus, in theory, an equol producer will be more likely to be affected by soy isoflavone exposure than non-producer when the same amount of soy isoflavones is taken, although there are studies which are not in line with this (e.g., Vafeiadou et al., 2006).

The comparison with national representative concentrations in the US males (Valentín-Blasini et al., 2005; Barr et al., 2010; CDC, 2010) showed that the geometric mean concentration was higher in the present subjects for MMP (1.17 ng/mL in the US vs 7.98 ng/mL in the present), MnBP (19.8 ng/mL vs 62.4 ng/mL), daidzein (0.0896 μg/mL vs 0.943 μg/mL), equol (0.00914 μg/mL vs 0.047 μg/mL) and cadmium (0.195 ng/mL vs 0.558 ng/mL). Higher concentration of soy isoflavone metabolites and cadmium in our subjects was most likely due to greater daily consumption of soy products and rice in Japanese, respectively. On the contrary, urinary MEP concentration was lower in the present study (12.8 ng/mL) than in the US (162 ng/mL) by one order of magnitude. The difference observed between the urinary concentrations of phthalate metabolites in the two countries are not clear but difference in lifestyle including use of personal care products may be the reason. When this different exposure situation is considered, it was expected that combined effect of each chemical to semen quality, if any, can be different between the people in the US and Japan.

In the multiple regression analyses, selected variables for explaining the variance of semen quality included exposure to two environmental chemicals (di-n-butyl phthalate ester and pyrethroid insecticide), exposure to phytoestrogen (soy isoflavones), smoking and dietary habits (Table 3).

Table 3
Result of multiple regression analysis.

	Standardized β	<i>t</i>	<i>p</i>
Dependent variable: sperm concentration $R^2 = 0.557$			
Consumption of fruits ^a	0.599	5.378	<0.001
Consumption of coffee ^b	-0.375	-3.362	<0.01
Urinary daidzein	-0.463	-3.769	<0.001
Urinary MnBP	0.294	2.384	<0.05
Dependent variable: motility $R^2 = 0.478$			
Consumption of coffee ^b	-0.469	-3.908	<0.001
Detectability of equol ^c	-0.315	-2.639	<0.05
Current smoker ^d	0.474	3.713	<0.001
Urinary 3-PBA	-0.374	-2.942	<0.01

^a Less than once a week: 0; more than once a week: 1.^b Less than one cup a day: 0; more than one cup a day: 1.^c Equol not-detected (<0.005 μg/mL): 0; equol detected (≥0.005 μg/mL): 1.^d Non smoker: 0; current smoker: 1.

The statistical association between semen quality and urinary pyrethroid metabolite concentration was reported previously in China (Xia et al., 2008) and in the US (Meeker et al., 2008) and the present result, i.e., lower motility in subjects with higher urinary 3-PBA (Table 3), was consistent with these reports. Consistent results from different settings strengthen the causality of the involvement of pyrethroid insecticides at environmental exposure level in male reproductive health. This causality was supported by the observed association between pyrethroid exposure and reproductive hormones in non-occupationally exposed male Chinese (Han et al., 2008) as its mechanistic explanation. Selection of urinary MnBP for sperm concentration with positive β was contrasted with the previous findings of human studies (Duty et al., 2003; Hauser et al., 2006) in which urinary MnBP concentration was associated with poorer semen quality. The reason is not clear with regard to the inverse relationship obtained in this study.

Sperm concentration was negatively associated with urinary daidzein concentration and motility was lower in equol detectable subjects than in non-detectable subjects in the present study (Table 3). Since soy isoflavones are known to possess weak estrogenic activity via binding to estrogen receptor, it may be reasonable to expect that poorer semen quality in subject with greater soy isoflavone intake. Chavarro et al. (2008) suggested that lower sperm concentration was associated with higher intake of soy products in subfertile couples in the US. The present study may be the first one that indicated the relationship by the biomarker of intake of soy products, i.e., urinary daidzein and equol. Although the estrogenic activity may be weak, intake of soy isoflavone in general Japanese people is much greater than the people in western countries, absolute estrogenicity derived from soy isoflavones may not be ignored.

It was reported that dietary habit affects human semen quality: frequent intake of vegetable and fruits was associated with better semen quality in a Spanish case-control study (Mendiola et al.,

2009). This result was inferred that antioxidant nutrients in vegetable and fruits had protective effect to semen quality. The present study also suggested more frequent intake of fruits and vegetable was associated with higher sperm concentration, and the former was statistically significant when controlled for other covariates (Table 3).

Recently Li et al. (2011) reported the results of meta-analyses on the association between sociological, psychological and behavioral factors and semen quality. In that study, higher age, alcohol consumption, smoking and psychological stress were found to be risk factor of deteriorated semen quality. However, two of the 4 suggested factors (age and alcohol) were not significant for semen quality in the present study and effect of smoking was in the inverse direction. In addition, coffee consumption was found not to be associated with semen quality in Li et al., but in the present study, it had negative effect on sperm concentration and motility. Thus our results were in many aspects inconsistent with Li et al. (2011).

The present study was a pilot design and involves a small sample size and non-standard semen examination procedure. It was not possible to strictly follow the guideline of WHO for sampling, transportation and analysis of semen because subjects' convenience had to be prioritized under clinical situation and this is one of the serious limitation of this study. Therefore, some of the observed associations between semen parameters and urinary biomarkers or other factors in the present study might not be valid enough. Some of them could be by chance because a number of statistical comparisons and analyses were carried out. Nevertheless, this pilot study suggested the necessity to include soy isoflavone intake as well as other dietary habit as relevant covariate in the future study on the effect of endocrine disrupting chemicals on human semen quality.

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