Original Article

Urinary Bisphenol-A Concentration in Infertile Japanese Women and Its Association with Endometriosis: A Cross-Sectional Study

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Abstract

Objectives: Bisphenol A (BPA), a raw material commonly used in the manufacture of resins such as polycarbonate and epoxy, is a possible xenoestrogen that is hypothesized to disrupt the human endocrine system. Humans are widely exposed to BPA. We investigated the urinary concentration of BPA in infertile Japanese women and its possible association with endometriosis.

Materials and Methods: We recruited 166 women (aged 20–45) who had complained of infertility and visited a university hospital in Tokyo. The subjects were interviewed and their urine samples were obtained prior to a laparoscopic diagnosis of endometriosis between January 2000 and December 2001. Urinary total BPA concentration in 140 eligible urine samples was then measured using enzymatic deconjugation of glucuronide and sulfate and high-performance liquid chromatography isotope-dilution tandem mass spectrometry.

Results: Median (25th–75th percentile) unadjusted and creatinine-adjusted urinary BPA concentrations were 1.6 (0.69–2.8) μg/L and 0.80 (0.45–1.3) μg/g creatinine. No significant monotonic association of endometriosis with urinary BPA concentration was observed. Median urinary BPA concentration in women with stage 0–I endometriosis (0.74 μg/g creatinine) did not significantly differ from that in those with stage II–IV endometriosis (0.93 μg/g creatinine) (p for difference=0.24).

Conclusions: This study, based on a larger number of samples than those in previous studies in Japan and using the most reliable analytical method currently available, showed that urinary concentrations of BPA in women who consulted a physician for infertility were not higher than those in other populations. Moreover, no association between urinary BPA concentration and endometriosis was found in this cross-sectional study.

Key words: endocrine disruptor, HPLC-MS/MS, epidemiology, urine, xenoestrogen

Introduction

Bisphenol A (4,4'-isopropylidenediphenol, BPA) is a raw material commonly used in the manufacture of resins such as polycarbonate and epoxy. Polycarbonate plastics are used in certain tableware products and bottles, whereas epoxy resin is used as a protective coating on food and beverage cans (1, 2). Because of its widespread use, humans can be exposed to BPA on a daily basis. BPA has shown estrogenic activity in experimental studies, and is considered a possible xenoestrogen that is hypothesized to disrupt the human endocrine system (2). This in turn has led to scientific and public concern about its effects on human health (3–5), particularly on the issue of whether human exposure to BPA is associated with estrogen-dependent diseases such as endometriosis.

Urinary BPA level has been surveyed in various populations. BPA is frequently detected in urine, which, unlike blood, allows for estimates of daily intake. Most studies to date have been constrained by two problems, however. First, despite the finding that BPA sulfate is a major metabolite of BPA in women (6), to our knowledge only the most recent US study (n=30) employed BPA detection using both mass spectrometry and deconjugation of BPA sulfate (7). Second, sample sizes have been limited to smaller than 100. Thus, taking those conducted

in Japan as examples, in one study, BPA concentrations was measured in single spot urine collected from 48 female students using electrochemical detection (8); in another study, BPA concentration was measured in 56 pregnant women by enzyme-linked immunosorbent assay (9); and in another study, daily urinary BPA excretion was measured in 36 male university students after deconjugation of BPA glucuronide (10).

Here, we conducted a hospital-based, cross-sectional study to investigate urinary concentrations of BPA in a large sample of infertile Japanese women. The urinary total concentration of BPA was measured using high-performance liquid chromatography isotope-dilution tandem mass spectrometry (isotope-dilution LC-MS/MS). The data obtained were then used to investigate a possible association between urinary BPA concentration and endometriosis stage.

Materials and Methods

Subjects

Subjects were recruited from among 166 consecutive women aged 20 to 45 years who had complained of infertility and consulted the Department of Obstetrics and Gynecology of the Jikei University School of Medicine for treatment of infertility. A total of 148 agreed to participate and provided written informed consent. Women who had previously given birth or who had lactated, and those who had undergone surgery for endometriosis were excluded. One woman of non-Japanese race and another who had lived abroad were also excluded, finally leaving 142 women eligible to participate in this study. Of these, 140 who submitted an eligible single spot urine sample and who underwent laparoscopic examination between January 2000 and December 2001 were available for analysis. Nine patients did not actually complain of infertility according to their questionnaire responses but were included to increase statistical power. The present study was approved by the Institutional Review Boards of the Jikei University School of Medicine and National Cancer Center.

The severity of endometriosis was diagnosed using laparoscopy and then classified into five stages on the basis of the revised American Fertility Society classification (11): stage 0 (n=60), I (n=21), II (n=10), III (n=24), and IV (n=25).

In addition, participants were interviewed before laparoscopic examination by a single trained interviewer using a structured questionnaire to collect information on demographic factors, age, height, weight, personal and family medical, reproductive and menstrual histories, oral contraceptive use, food- and alcohol-consumption frequencies, and smoking history. The items in the questionnaire and participant profile have been described in detail elsewhere (12, 13). No statistically significant differences between stage 0–I and II–IV patients were observed among these profile items except with regard to the regularity of menstrual cycle and average menstrual cycle length.

The participants also collected a first-morning urine sample into a paper cup, which was then transferred into a plastic tube, before laparoscopic examination. Four urine samples were not first-morning urine but were included. Samples were stored at −80°C for about 5 years until analysis

but were thawed and refrozen several times during this period.

Urinary BPA analysis

We analyzed urine samples without information on the participant's endometriosis status. Urinary BPA was deconjugated using a hydrolytic enzyme, β-glucuronidase/sulfatase (from *Helix pomatia*), separated using solid-phase extraction and high-performance liquid chromatography, and detected and measured using isotope-dilution tandem mass spectrometry. The analytical method is described in detail in the Appendix. The lower limits of detection (LODs) were 0.30–0.55 μg/L. Accuracy was assured by analyzing blank and quality control samples (2.4 μg/L) along with unknown samples in each analytical batch. Intraday and interday reproducibilities (CV= 8.8% and 19%, n=5, respectively) were previously checked by repeated analyses. The mean total surrogate recovery was 72% in one batch (n=5). In addition, an aliquot of each urine sample was shipped to a commercial clinical examination center, SRL (Tokyo, Japan), for measurement of creatinine concentration. Creatinine was detected in all samples. Urinary BPA concentration [μg/L] was divided by individual creatinine concentration to correct variability in urine dilution, and finally converted into daily BPA intake using equations 1–3 (14).

$$Intake_{BPA} = \frac{(C_{BPA} / C_{creatinine}) \times CE}{f} \times \frac{MW_{dosed}}{MW_{excreted}} \qquad (1)$$

$$CE = PRCr / \text{body weight} / 1000 \qquad (2)$$

$$PRCr = -4.72 \times \text{age} + 8.58 \times \text{body weight} + 5.09 \times \text{height} - 74.95 \qquad (3)$$

where $Intake_{BPA}$ [μg/kg body weight/day] is BPA daily intake; C_{BPA} [μg/L] and $C_{creatinine}$ [g/L] are the individual urinary raw concentrations of BPA and creatinine, respectively; CE [g/kg body weight/day] is the individual urinary daily creatinine-excretion rate to body weight [kg]; MW_{dosed} and $MW_{excreted}$ are the molecular weights of ingested and excreted BPA, respectively; and f is the molar fraction of urinary excreted BPA relative to ingested BPA. CE was calculated using equation 2 on the basis of the individual daily urinary creatinine excretion rate (*PRCr* [mg/day]), which was predicted using participants' individual age [years], body weight [kg], and height [cm], along with equation 3, an existing multiple regression model (15). Because urinary conjugated BPAs were enzymatically deconjugated to the free form, $MW_{dosed}/MW_{excreted}$ is equal to 1. For BPA, $f=1$ was also assumed on the basis of the results of two studies of BPA oral administration in humans (16, 17). Daily urinary BPA excretion is considered as average daily BPA intake (3, 10). These unadjusted and creatinine-adjusted concentrations and estimated daily intake were used in statistical analyses.

Statistical analysis

For measurement concentrations of BPA below the LOD, we assigned a value equal to the LOD divided by the square root of 2 (18). For other missing information, we performed list-wise deletion in every analysis. All statistical tests were

two-sided. Statistical analyses were performed using statistical analysis software, SAS version 9.1 (SAS Institute, Cary, NC).

Results

The participants were urban residents aged 24–43 years (median, 32). The median body weight was 51 kg. Among other variables, 63% were white collar workers, 15.7% were current smokers, 10.0% were daily alcohol drinkers, 90.1% had a history of menstrual pain, 57.8% had a history of dyspareunia, 30.8% had a history of hypermenorrhea, and 38.2% had a history of myoma of the uterus. No women had a history of cervical cancer, galactorrhea, adrenal disorder, or diabetes. Endometriosis stage was not associated with any factor except menstrual cycle length, regularity of menstrual cycle, and history of dyspareunia.

BPA was detected in 93% of urine samples. Urinary concentrations of unadjusted and creatinine-adjusted BPA and estimated daily BPA intake are summarized in Table 1, with median values (25th–75th percentiles) of 1.6 (0.69–2.8) µg/L, 0.80 (0.45–1.3) µg/g creatinine, and 0.016 (0.010–0.026) µg/kg body weight/day, respectively. Urinary BPA concentration showed a skewed distribution (Fig. 1), and no association was observed for any of the lifestyle factors described above, such as occupation or smoking status.

Table 1 also shows a cross-sectional comparison of urinary BPA concentration with the stage of endometriosis. Higher un-adjusted concentrations were associated with a more advanced stage of endometriosis, although this association was without statistical significance (p for difference=0.08) and became null after division of unadjusted concentrations by urinary creatinine concentration. Overall, we observed no association between endometriosis and any measured concentration of BPA.

Discussion

We measured the urinary concentration of BPA in women consulting a physician for infertility, and found no association between urinary BPA concentration and endometriosis. To our knowledge, this cross-sectional study has the largest sample size for the investigating urinary concentrations of BPA in Japan. It is further notable for its measurement of urinary total concentration of BPA by enzymatic deconjugation of both its
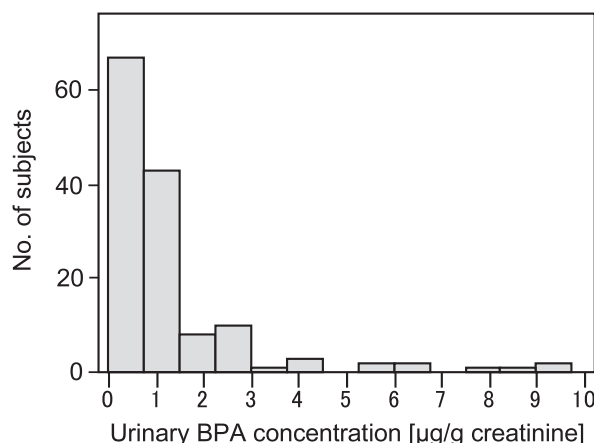

Fig. 1 Distribution of urinary BPA concentration (creatinine-adjusted; n=140).

glucuronide and sulfate and isotope-dilution LC-MS/MS. In contrast, most previous studies were carried out by either mass spectrometry or deconjugation of BPA sulfate alone.

Urinary concentrations of BPA in these women who consulted a physician for infertility were not higher than those in the previously surveyed populations. Our data are similar to those previously obtained at environmental concentrations in Japan (Table 1), albeit that these other studies used instrumental analyses for measurement, and some failed to consider BPA sulfate, although admittedly this is only a minor metabolite of BPA, in men at least (6). Specifically, our median concentrations (25th–75th percentiles) of unadjusted and creatinine-adjusted BPA of 1.6 (0.69–2.8) µg/L and 0.80 (0.45–1.3) µg/g creatinine, respectively, compare well with concentrations ranging from 0.1 to 11.9 (median of 0.77) µg/g creatinine in single spot urine samples collected from 48 female university students in Japan (8), and concentrations ranging from 0.14 to 5.47 (0.81 on average) µg/L in pooled urine samples in 46 male and 23 female volunteers (16).

For reference, one study showed median (25th–75th percentiles) BPA concentrations of 1.27 (0.49–2.46) µg/L and 1.77 (0.72–2.95) µg/g creatinine in urine from 210 women in the US (19), while another showed a median concentration (range) of 14.93 (0.0005–243.43) µg/g creatinine in urine from 160 Korean subjects and a geometric mean concentration of 5.01 µg/L in urine from 79 Korean females who were not

Table 1 Urinary BPA concentrations and endometriosis stages (n=140)

Endometriosis stage	No. of subjects	Urinary BPA concentration								
		Unadjusted [µg/L]			Creatinine-adjusted [µg/g creatinine]			Estimated daily intake [µg/kg body weight/day]*		
		Median	25th percentile	75th percentile	Median	25th percentile	75th percentile	Median	25th percentile	75th percentile
0–IV	140	1.57	0.69	2.78	0.80	0.45	1.29	0.016	0.010	0.026
0–I	81	1.32	0.63	2.40	0.74	0.45	1.21	0.014	0.009	0.025
II–IV	59	1.87	0.87	3.28	0.93	0.53	1.48	0.019	0.012	0.027
p for difference†				0.08			0.24			0.25

* n=131.

† Wilcoxon rank-sum test based on normal approximation.

occupationally exposed to BPA (20). Thus, our present values are similar to those obtained in other studies in Japan but not as high as those in Korean subjects.

In addition to BPA concentrations in spot urine, daily urinary BPA excretion rates based on 24-hour urine sampling has been measured (10, 16). Of interest, the rates of daily urinary BPA excretion are in direct accord with estimated values for daily BPA intake. The following values are also consistent with the daily BPA intake observed here (Table 1). The daily urinary excretion rates of BPA based on 24-hour urine samples collected from 36 male university students (mean age, 24.7 years) in 2003 ranged from <0.003 to 0.23 (median, 0.02) μg/kg body weight/day (10). Another study in which values were determined from whole-day urine samples from 11 male and 11 female volunteers showed daily urinary BPA excretion rates ranging from 0.48 to 4.5 (average, 1.68) μg/day (16). Furthermore, the observed range of estimated daily BPA intake (Table 1) was much lower than the tolerable daily intake of 50 μg/kg body weight/day established by the European Food Safety Authority in 2007 (21).

A change in BPA exposure over time has also been reported. Matsumoto et al. (22) measured BPA concentration in urine samples collected from university students in Japan and showed that the median of total BPA concentrations in 1992 was 2.2-fold higher than that in 1999. Furthermore, daily dietary BPA intake rates determined by measuring BPA concentrations in hospital-meal samples in Japan were in the range of 0.15–1.34 (average, 0.64) μg/day in 2000 but were only in the range of 0.06–0.68 (average, 0.20) μg/day in 2001 (23). Given the frequent detection of BPA in canned foods and retort foods (24), these reductions may have partly resulted from changes in the protective coating of epoxy on food and beverage cans (3). This finding may be useful in interpreting the meaning of urinary BPA levels in respective studies.

In addition to our exposure survey, we also explored whether urinary BPA concentration is associated with endometriosis in infertile Japanese women. Analysis of these data showed the absence of association. Although the pathogenesis of endometriosis is poorly understood, epidemiological evidence has suggested its association with several estrogen-dependent factors, namely early menarche, shorter menstrual cycle length, and lower parity (25–27). Based on this, endometriosis probably represents a sensitive detector of the effect of xenoestrogens in humans. However, two recent human experimental studies showing that orally administered BPA is quickly recovered in urine suggest that accurate exposure assessment based on urinary excretion is problematic, because it likely reflects recent rather than cumulative or long-term exposure (16, 17). The probable importance of chronic over recent exposure may partly explain our finding of the lack of association between BPA exposure and endometriosis, and future investigation should focus on the measurement of cumulative exposure to BPA.

Several other suggestions to improve studies of the association between BPA exposure and endometriosis can be made. In addition to accounting for cumulative exposure, investigation would be facilitated by a better study design, such as prospective cohort rather than cross-sectional studies. Measurement of free urinary BPA would be informative, providing its accuracy

is confirmed, because BPA glucuronide shows no significant estrogenic activity, in vitro at least (2). Furthermore, a previous prospective cohort study showing that the risk of endometriosis is associated with exposure to diethylstilbestrol in utero indicates that early-life exposure to BPA, including fetal exposure, might also be critical to the development of endometriosis (28). Additionally, measurement of endogenous estrogen level would allow evaluation of the interaction between serum endogenous estrogens and BPA; this was not possible in the present study because individual endogenous estrogen level in individual premenopausal women periodically fluctuates in accordance with their menstrual cycle.

At a more basic concentration, studies of the effect of BPA on animals have also been inconclusive. BPA showed estrogenic activity generally 10^{-5}–10^{-3} times that of 17β-estradiol in a MCF-7 cell assay in a human breast cancer cell line (2). Furthermore, oral administration of BPA at 200 mg/kg body weight/day to a strain of immature rats resulted in an increase in uterine weight (2). In contrast, BPA showed no clear endocrine disrupting effect on rodents at estimated human exposure doses (5). Moreover, experimental results for BPA have been shown to depend on a number of factors, namely measurement endpoint, cell line, animal species and strain, and conjugate form and dose (2).

The strength of our study includes the use of a reliable biomarker measurement method and detailed information on subjects. In particular, we employed the most reliable analytical method currently available, namely, enzymatic deconjugation of both BPA sulfate and glucuronide and isotope-dilution LC-MS/MS measurement. Moreover, to monitor and control sample contamination, method blank tests were conducted in parallel with unknown sample analysis for all measurements, in addition to the rinsing of glassware and plastic tubing with methanol or acetonitrile before each test.

Two limitations of our study warrant mention. First, our subjects were restricted to urban residents of reproductive age, as well as other characteristics; thus, the generalizability of our results may also be limited. Second, intraindividual variation in BPA exposure and uncertainty in laboratory analysis may have contributed to urinary BPA concentration measurement errors. One study in which between-day variation in daily urinary BPA excretion was examined suggested that the magnitude of intraindividual variation is comparable to that in interindividual variation (10). If present, however, such errors would at least not tend to bias our results toward one side; thus, the median or geometric mean urinary BPA concentration would have been properly estimated. In contrast, interindividual variation, such as geometric standard deviation, might have been overestimated and thus remain to be corrected.

In conclusion, we report urinary concentrations of BPA in Japanese women who consulted a physician for infertility. Values were derived from the largest subject sample size studied in Japan to date and were obtained using the most reliable analytical method. Results showed that urinary BPA concentrations in these women were not higher than those in other populations. Moreover, this cross-sectional study revealed no association between urinary BPA concentration and endometriosis. Further study is required to confirm this result.

Appendix: Urinary BPA analysis

In the present study, we measured urinary bisphenol A (BPA) concentration. Here, we detail the methods used to measure urinary BPA concentration.

We analyzed urine samples without information on the participant's endometriosis status. Urinary BPA was separated, detected and measured using enzymatic deconjugation, offline solid-phase extraction, and high-performance liquid chromatography isotope-dilution tandem mass spectrometry (isotope-dilution LC-MS/MS). In addition to urinary BPA glucuronide, the recently identified compound urinary BPA sulfate has also been included in urinary BPA analysis in recent years (6–8, 29, 30). To deconjugate these BPAs into their free forms, we used β-glucuronidase/sulfatase (from *Helix pomatia* H-1, 492,000 units/g solid, Sigma Aldrich, St. Louis, MO, USA), a hydrolytic enzyme with sulfatase activity, as described in most recent studies (7, 29).

We purchased the native BPA standard (99.8%, for environmental analysis) and isotopically labeled standard (BPA-d_{16}, 99.9%, for environmental analysis) from Kanto Chemical Co., Inc. (Tokyo, Japan). Methanol (≥99.8%, residual-pesticide analysis grade 5000) and acetonitrile (≥99.8%, HPLC grade) were purchased from Sigma Aldrich Japan (Tokyo, Japan). Ammonium acetate (≥97.0%), sodium acetate trihydrate (≥99.0%), formic acid (≥98.0%), and acetic acid (≥99.7%) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). We used ultrapure water (Milli-Q Gradient A10, Millipore, Bedford, MA). To prepare 0.1 M acetate buffer, we diluted sodium acetate trihydrate (0.5 mmol) and acetic acid (0.5 mmol) with water to 10 ml. Standard substances were measured by weight and then dissolved in acetonitrile. They were serially diluted with acetonitrile:water=2:3 (v/v) to their respective target concentrations. Except for unused vials, spatula and glassware were previously rinsed with methanol three times and dried.

Frozen urine was thawed at room temperature. A 0.2-mL aliquot was decanted into a 2-mL silanized glass vial. We dissolved β-glucuronidase/sulfatase (0.2 mg) in 0.1 M acetate buffer solution (0.1 mL). This hydrolytic enzyme solution and 20 μL of the surrogate solution (50 μg/L) were added to the vial. This sample was mixed well and then incubated at 37°C overnight, as described in recent studies (7, 29).

A solid-phase extraction (SPE) cartridge (FOCUS Versa-Plate Tube, 10 mg/1.8 mL, Varian, Lake Forest, CA, USA) was preconditioned with 1-mL methanol followed by 1-mL water. After dilution with 1 M ammonium acetate aqueous solution (1 mL), the urine sample was vortex-mixed, and then loaded onto this SPE column. The SPE column was then washed with 1-mL water followed by acetonitrile:water=1:4 (v/v; 1 mL). Analytes were finally eluted from the SPE column using 1-mL solvent (methanol:acetonitorile:water:formic acid=6:3:1:0.01, v/v/v/v). A plastic tube (1.1 mL) into which the SPE eluate was collected was previously washed with acetonitrile. The eluate was dried out using a vacuum centrifuge (100 min) and the residue was reconstituted with acetonitrile:water=2:3 (v/v; 200 μL). The resulting solution was decanted into a silanized-glass insert.

We then measured BPA concentration in the extracted urine sample using a tandem quadrupole mass spectrometer (API 2000, Applied Biosystems, Foster City, CA, USA) connected to a high-performance liquid-chromatography (HPLC) system (LC-10 AD_{VP} pomp, SIL-10 AD_{VP} autosampler, CTO-$10AC_{VP}$ column oven, and SCL-10A$_{VP}$ system controller, Shimadzu, Kyoto, Japan) equipped with a C_{18} column (S-3 μm, 8 nm, 2.0 mm i.d.×100 mm, YMC-pack Pro RS, YMC Co., Ltd., Kyoto, Japan). We used 0.1-mL acetic acid in 1-L water (solvent A) and 0.1-mL acetic acid in 1-L acetonitrile (HPLC grade) (solvent B) as HPLC mobile phases with a constant flow rate (0.2 mL/min). A 20-μl aliquot of the sample was injected into the HPLC column using an auto sampler. The sample vial was cooled at 4.0°C. The percentage of solvent B was elevated from 40% (0 min) to 100% (5 min) for separation, kept at 100% (5–8 min) for flushing, and then kept at 40% (8.01–20 min) for re-equilibrium. The temperature of the HPLC column was kept constant (40.0°C). We used electrospray ionization and multiple-reaction monitoring to produce the combinations of the precursor and product ion of m/z 227.1 and 132.9 for BPA, and m/z 241.2 and 141.8 for BPA-d_{16}, respectively. The ion source temperature and collision energy were 500°C and −30 volts, respectively. MS/MS parameters on API 2000 were automatically optimized using a personal computer-based instrument software program (Analyst version 1.4, Applied Biosystems, Foster, CA, USA), which was also used to acquire and process the data obtained. To obtain a calibration curve (0.6–20 μg/L), each calibration point was weighted by the reciprocal of concentration (1/X), and the origin was ignored. In each analytical batch, one quality control material, all portions of which were obtained from a single sample of pooled urine (2.4 μg/L), and five method blanks were also analyzed along with unknown samples to ensure the accuracy of analysis. Intraday and interday reproducibilities (CV=8.8% and 19%, n=5, respectively) were previously checked by repeated measurements. In the batches where no method blank was observed, the standard deviation derived from six repeated measurements of a low-concentration standard solution (1 μg/L) was employed as the standard deviation at concentration zero (S_0). In the batch where method blanks were observed, S_0 was calculated from the standard deviation of the method blanks. We defined $3S_0$ as the analytical limits of detection (LODs), which were 0.30–0.55 μg/L. Mean total surrogate recovery was 72% in one batch (n=5). Any loss of BPA through analysis was automatically corrected on the basis of individual surrogate recovery. The mean of the method blank values was subtracted from the BPA measurement value in each batch.

Acknowledgements

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