Human Semen Quality, Sperm DNA Damage, and the Level of Reproductive Hormones in Relation to Urinary Concentrations of Parabens

Joanna Jurewicz, PhD, Michał Radwan, PhD, Bartosz Wielgomas, PhD, Emila Dziewirska, MSc, Anetta Karwacka, MD, Anna Klimowska, MSc, Paweł Kałużny, PhD, Paweł Radwan, PhD, Michał Bochenek, PhD, and Wojciech Hanke, MD, PhD

Objective: The aim of this study was to evaluate the association between environmental exposure to parabens and semen quality parameters [main semen parameters, computer-aided semen analysis (CASA parameters], sperm chromatin structure, and the level of reproductive hormones in men [follicle-stimulating hormone (FSH), testosterone, estradiol]. **Methods:** Urine samples collected from 315 men who attended the infertility clinic for diagnostic purposes with normal semen concentration of 15 to 300 mln/mL were analyzed for five parabens concentrations using a validated gas chromatography ion-tap mass spectrometry method. Participants were interviewed and also provided a semen, saliva, and blood samples. **Results:** Urinary parabens concentrations were significantly associated with an increase in the percentage of sperm with abnormal morphology, in sperm with high DNA stainability and a decrease in the percentage of motility and testosterone level. **Conclusions:** This is one of the first study on this topic, so the observation of the relationship between parabens and semen quality warrants further investigation.

Parabens are man-made chemicals used as preservatives in personal care and consumer products, food, beverages, and pharmaceuticals, because of broad-spectrum antimicrobial efficacy and chemical stability. Many products contain parabens, including some toothpastes, sunscreens, body lotions, facial lotions and cleansers, mascara, assorted lipsticks, hand soaps, as well as hair products including shampoos, conditioners, sprays, gels, and some food products.[1–3] Parabens are commonly used because they are relatively inexpensive and have low toxicity.[4]

Exposure to parabens may occur through dermal absorption, ingestion, and inhalation.[5] After absorption, all parabens are rather rapidly hydrolyzed by unspecific esterases to p-hydroxybenzoic acid (PHBA), which is conjugated with sulfate, glucuronic acid, or glycine (p-hydroxyhippuric acid, PHHA) before being excreted in urine.[6–8] Thus, the main urinary metabolite of all parabens is unspecific PHBA and its conjugates.[6–8]

From the Department of Environmental Epidemiology, Nofer Institute of Occupational Medicine, Lodz, Poland (Drs Jurewicz, Dziewirska, Kałużny, Hanke); Department of Gynecology and Reproduction, ''Gameta'' Hospital, Rzgów, Poland (Drs Radwan, Karwacka, Radwan); Department of Toxicology, Medical University of Gdańsk, Gdańsk, Poland (Drs Wielgomas, Klimowska); and Department of Biotechnology of Animal Reproduction, National Research Institute of Animal Production, Kraków-Balice, Poland (Dr Bochenek).

This study was performed under the project ''The association between environmental exposure to widespread man made endocrine disrupting chemicals and level of hormones associated with the activity of hypothalamic pituitary testicular axis among young men'' supported by National Science Centre in Poland from grant no. UMO-2014/13/B/NZ7/02223 and the project financed with a grant for statutory activity IMP 10.23/2016.

The authors declare no conflict of interest.

Address correspondence to: Joanna Jurewicz, PhD, Department of Environmental Epidemiology, Nofer Institute of Occupational Medicine, 8 Teresy St, 91-362 Lodz, Poland (joanna.jurewicz@imp.lodz.pl).

DOI: 10.1097/JOM.0000000000001106

The use of parabens as preservatives has raised some concerns because of possible endocrine disrupting properties. Parabens are suspected endocrine disruptors that are weakly estrogenic[4,9,10] and anti-androgenic.[11]

Limited animal studies have reported adverse effects on sperm production and testosterone levels following oral exposure to parabens with longer sidechains, for example, and propylparaben.[12,13] Furthermore, parabens are known to be estro-genic in vitro and in uterotrophic assays in vivo, and estrogenicity appears to increase with side chain length.[11] Whereas Hoberman et al[14] did not find an association between methyl paraben (MP) or butyl paraben (BP) and reproductive markers in rats.

To our knowledge, only one human study has investigated the relationship between paraben exposure and male fertility. Meeker et al[15] found that categories of urinary BP concentration (0.2 to 0.6 and >0.6 μg/L) were not associated with semen quality parameters and hormone levels. The statistically significant positive association was observed between urinary BP levels and sperm DNA damage (P for trend $= 0.03$). The relatively small sample size in this study limited the ability to detect the relationship between urinary parabens and male fertility. This study adds more statistical power to the previous human semen quality studies of parabens exposure, by including five parabens: ethyl paraben (EP), BP, MP, iso-butyl paraben (iBu-P), and by assessment of semen quality parameters, sperm DNA damage, and reproductive hormones among larger number of subjects. To our knowledge, no human studies have assessed the environmental exposure to five different parabens. In the present study, the environmental exposure to parabens and main semen quality parameters, sperm chromatin structure, and the level of reproductive hormones were evaluated.

METHODS

Participant Recruitment and Biological Samples Collection

Participants were men who attended an infertility clinic in Lodz, Poland, for diagnostic purposes with normal semen concentration of at least 15 mln/mL or (WHO, 2010)[16] (Jurewicz et al)[17] between 2008 and 2011 from the study ''Environmental factors and male infertility.'' The study was performed according to the Declaration of Helsinki guidelines, and the procedures employed were approved by the The Nofer Institute of Occupational Medicine Bioethical Committee Board had approved the study [Resolution No 9/2007 (June 4, 2007)] and written informed consent was obtained from all subjects before their participation. Men under 45 years of age were eligible for study inclusion. The study participation rate was 59% ($N = 315$). Study participants completed questionnaire and provided urine, saliva, blood, and semen samples on the same day of their clinic visit. The smoking status was verified by measuring cotinine level in the saliva using high-performance liquid chromatography coupled with tandem mass spectrometry/positive electrospray ionisation (LC-ESI + MS/MS) and the isotope dilution method as previously described (Jurewicz et al).[18]

Main Semen Quality Parameters, Sperm Chromatin Structure, and Level of Reproductive Hormones Analysis

Semen samples were collected following masturbation into a sterile a standard plastic container and were liquefied at 37°C for 30 minutes. Analysis details for semen parameters have been previously described (Jurewicz et al).[17] Semen samples were analyzed for sperm concentration, motility, and motion parameters using a computer-aided semen analysis (CASA) (Hamilton-Thorne Version 10HTM-IVOS, Hamilton-Thorne Research, Danvers, MA). Out of seven CASA motion variables measured, three principal parameters for the vigor and pattern of sperm motion were included in the analysis [straight-line velocity (VSL), curvilinear velocity (VCL), linearity (LIN)]. Sperm morphology was quantified using strict Kruger criteria to classify men as having normal or below normal morphology (Jurewicz et al).[17]

To assess sperm DNA damage, sperm chromatin structure assay (SCSA) was performed using flow cytometry (DAKO Galaxym DAKO, Glostrup, Denmark).[19] The DNA Fragmentation Index (DFI) was calculated according to the formula: DFI = (cells with a shift of the alpha-t parameter/all cells) x 100 each analyzed sperm cell and was shown on a histogram. The full details of the method used for sperm chromatin structure assessment are presented elsewhere (Jurewicz et al).[17]

Levels of follicle-stimulating hormone (FSH), testosterone, and estradiol were determined in human plasma using a Chemiluminescent Microparticle Immunoassay (ARCHITECT System; Abbott, Longford, Ireland) as previously described (Jurewicz et al).[18] The results are expressed as IU/L for FSH, pg/mL for estradiol, and ng/mL for testosterone.

Urinary Parabens Analysis

Urine samples were frozen at −20°C and sent to the laboratory in Department of Toxicology, Medical University of Gdańsk for analysis.

Standards and Materials

Analytical standards were purchased from Fluka (Steinheim, Germany): [butylparaben (purity 99.96%) and ethylparaben (99.6%)], Sigma-Aldrich (Steinheim, Germany): [iso-butylparaben (97%) and propylparaben (99.96%), Supelco (Bellefonte, PA): methylparaben (99.9%)]. Mixture of labeled analogs of parabens (methyl-, ethyl-, propyl-, and butylparaben, ring-13C6, 50 μg/mL in acetone, >95%) was obtained from Fluka (Merck, Steinheim, Germany) and they were used as internal standards.

Hexane (HEX, ≥99%), tert-butyl methyl ether (MTBE, ≥99%), and mixture of N,O-bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (BSTFA:TMCS, 99:1, BSTFA 99.5%, TMCS 99.2%) were purchased from Sigma-Aldrich (Steinheim, Germany). Formic acid (80%), magnesium sulfate anhydrous (98.5%), sodium acetate (99%), and primary secondary amine sorbent (PSA) were obtained from POCH (Gliwice, Poland) and Scharlab (Barcelona, Spain), respectively. β-Glucuronidase (Type HP-2 from Helix pomatia, activity 184,973 U/mL) was obtained from Supelco (Bellefonte, PA).

Standard stock solutions were prepared in acetonitrile at concentration of 10 mg/mL (except ethylparaben, 1 mg/mL) and then were used to prepare working solutions of 100 and 10 μg/mL. Working solutions were applied to prepare sequential dilutions for method calibration. Internal standard solution was prepared in acetonitrile at a final concentration level of 1 μg/mL. All solutions were stored at −20°C.

Acetate buffer solution (1 M, pH 5.0) containing 230 U of β-glucuronidase per 750 μL was prepared freshly for each analytical batch.

Sample Preparation

Three milliliters of urine were placed in 10-mL screw-cap glass tube followed by 50 μL of mixed internal standard solution and 750 μL of freshly prepared acetate buffer (1 M, pH 5.0) containing 230 U of β-glucuronidase were added. Overnight incubation (at least 12 hours) at 37°C was performed. Then, the sample was acidified with 450 μL of 80% formic acid and 3 mL of HEX:MTBE mixture (3:1, v:v) was added to the sample and tube was shaken for 10 minutes. After centrifugation, the organic layer was transferred into open glass tube and the extraction was repeated. Combined extracts were cleaned-up with 200 mg of MgSO4 and 10 mg of PSA by shaking in hands for 1 minute. Then, 5 mL of cleaned extract was transferred into new open glass tube and evaporated to dryness under stream of nitrogen at 35°C. The residue was dissolved with 50 μL of BSTFA:TMCS (99:1) and derivatized for 30 minutes at 40°C. One microliter of final extract was analyzed by GC-MS/MS.

GC-MS/MS Conditions

Analyses were performed using gas chromatography (Varian GC-450) coupled with tandem mass spectrometry (Varian 220-MS, ion-trap mass spectrometer). Separation was achieved on a VF-5 ms (Varian, Palo Alto, CA) low bleed capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness with integrated 10 m guard column) using the following temperature oven program: 60°C for 3 minutes, 60°C to 140°C (120°C/min), 140°C to 290°C (17°C/min), 280°C—held for 13 minutes. The flow rate of carrier gas, helium, was 1.0 mL/min. Temperature of the manifold, trap, and transfer line was 45°C, 200°C, and 290°C, respectively.

One microliter of the sample extract was injected in a splitless mode into a 1177 split/splitless injector (injector temperature 280°C).

The limit of detection (LOD) was 0.5 μg/L.

Statistical Analysis

R statistical software (ver.3, R Foundation for Statistical Computing, Vienna, Austria) was used to perform statistical analysis.[20] Descriptive statistics for subjects grouped by demographic characteristics were calculated, along with the distributions of urinary parabens, and main semen quality parameters, sperm chromatin structure parameters, and reproductive hormone levels. The frequency of samples below the LOD was as follows: MP was 1.3%, for EP 57.8%, for PP 10.8%, for BP 89.5%, and for iBuP 84.1%. Spearman correlation coefficient was used as nonparametric measure of associations between concentrations of different parabens. Multiple least squares linear regression models were used to quantify the associations of urinary parabens (explanatory variables) with selected sperm quality measures and the concentrations of reproductive hormones as dependent variables. Motility, % of sperm with abnormal morphology, FSH, estradiol, testosterone, DFI, and high DNA stainability (HDS) were subjected to shifted natural log-transformation (ln) log(1+x), to obtain more systematic quasi-normal distributions. In basic regression model, with the continuous explanatory (independent) variables, paraben concentrations also had to be transformed by shifted natural log-transformation to obtain approximately symmetric, quasinormal distribution.

For parabens concentrations < LOD, a value LOD/2 was imputed.

Creatinine-adjusted parabens, MP and PP, were categorized into four groups; the first one consisted of values below LOD to 25th percentile value, second greater than the 25th percentile value to the median, third greater than the median to 75th percentile value, while the fourth group consisted of values greater than the 75th percentile. In addition, urinary concentrations of MP and PP were presented as continuous variables. Because of the high proportion of samples with BP, iBuP, and EP values < LOD, this parabens concentrations were categorized as above and below LOD.

TABLE 1. Characteristics of the Study Population $N = 315$

Characteristics	
Education, *n* (%)	
Primary and vocational	65 (20.6)
Secondary	119 (37.8)
Higher	131 (41.6)
Smoking determined by cotinine level, *n* (%)	
No	224 (71.1)
Yes	91 (28.9)
BMI, kg/m^2, *n* (%)	
<25	106 (33.7)
≥25	209 (66.4)
Mean (sd)	26.8 ± 3.4
Median (min-max)	27.6 (18.3–39.5)
Duration of couple's infertility, years, *n* (%)	
1–2	120 (38.1)
2–3	104 (33.0)
3–5	45 (14.3)
>5	46 (14.6)
Past diseases, which may have an impact on semen quality, *n* (%)	
No	276 (87.6)
Yes	39 (12.4)
Abstinence, days, *n* (%)	
<3	11 (3.5)
3–7	242 (76.8)
>7	16 (5.1)
Missing data	46 (14.6)
Mean (sd)	5.0 ± 2.3
Median (min-max)	5.0 (0.0–20.0)
Age, years	
Mean (sd)	32.14 (4.2)
Median (min-max)	31.60 (22.0–44.3)
Alcohol use, *n* (%)	
None or <1 drink/week	104 (33.0)
1–3 drinks /week	163 (51.8)
Everyday	48 (15.2)

n, number of participants; Past diseases that may have an impact on semen quality—mumps, cryptorchidism, testes surgery, testes trauma.
BMI, body mass index.

Inclusion of covariates in the multivariable regression models was based on biological and statistical considerations. Sexual abstinence (days) and age (years) were included in the models as continuous variables. Smoking (yes/no based on cotinine level in saliva), past diseases (which may have impact on semen quality—mumps, cryptorchidism, testes surgery, testes trauma) (yes/no), alcohol consumption (None or <1 drink/week, 1 to 3 drinks/week, 4 to 7 drinks per week) were considered as categorical variables.

Missing values in abstinence variable were imputed with an auxiliary regression model using sperm volume as a predictor.

RESULTS

Demographic and Clinical Participant Characteristics

Demographic characteristics of participants are presented in Table 1. Briefly, most men had higher (41.6%) or secondary (37.8%) education and were nonsmokers (71.1%). The mean (±SD) age and body mass index (BMI) were 32.1 ± 4.2 years and 26.8 ± 3.4 kg/m^2, respectively. Most of the study men drank one to three drinks per week (51.8%) and 12% of study participants reported past diseases that may have an impact on semen quality (eg, mumps, cryptorchidism, testes surgery, testes trauma). The mean (±SD) values for sperm concentration and motility were 50.6 mln/mL (52.4) and 56% (19.9), respectively. The percentages of sperm with abnormal morphology, sperm DNA damage, and HDS were as follows (mean ± SD): 53.7 ± 23.9, 16.5 ± 11.4, and 7.7 ± 3.9, respectively. The distribution of CASA parameters and the level of reproductive hormones are presented in Table 2.

Parabens Levels in Urine

Summary statistics for urinary parabens concentrations are summarized in Table 3. The unadjusted geometric mean ± SD and median urinary concentrations of parabens were as follows: MP 14.7 μg/L ± 5.35 (median 15.6 μg/L), PP 4.3 μg/L ± 5.97 (median 3.7 μg/L), EP 1 μg/L ± 7.02 (median 9.39 μg/L), iBuP 0.4 μg/L ± 2.76 (median 2.27 μg/L), and BP 0.3 μg/L ± 2.66 (median 3.48 μg/L).

Table 4 summarizes correlation coefficients and *P* values for measured parabens. Examined parabens were highly correlated: MP

TABLE 2. Semen Quality and the Level of Reproductive Hormones Among Study Participants

Semen Quality	Selected Descriptive Statistics								
	Min	25%	50%	75%	95%	Max	Mean	SD	*N*
Main semen parameters									
Concentration, mln/mL	15	22.5	32.6	85.77	125	360.0	50.6	52.4	315
Motility (%)	4	46	55.0	66	83	99.0	55.7	19.9	315
Sperm with abnormal morphology [%]	11	31.22	50.0	69.51	75.25	96.0	53.7	23.9	315
CASA parameters									
VSL, μm/s	14.9	37	43.1	50.7	61.41	77.1	43.6	10.3	315
VCL, μm/s	20.8	37.5	79	92.0	108	146	78.1	16.8	315
LIN (%)	20.0	51	56	61	66.1	74	56	6.4	315
Level of reproductive hormone									
FSH, IU/L	0.1	2.50	3.50	5.30	10.4	25.2	4.4	3.0	315
Estradiol, pg/mL	2.6	21.76	27.4	33.67	45.53	75.2	28.3	10.0	306
Testosterone, ng/mL	1.5	3.3	4.6	5.8	8.30	28.3	4.8	2.4	315
DNA fragmentation index									
DFI	2.7	8.34	14.0	20.43	41.18	68.7	16.5	11.4	262
HDS	0.7	5.22	7.16	9.31	13.46	30.70	7.7	3.9	262

DFI, DNA fragmentation index; FSH, follicle-stimulating hormone; HDS, high DNA stainability; LIN, linearity; N, number of participants; VCL, curvilinear velocity; VSL, straight-line velocity.

TABLE 3. Parabens Concentration in Urine

Parabens in Urine	Statistical Variables								
Unadjusted, μg/L	**25%**	**50%**	**75%**	**95%**	**Max**	**GM ± SD**	**N**	**<LOD**	**Frequency of Detection**
MP	4.88	15.6	50.74	204.88	568.1	14.7 ± 5.35	315	4	98.7%
EP	3.22	9.39	22.99	73.12	229.3	1.0 ± 7.02	315	182	42.2%
PP	1.12	3.7	15.03	117.12	598.5	4.3 ± 5.97	315	34	89.2%
BP	1.60	3.48	4.07	8.49	99.9	0.3 ± 2.66	315	282	10.5%
iBuP	0.87	2.27	5.23	5.71	22.0	0.4 ± 2.76	315	265	15.9%
CR-adjusted, μg/g creat									
MP	4.82	15.2	44.33	200.32	564.2	14.2 ± 4.21	315	4	98.7%
EP	2.99	0.24	21.13	72.11	228.2	1.0 ± 6.28	315	182	42.2%
PP	1.09	3.1	15.01	116.15	587.9	4.2 ± 4.33	315	34	89.2%
BP	1.30	0.24	8.42	4.05	87.6	0.4 ± 2.12	315	282	10.5%
iBuP	0.55	0.24	5.20	5.14	21.7	0.3 ± 2.55	315	265	15.9%

AM, arithmetic mean; BP, butyl paraben; EP, ethyl paraben; GM, geometric mean; iBuP, isobutyl paraben; LOD, limit of detection; MP, methyl paraben; n, number of participants; PP, propyl paraben; SD, standard deviation; SD, standard deviation.

with EP, PP, iBuP, and BP with iBuP ($P < 0.0001$) and BP with MP and EP ($P = 0.013$, $P = 0.033$, respectively) and iBuP with EP ($P = 0.012$) (Table 4), whereas no correlations were found between PP and BP, iBuP, and EP (Sperman correlation coefficient = 0.07, 0.08, 0.09, respectively).

Urinary Parabens Concentration and Male Fertility

In multivariate linear regression models adjusted for age, smoking, alcohol consumption, sexual abstinence, and past diseases, the negative association was observed between urinary concentrations of BP ≥LOD and motility ($P = 0.026$) and testosterone level ($P = 0.031$) (Table 5). Urinary level of EP and BP (≥LOD) increases the percentage of sperm with abnormal morphology ($P = 0.048$ and $P = 0.03$, respectively). In addition, the level of iBuP in urine increases HDS ($P = 0.03$) (Table 5). Neither categories of urinary concentrations of parabens nor continuous concentrations of parabens were associated with the level of reproductive hormones (Table 5). In addition, urinary concentrations of MP and PP were not related to any of the examined semen quality parameters, sperm DNA damage, or the level of reproductive hormones (Table 5).

DISCUSSION

In this study, we found relationships between urinary parabens and semen quality. The statistically significant associations were found between urinary parabens concentrations and an increase the percentage of sperm with abnormal morphology and percentage of sperm with HDS and decrease in percentage of motility.

To our knowledge, only one human study has investigated the association between urinary paraben concentrations and male fertility. Our findings are in line with the study performed by Meeker et al.[15] In this study, BP concentrations in urine were associated with sperm DNA damage, whereas no statistically significant associations were observed between MP, PP and BP and conventional semen quality parameters in US study. On the contrary, in the present study, BP concentrations were significantly associated with an increase in the percentage of sperm with abnormal morphology and a decrease in the percentage of motility. Also, exposure to EP was related to abnormal sperm morphology. The inconsistency between the study results may be associated with the fact that smaller sample size was examined in US study. In addition, lower levels of BP in urine among participants from the study performed by Meeker et al, 2011 may affect the study results. Some parabens, EP and iBuP, were assessed for the first time in our study. Different confounding factors used in the studies, creatinine adjustment in our study, and specific gravity adjustment in Meeker's study may also impact on the final results.

TABLE 4. Spearman Correlation Between Parabens

	MP	**EP**	**PP**	**BP**	**iBuP**
MP	1	0.240	0.240	0.140	0.210
EP	<0.0001	1	0.090	0.120	0.140
PP	<0.0001	0.112	1	0.070	0.080
BP	0.013	0.033	0.245	1	0.440
iBuP	<0.0001	0.012	0.134	<0.0001	1

Below diagonal—*P* values, above diagonal—correlations.
EP, ethyl paraben; MP, methyl paraben; PP, propyl paraben.

Limited animal studies have explored relationships between exposure to parabens and semen quality. Rodent exposure to butylparaben[11,12] and propylparaben[21] adversely affected testosterone synthesis and male reproductive function. On the contrary, a study performed by the same author exhibited contrary results for methyl- and ethylparaben.[22] In addition, Hoberman et al[14] and Daston[23] did not observe any significant changes on reproductive organ weights, sperm motility, sperm count, and hormone level. Therefore, the study suggests that the rapid metabolism of parabens by esterases may be the reason for why these compounds do not induce in vivo effects when administered by oral route, whereas the dermal exposure is the main route of exposure in humans.

The observation that statistically significant relationships involved BP is consistent with in vitro and animal data that suggest BP has greater reproductive toxicity potential than the other parabens examined in this study.[13] Also, Routledge et al[10] found that BP is more hormonally active than other individual parabens.

Urinary concentrations of parabens in our study subjects were comparable with those reported in a national sample of US men in the fourth report of the National Health and Nutrition Examination Survey (NHANES)[24] but only for PP. Median and 95th percentile concentrations recently reported in males participating in the NHANES for 2009 to 2010 were, respectively, 2.0 and 134 μg/L for PP, compared with 3.7 and 117 μg/L in the present study. The urinary concentration of MP was higher in NHANES than in our study, 25.3 and 727 μg/L and 15.6 and 205 μg/L, respectively. On the contrary, the levels of BP in urine were higher in the present study than among US general population. Median and 95th percentile concentrations were 3.48 and 8.49 μg/L in Poland

TABLE 5. Parabens Concentration in Urine and Semen Quality and the Level of Reproductive Hormones—Categories of Urinary Parabens Concentrations

	Exposure	MP			PP				EP			BP			iBP		
	Percentile of Exposure	Coef	95% CI	*P*	Coef	95% CI	*P*	Detection	Coef	95% CI	*P*	Coef	95% CI	*P*	Coef	95% CI	*P*
Conc	≤25th		Ref			ref		< LOD	Ref			Ref			Ref		
	25th–50th	−0.10	−0.43 to 0.24	0.56	−0.26	−0.60 to 0.08	0.13										
	50th–75th	0.22	−0.12 to 0.55	0.20	−0.10	−0.43 to 0.24	0.58										
	>75th	0.73	−0.06 to 0.61	0.11	0.08	−0.25 to 0.42	0.63	≥ LOD	0.13	−0.11 to 0.38	0.29	0.08	−0.30 to 0.46	0.67	0.09	−4.46 to 4.63	0.97
	Cont	0.05	−0.03 to 0.14	0.21	0.26	−0.06 to 0.11	0.54										
Motility	≤25th		Ref			Ref		< LOD	Ref			Ref			Ref		
	25th–50th	1.21	−4.55 to 6.96	0.68	−0.08	−5.88 to 5.73	0.98										
	50th–75th	2.66	−3.74 to 9.08	0.41	−0.55	−6.94 to 5.83	0.86										
	>75th	4.60	−1.26 to 10.46	0.12	4.14	−1.71 to 9.98	0.17	≥ LOD	1.97	−2.88 to 6.82	0.43	−1.20	−1.37 to −0.02	0.026	3.80	−2.53 to 10.13	0.24
	Cont	1.20	−0.40 to 2.79	0.14	1.37	−0.24 to 2.98	0.10										
Morph	≤25th		Ref			Ref		< LOD	Ref			Ref			Ref		
	25th–50th	3.01	−4.66 to 10.67	0.44	−5.95	−13.69 to 1.79	0.13										
	50th–75th	−2.73	−10.46 to 4.99	0.49	−8.12	−15.80 to 1.44	0.35										
	>75th	−1.32	−9.02 to 6.38	0.74	−3.41	−11.11 to 4.29	0.38	≥ LOD	6.11	0.05 to 12.16	**0.048**	9.51	0.80 to 18.21	**0.03**	1.49	−5.94 to 8.92	0.69
	Cont	−0.61	−2.52 to 1.31	0.53	−1.20	−3.14 to 0.73	0.22										
VSL	≤25th		Ref			Ref		< LOD	Ref			Ref			Ref		
	25th–50th	1.97	−0.96 to 4.90	0.19	1.82	−1.11 to 4.75	0.22										
	50th–75th	1.20	−2.13 to 4.54	0.48	−0.79	−4.11 to 2.53	0.64										
	>75th	0.10	−2.88 to 3.08	0.95	0.33	−2.62 to 3.29	0.83	≥ LOD	−0.30	−2.76 to 2.15	0.81	−2.80	−6.58 to 0.99	0.15	−0.87	−4.09 to 2.34	0.59
	Cont	0.04	−0.80 to 0.89	0.92	−0.45	−1.29 to 0.40	0.30										
VCL	≤25th		Ref			Ref		< LOD	Ref			Ref			Ref		
	25th–50th	2.58	−0.56 to 5.73	0.11	1.8	−1.35 to 4.95	0.26										
	50th–75th	2.09	−0.70 to 4.88	0.14	0.96	−1.85 to 3.76	0.50										
	>75th	0.34	−2.86 to 3.55	0.83	0.42	−2.77 to 3.6	0.80	≥ LOD	−0.03	−2.67 to 2.61	0.98	−2.49	−6.58 to 1.61	0.23	−0.94	−4.41 to 2.53	0.59
	Cont	0.15	−0.76 to 1.06	0.74	−0.43	−1.34 to 0.48	0.35										
LIN	≤25th		Ref			Ref		< LOD	Ref			Ref			Ref		
	25th–50th	−2.32	−4.37 to 1.27	0.27	−0.88	−2.77 to 1.01	0.36										
	50th–75th	−0.80	−2.87 to 1.26	0.44	−0.58	−2.65 to 1.49	0.58										
	>75th	−0.86	−2.91 to 1.19	0.41	−0.74	−2.63 to 1.15	0.44	≥ LOD	0.60	−0.97 to 2.17	0.46	0.85	−1.60 to 3.29	0.50	0.85	−1.19 to 2.90	0.41
	Cont	−0.33	−0.86 to 0.21	0.23	−0.15	−0.69 to 0.39	0.59										
FSH	≤25th		Ref			Ref		< LOD	Ref			Ref			Ref		
	25th–50th	−0.11	−0.23 to 0.01	0.06	−0.06	−0.19 to 0.06	0.30										
	50th–75th	−0.01	−0.16 to 0.13	0.85	−0.09	−0.23 to 0.06	0.23										
	>75th	−0.02	−0.14 to 0.11	0.79	−0.09	−0.21 to 0.03	0.14	≥ LOD	0.04	−0.06 to 0.14	0.43	0.11	−0.05 to 0.26	0.16	−0.01	−0.15 to 0.12	0.85
	Cont	0.01	−0.03 to 0.04	0.72	−0.01	−0.05 to 0.02	0.50										
Estradiol	≤25th		Ref			Ref		< LOD	Ref			Ref			Ref		
	25th–50th	0.03	−0.05 to 0.11	0.48	0.05	−0.04 to 0.13	0.29										
	50th–75th	1.42	−1.86 to 4.69	0.40	0.06	−0.01 to 1.12	0.44										
	>75th	−0.05	−0.14 to 0.03	0.20	0.01	−0.08 to 0.09	0.93	≥ LOD	−0.02	−0.09 to 0.05	0.63	−0.07	−0.18 to 0.03	0.17	−0.04	−0.14 to 0.05	0.34
	Cont	−0.01	−0.04 to 0.01	0.26	−0.01	−0.03 to 0.01	0.43										
TES	≤25th		Ref			Ref		< LOD	ref			ref			ref		
	25th–50th	0.08	−0.02 to 0.17	0.10	0.03	−0.07 to 0.12	0.61										
	50th–75th	0.08	−0.03 to 0.19	0.17	0.04	−0.07 to 0.16	0.45										
	>75th	0.05	−0.05 to 0.14	0.36	0.01	−0.09 to 0.10	0.95	≥ LOD	−0.03	−0.11 to 0.05	0.47	−0.13	−0.26 to 0.01	**0.031**	−0.04	−0.14 to 0.07	0.49
	Cont	0.01	−0.02 to 0.03	0.68	0.004	−0.02 to 0.03	0.77										
DFI	≤25th		Ref			Ref		< LOD	Ref			Ref			Ref		
	25th–50th	−0.20	−0.46 to 0.07	0.15	−0.11	−0.39 to 0.16	0.42										
	50th–75th	−0.14	−0.45 to 0.17	0.38	−0.14	−0.44 to 0.16	0.35										
	>75 th	0.13	−0.12 to 0.38	0.31	0.08	−0.18 to 0.34	0.55	≥ LOD	0.02	−0.21 to 0.25	0.86	0.14	−0.19 to 0.48	0.40	0.07	−0.21 to 0.36	0.61

TABLE 5. (*Continued*)

Exposure		MP			PP				EP			BP			iBP		
	Percentile of Exposure	Coef	95% CI	*P*	Coef	95% CI	*P*	Detection	Coef	95% CI	*P*	Coef	95% CI	*P*	Coef	95% CI	*P*
HDS	Cont	0.03	−0.05 to 0.10	0.44	0.02	−0.06 to 0.09	0.67										
	≤25th		Ref			Ref		< LOD	Ref			Ref			Ref		
	25th–50th	−0.01	−0.17 to 0.16	0.94	−0.01	−0.18 to 0.16	0.91										
	50th–75th	−1.26	−3.13 to 0.61	0.18	−0.79	−3.69 to 0.16	0.08										
	>75th	−0.04	−0.20 to 0.12	0.65	−0.01	−0.16 to 0.16	0.99	≥ LOD	0.02	−0.12 to 0.19	0.80	−0.13	−0.33 to 0.08	0.22	3.52	1.02–16.03	**0.03**
	Cont	−0.01	−0.06 to 0.04	0.70	−0.01	−0.05 to 0.03	0.66										

Statistically significant at the level 0.05. Multivariate model adjusted for sexual abstinence, age, smoking, alcohol consumption, past diseases.

BP, butyl paraben; coef, ß coefficient; Conc, sperm concentration; DFI, DNA fragmentation index; EP, ethyl paraben; FSH, follicle-stimulating hormone; HDS, high DNA stainability; iBuP, isobutyl paraben; LIN, linearity; LOD, limit of detection; Morph, % of sperm with abnormal morphology; MP, methyl paraben; PP, propyl paraben; TES, testosterone; VCL, curvilinear velocity; VSL, straight-line velocity.

than in US <LOD and 4.1 μg/L. The urinary concentration of iBuP was not measured in the US survey. The iBuP level was assessed in an urban community of Western Canada where the urinary concentrations were lower (median 0.22 μg/L)[25] than the present study (median 2.27 μg/L). The differences in the level of iBuP may be associated with the fact that in the study in Canada, the exposure assessment was performed only among 11 men.

The mechanism of the impact of parabens on male fertility is based on animal toxicology studies, which suggests that parabens act like endocrine disruptors, exogenous chemicals that often interfere with the normal hypothalamo–pituitary–gonadal axis, mimicking hormones, blocking hormonal action, or triggering inappropriate hormone activity.[26] Glander et al,[27] who studied primary microbiological contamination in human ejaculates, and also secondary contamination after cryopreservation using methylparaben, found that not only methylparaben reduced microbiological contamination of the cryoprotective medium but also decreased human sperm motility. The study regarding in vitro spermicidal activity of methyl-, ethyl-, propyl-, and butylparaben in human subjects found that, in fact, these parabens are effective spermicides.[28] Song et al[29] showed that butylparaben exerts an inhibitory effect on the acrosomal enzyme acrosin, and impairs sperm membrane function, indicating that it can potentially be used as a contraceptive, and suggesting another target for paraben action on sperm.

Our study was limited because we used a single urine sample to assess parabens exposure, a single serum sample to describe the level of reproductive hormones, and a single semen sample to assess semen quality. Although parabens are nonpersistant chemicals that are excreted from the body within hours after exposure,[7] Meeker et al[15] found that temporal reliability was greater for concentrations of urinary metabolites of phthalates and nonpersistent pesticides than for parabens among the same men. In addition, consistent individual time-activity patterns may lead to stable concentrations over long periods of time.[30] In the study performed by Smith et al,[31] within-person variability was found moderate in MP and PP. Two reports[32,33] provide evidence that one semen sample may be representative of semen quality over several weeks in epidemiological studies. In addition, in the present study, all subjects were recruited in the same center, and all semen samples were collected and analyzed using a standardized protocol. Similarly, a single sample can be used to assess men's reproductive hormones.[34] As the present study was conducted among men recruited through an infertility clinic, it may limit the ability to generalize the results to the general population. However, we tried to overcome this disadvantage by selecting among infertility male patients only men with normal semen parameters according to WHO classification (WHO, 2010).[16] We are not aware of evidence showing that men from infertility clinic would have to respond differently to parabens compared with men from general population. Thus, our results may apply to general population samples as well. Because a large number of analyses were performed in the present study, some of our observations could be chance findings due to multiple testing. On the contrary, these findings may be of concern because of increased use of parabens that results in widespread exposure among the general population and lack of human epidemiologic studies.

Strengths of the present study include verification of smoking status using the level of cotinine in saliva and detailed information on demographics, medical, and lifestyle risk factors received from the participants allowed for control of confounding in the statistical models. In conclusion, human environmental exposure to parabens may be associated with poorer semen quality and increased sperm DNA damage. Because this is the first study, which assesses the level of five different parabens in urine and semen quality, sperm DNA measures, and the level of reproductive hormones, further epidemiological studies will be helpful to better understand the

effects of parabens on reproductive outcomes. Future studies should also measure paraben concentrations in multiple urine samples collected over the exposure window of interest to reduce exposure measurement error.

REFERENCES

1. Yazar K, Johnsson S, Lind ML, Boman A, Lidén C. Preservatives and fragrances in selected consumer-available cosmetics and detergents. *Contact Dermatitis*. 2011;64:265–272.
2. Witorsch RJ, Thomas JA. Personal care products and endocrine disruption: a critical review of the literature. *Crit Rev Toxicol*. 2010;40:1–30.
3. Wang L, Zhang X, Wang Y, Wang W. Simultaneous determination of preservatives in soft drinks, yogurts and sauces by a novel solid-phase extraction element and thermal desorption-gas chromatography. *Anal Chim Acta*. 2006;577:62–67.
4. Soni MG, Carabin IG, Burdock GA. Safety assessment of esters of p-hydroxybenzoic acid (parabens). *Food Chem Toxicol*. 2005;43:985–1015.
5. Moos RK, Angerer J, Dierkes G, Brüning T, Koch HM. Metabolism and elimination of methyl, iso- and n-butyl paraben in human urine after single oral dosage. *Arch Toxicol*. 2015;90:2699–2709.
6. Abbas S, Greige-Gerges H, Karam N, Piet MH, Netter P, Magdalou J. Metabolism of parabens (4-hydroxybenzoic acid esters) by hepatic esterases and UDP-glucuronosyltransferases in man. *Drug Metab Pharmacokinet*. 2010;25:568–577.
7. Janjua NR, Frederiksen H, Skakkebaek NE, Wulf HC, Andersson AM. Urinary excretion of phthalates and paraben after repeated whole-body topical application in humans. *Int J Androl*. 2008;31:118–130.
8. Ye X, Kuklenyik Z, Needham LL, Calafat AM. Automated on-line column-switching HPLC-MS/MS method with peak focusing for the determination of nine environmental phenols in urine. *Anal Chem*. 2005;77:5407–5413.
9. Golden R, Gandy J, Vollmer G. A review of the endocrine activity of parabens and implications for potential risks to human health. *Crit Rev Toxicol*. 2005;35:435–458.
10. Routledge EJ, Parker J, Odum J, Ashby J, Sumpter JP. Some alkyl hydroxy benzoate preservatives (parabens) are estrogenic. *Toxicol Appl Pharmacol*. 1998;153:12–19.
11. Darbre PD, Harvey PW. Paraben esters: review of recent studies of endocrine toxicity, absorption, esterase and human exposure, and discussion of potential human health risks. *J Appl Toxicol*. 2008;28:561–578.
12. Oishi S. Effects of butylparaben on the male reproductive system in rats. *Toxicol Ind Health*. 2001;17:31–39.
13. Oishi S. Effects of propyl paraben on the male reproductive system. *Food Chem Toxicol*. 2002;40:1807–1813.
14. Hoberman AM, Schreur DK, Leazer T, et al. Lack of effect of butylparaben and methyl-paraben on the reproductive system in male rats. *Birth Defects Res B Dev Reprod Toxicol*. 2008;83:123–133.
15. Meeker JD, Yang T, Ye X, Calafat AM, Hauser R. Urinary concentrations of parabens and serum hormone levels, semen quality parameters, and sperm DNA damage. *Environ Health Perspect*. 2011;119:252–257.
16. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction*. 5th ed. Cambridge, UK: World Health Organization, Cambridge University Press; 2010.
17. Jurewicz J, Radwan M, Sobala W, et al. Lifestyle and semen quality: role of modifiable risk factors. *Syst Biol Reprod Med*. 2014;60:43–51.
18. Jurewicz J, Radwan M, Sobala W, et al. Human urinary phthalate metabolites level and main semen parameters, sperm chromatin structure, sperm aneuploidy and reproductive hormones. *Reprod Toxicol*. 2013;42:232–241.
19. ASRM Practice Committee. Report on optimal evaluation of the infertile male. *Fertil Steril*. 2006;86:202–209.
20. R Core Team. R: A language and Environment for Statistical Computing, ISBN 3-900051-07-0. Vienna, Austria: R Foundation for Statistical Computing; 2016. Available at: http://www.R-project.org/.
21. Oishi S. Effects of propylparaben on the male reproductive system. *Food Chem Toxicol*. 2002;40:1807–1813.
22. Oishi S. Lack of spermatotoxic effects of methyl and ethyl esters of p-hydroxybenzoic acid in rats. *Food Chem Toxicol*. 2004;42:1845–1849.
23. Daston GP. Developmental toxicity evaluation of butylparaben in Sprague–Dawley rats. *Birth Defects Res B Dev Reprod Toxicol*. 2004;71:296–302.
24. NHANES, 2012. Fourth National Report on Exposure to Environmental Chemical Exposure. Available at: http://www.cdc.gov/exposurereport/pdf/FourthReport.pdf. Accessed February 15, 2017.
25. Genuis SJ, Birkholz D, Curtis L, Sandau C. Paraben levels in an urban community of Western Canada. *ISRY Toxicol*;. 2013. Article ID 507897; 8 pages. Available at: http://dx.doi.org/10.1155/2013/507897. Accessed February 15, 2017.
26. Tavares RS, Martins FC, Oliveira PJ, Ramalho-Santos J, Peixoto FP. Parabens in male infertility: is there a mitochondrial connection? *Reprod Toxicol*. 2009;27:1–7.
27. Glander HG, Rytter M, Schonborn C. Studies on the mycotic and bacterial risk of contamination and the use of nipagin in the artificial insemination of cryosperm. *Zentralbl Gynakol*. 1984;106:573–584.
28. Song BL, Li HY, Peng DR. In vitro spermicidal activity of parabens against human-spermatozoa. *Contraception*. 1989;39:331–335.
29. Song BL, Peng DR, Li HY, et al. Evaluation of the effect of butyl p-hydroxybenzoate on the proteolytic activity and membrane function of human spermatozoa. *J Reprod Fertil*. 1991;91:435–440.
30. Meeker JD, Barr DB, Hauser R. Human semen quality and sperm DNA damage in relation to urinary metabolites of pyrethroid insecticides. *Hum Rep*. 2008;23:1932–1940.
31. Smith KW, Braun JM, Williams PL, et al. Predictors and variability of urinary paraben concentrations in men and women, including before and during pregnancy. *Environ Health Perspect*. 2012;120:1538–1543.
32. Francavilla F, Barbonetti A, Necozione S, et al. Within-subject variation of seminal parameters in men with infertile marriages. *Int J Androl*. 2007;30:174–181.
33. Stokes-Riner A1, Thurston SW, Brazil C, et al. One semen sample or 2? Insights from a study of fertile men. *J Androl*. 2007;28:638–643.
34. Bjørnerem A, Straume B, Oian P, Berntsen GK. Seasonal variation of estradiol, follicle stimulating hormone, and dehydroepiandrosterone sulfate in women and men. *J Clin Endocrinol Metab*. 2006;91:3798–3802.

