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ORIGINAL ARTICLE

LINE-1 hypomethylation in spermatozoa is associated with Bisphenol A exposure

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SUMMARY

Bisphenol A (BPA) is an endocrine disruptor with potentially harmful effects on humans. However, epigenetic mechanisms that modulate the effects of BPA remain unclear. Methylation of long interspersed nucleotide elements (LINE-1) is a marker of genome-wide methylation status. This study aims to examine whether BPA exposure was associated with LINE-1 methylation changes in men. Male factory workers in Hunan, China (N = 149) were studied, 77 with BPA exposure in workplace (BPA-exposed group) and 72 without BPA exposure in workplace (control group). Pre-shift and post-shift urine samples were collected from the BPA-exposed group and spot urine samples were collected from the control group. Urine samples were assessed for BPA. In addition, blood and semen samples were collected from both groups for LINE-1 methylation analysis. In multivariate analysis adjusted for age, education, smoking habits and alcohol consumption, sperm LINE-1 methylation level was significantly lower in BPA exposed workers ($p < 0.001$) compared to that in the unexposed workers. Linear regression analysis also showed that log-transformed urine BPA levels were inversely associated with sperm LINE-1 methylation ($p < 0.0001$), but not peripheral blood cell LINE-1 methylation. Moreover, the association between urine BPA level and semen quality was not attenuated after adjustments for LINE-1 level. In summary, the observed independent relationship between BPA exposure and LINE-1 methylation may have public health implications on reproductive health in men because of ubiquitous exposure to BPA.

INTRODUCTION

Bisphenol A (BPA) is a synthetic endocrine disrupter with weak oestrogenic and strong anti-androgenic effects (Kuehn, 2007). Exposure to BPA is common, as it is routinely used to make polycarbonate plastic and epoxy resins (Calafat *et al.*, 2008). Adverse effects of BPA in animal studies, even at low environmentally relevant doses, are well established, including impaired fertility, increased risks of cancer, obesity and insulin-resistant diabetes (Lee *et al.*, 2003; Wetherill *et al.*, 2007; Chapin *et al.*, 2008; Salian *et al.*, 2009). In contrast, the effects of BPA on humans are less well characterized, and previous studies have yielded inconsistent results (Carwile & Michels, 2011; Wang *et al.*, 2012; Lang *et al.*, 2008; Mendiola *et al.*, 2010). We have previously reported reduced male sexual function after exposure to high levels of BPA (Li *et al.*, 2010a,b). Moreover, exposure to BPA led to poor semen quality (Li *et al.*, 2011). Recently, higher urine BPA concentrations have been associated with reduced ovarian response and early reproductive outcomes in women undergoing IVF (Ehrlich *et al.*, 2012). The molecular mechanisms behind these observations remain unclear.

Epigenetic changes have been associated with different exogenous chemicals including BPA (Dolinoy *et al.*, 2007; Rusiecki *et al.*, 2008; Bromer *et al.*, 2010; Doshi *et al.*, 2011; Manikkam *et al.*, 2013). For example, DNA hypomethylation affecting the developmental programming of uterine oestrogen response was observed after in utero BPA exposure in mice (Bromer *et al.*, 2010). Epigenetic changes were also seen in the testes of BPA-exposed mice (Doshi *et al.*, 2011). In humans, lower methylation at promoter CpG islands of the TSP50 gene was observed in women with higher BPA exposure (Hanna *et al.*, 2012).

About 55% of the human genome consists of repetitive elements, including long interspersed nucleotide elements

(LINE-1) and Alu repetitive elements, which are normally heavily methylated (Yang *et al.*, 2004). Hypomethylation of LINE-1 and Alu elements increase their activity as retrotransposable sequences, which may induce genomic alterations by insertion and/or homologous recombination, and deregulate gene transcription (Han *et al.*, 2004; Zhang *et al.*, 2011). LINE-1 methylation is modifiable to the environmental factors including air pollution (Baccarelli *et al.*, 2009; Peluso *et al.*, 2012) heavy metals (Hossain *et al.*, 2012; Lambrou *et al.*, 2012; Tajuddin *et al.*, 2013) and medication (Doehring *et al.*, 2013), as well as nutritional factors such as folate (Dolinoy *et al.*, 2007; Haggarty *et al.*, 2013). However, only peripheral blood samples were examined in these studies, and sensitiveness of LINE-1 methylation to environmental challenge has been poorly studied in other human tissues, as a result of difficulties in collecting and storing these samples.

Identifying the relationship between BPA and global DNA methylation may help understand potentially underlying mechanisms of observed adverse effects of BPA exposure on male reproductive organs including semen quality. This study examined whether BPA exposure led to alternations of global DNA methylation in the male reproductive system by analysing semen and peripheral blood LINE-1 methylation.

METHODS

Participants

One hundred and forty-nine male factory workers in Hunan province were studied. They have been previously described (Li *et al.*, 2010a,b, 2011). In brief, male workers were recruited from manufacturers of epoxy resin (BPA-exposed group) between 2004 and 2008. Unexposed male workers were recruited from a variety of industries without BPA exposure in the same area during the same period. The study was approved by the research ethics committees of all participating institutes. All participants gave written informed consent before participating in the study.

Among the five sites included in the initial studies, only samples from one site were analysed for LINE-1 methylation because of limited funding (n = 149). This site was chosen because all workers were local residents, which minimized the potential impact of regional genetic and environmental differences on LINE-1 methylation status (Rusiecki *et al.*, 2008; Weng *et al.*, 2010; Tajuddin *et al.*, 2013). The participation rates were 72.88% for the exposed group and 52.38% for the unexposed group.

Data collection

Information on demographics, lifestyle factors (smoking, alcohol consumption) and employment history was obtained through face-to-face interviews by trained Chinese-speaking researchers.

Semen quality measurement

Collection and examination of semen specimens were in accordance to the standards set by the World Health Organization (1999). In brief, participants were asked to abstain from sexual activities for at least 2 days (but not exceeding 7 days) before semen collection. Specimens were collected through masturbation and ejaculation into a clean, wide-mouthed container in a private room with a temperature of 20–28 °C. The semen samples were analysed within 1 h of ejaculation. Both macroscopic (e.g. liquefaction, appearance, viscosity, volume and pH) and microscopic analyses (e.g. concentration, motility, vitality and morphology) were performed according to the WHO manual (World Health Organization, 1999). All semen analyses were conducted by the same technician to ensure consistency.

Adjusted urinary BPA measurements

Pre-shift and post-shift urine samples were collected from each worker in the BPA-exposed group (varied between 07.00 and 12.00 h depending on the schedule of shifts). Mean BPA concentrations were calculated and used for the statistical analyses performed. One spot urine sample was collected from each worker in the unexposed group (varied between 08.00 and 16.00 h).

Total (free and conjugated) urinary concentrations of BPA were measured through high-performance liquid chromatography as previously described (Li *et al.*, 2010b). The limit of detection for BPA level was 0.31 μg/L. BPA levels were adjusted for corresponding urinary creatinine levels to account for urine dilution.

DNA extraction and bisulphite treatment

The sperm DNA was extracted by treating sperm pellets with the lysate (4 M guanidine hydrochloride, 25 mM sodium citrate, 1% NP-40 and 1% beta-mercaptoethanol) for 30 min. The DNA was precipitated with two volumes of ethanol at −20 °C for more than 30 min. Frozen pulverized powders of the sperm DNA were re-suspended with 0.4 ml of lysis buffer (10 mM Tris-HCl pH 7.5, 20 mM EDTA pH 8.0, 0.5% SDS, 100 mM NaCl). The crude sperm DNA was treated with proteinase K (Sigma, St. Louis, MO, USA, 200 μg/mL) at 55 °C for 3–5 h.

Extraction with alcohol (Phenol, Chloroform and Isoamyl in a ratio 1 : 1 : 0.04) was repeated until no interphase was visible after centrifugation. DNA was precipitated from the aqueous phase in the presence of one volume of 7.5 M NH_4Ac and three volumes of ethanol. The DNA pellet was washed once with 75% ethanol and dissolved at 65 °C for 10 min with 0.2–0.4 mL TE (10 mM Tris-HCl pH 7.4 and 1 mM EDTA), followed by storage at −20 °C until further use. The DNA concentrations were determined by OD260 nm measurements.

The total DNA of peripheral blood was extracted using the FlexiGene DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

For bisulphite conversion, DNA from spermatozoa and whole blood was treated using Methylamp DNA Modification Kit (Base Catalog #P-1001; Epigentek, New York, NY, USA). The final elution was performed with 30 μL of M-Elution Buffer, according to the manufacturer's protocol.

Quantitative methylation analysis of LINE-1

LINE-1 methylation was measured with a methylation-specific real-time polymerase chain reaction (PCR) assay as previously described (Iacopetta *et al.*, 2007; Zhang *et al.*, 2011).

The following primers were used: unmethylated LINE-1 forward primer, TGTGTGTGAGTTGAAGTAGGGT; unmethylated LINE-1 reverse primer, ACCCAATTTTCCAAATACAACCATCA; methylated LINE-1 forward primer, CGCGAGTCGAAGTAGGGC and methylated LINE-1 reverse primer, ACCCGATTTTCCAA ATACGACCG.

Polymerase chain reaction products for unmethylated and methylated LINE-1 sequences were cloned with pGEM-T Easy

Vector system (Tiangen, Beijing, China). This construct was used as a standard for the measurement of both unmethylated and methylated LINE-1. Serial dilutions were used to validate the results. Real-time PCR was conducted using the Opticon Monitor 3 System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and SYBR Green Real-time PCR Master Mix (Code:QPK-201; Toyobo, Osaka, Japan). Real-time reactions for unmethylated and methylated LINE-1 sequence were performed simultaneously. The percentage of methylated LINE-1 was calculated using the formula: 100 × methylated reaction/(unmethylated reaction + methylated reaction).

Statistical analysis

Descriptive statistics are presented as mean (standard deviation) and median (inter-quartile range) as appropriate. Multivariate linear regression was used to examine the relationships between BPA and LINE-1 methylation (in peripheral blood and spermatozoa) after adjusting for potential confounders including age, smoking, drinking and history of disease. Comparison between groups was analysed according to exposure status and urine BPA levels (divided by tertiles) using linear regression. Log-transformed BPA levels were used to examine the linear association between BPA and methylation. Linear regression model was also used to explore the relationship between BPA and semen quality when (i) LINE-1 methylation level was added to the regression model; and (ii) stratified by LINE-1 methylation level. Data analysis was performed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Urine BPA levels and characteristics of each group are described in Tables 1 and 2 respectively. The median adjusted urine BPA level was significantly higher in BPA-exposed group (26.31 μg/g in the exposed group vs. 0.89 μg/g in the unexposed group). The two groups were similar in age, history of disease and smoking status. For the BPA-exposed group, the reported rates of alcohol consumption were higher though not significantly. While the differences in college education rates between two groups were statistically significant ($p = 0.047$), with more people had college education in unexposed group.

The results of LINE-1 methylation analyses are presented in Table 3. The BPA-exposed group had significantly lower spermatic LINE-1 methylation (median 0.74 vs. 0.79, respectively; $p < 0.001$). The highest tertile of urine BPA level was also associated with the lowest methylation rate in sperm LINE-1 (Table 3). For every unit change in the log-transformed urine BPA concentration, we observed a 0.01 (1%) reduction in spermatic LINE-1 methylation ($p < 0.0001$) (Table 4). To examine the relationship at low dose environmental exposure, analyses were repeated in the unexposed group only. Similar associations were observed (Table 4).

Table 1 Urine BPA level between groups (μg/g Cr)

	N	Mean (std)	Median (Q1, Q3)
By occupational exposure status			
BPA-exposed	77	36.23 (7.69)	26.31 (9.97, 120.3)
Unexposed	72	1.38 (6.89)	0.89 (0.22, 8.41)
By urine BPA level			
Low tertile	50	0.33 (2.14)	0.22 (022, 0.41)
Middle tertile	50	8.25 (1.77)	8.49 (5.26, 13.33)
Top tertile	49	93.69 (5.00)	52.46 (26.58, 202.35)

Table 2 Characteristics of workers in BPA-exposed and unexposed groups

	Exposed ($n = 77$)	Unexposed ($n = 72$)	*p*-value
Age			
22–29	30 (38.9)	22 (34.9)	0.572
30–35	24 (31.2)	25 (39.7)	
36–50	23 (29.9)	16 (25.4)	
History of disease[a]			
Yes	18 (23.4)	12 (19.1)	0.535
No	59 (76.6)	51 (80.9)	
Current smoker			
Yes	51 (66.2)	44 (69.8)	0.649
No	26 (33.8)	19 (30.2)	
Alcohol consumption			
Yes	18 (23.4)	9 (14.3)	0.175
No	59 (76.6)	54 (85.7)	
Education			
≤Middle school	14 (18.2)	10 (15.9)	0.047
High school	50 (64.9)	31 (49.2)	
≥College	13 (16.9)	22 (34.9)	
Years working			
≤5	33 (42.9)	17 (26.9)	0.086
5–10	21 (27.3)	27 (42.9)	
>10	23 (29.8)	19 (30.2)	

[a]Disease refers to any acute or chronic disease of liver, kidney or other organs.

Moreover, the observed association between urine BPA level and sperm LINE-1 methylation rate was unchanged after stratifying by smoking and alcohol consumption. Analyses after stratification by age showed trends towards stronger associations between urine BPA and sperm LINE-1 methylation in older groups; however, the test of interaction was not statistically significant (Table 4).

The above analyses were repeated to examine the relationship between urine BPA and peripheral blood LINE-1 methylation, and no significant association was found.

The comparison of semen characteristics between BPA-exposal and unexposal group was showed in Table S1. We also examined the beta coefficients of urine BPA level with semen quality parameters before and after adjusting for sperm LINE-1 methylation. The results showed that urine BPA remained associated with semen quality after adjusting for LINE-1 methylation. Stratification analyses showed that urine BPA was associated with poorer semen quality in high LINE-1 methylation groups, while the association in the low LINE-1 methylation group was not statistically significant. These results did not provide evidence that LINE-1 methylation play an intermediate role between urine BPA and semen quality (Table 5).

DISCUSSION

In this study, we found that BPA exposure (based on both occupational exposure history and urinary BPA levels) was inversely associated with sperm LINE-1 methylation, but not peripheral blood LINE-1 methylation. Similar relationships with urine BPA level was also observed in the unexposed group with only low environmental BPA exposure.

To our knowledge, this is the first study linking BPA exposure to altered sperm DNA methylation patterns. Chemicals including arsenic, benzene, persistent organic pollutant and lead exposure were previously reported to cause similar LINE-1 hypomethylation in humans at low doses (Bollati *et al.*, 2007;

Table 3 BPA exposure and methylation in LINE-1

	N	Mean (std)	Median	5th	25th	75th	95th	Min	Max	*p*-value[a]
Sperm LINE_1 methylation										
By occupational exposure status										
BPA-exposed	77	0.74 (0.07)	0.74	0.62	0.69	0.80	0.84	0.52	0.84	<0.001
Unexposed	72	0.78 (0.07)	0.79	0.66	0.75	0.83	0.88	0.59	0.89	
By urine BPA level										
Low tertile	50	0.78 (0.07)	0.80	0.65	0.76	0.83	0.88	0.59	0.88	<0.001
Middle tertile	50	0.76 (0.06)	0.76	0.67	0.71	0.80	0.84	0.62	0.85	
Top tertile	49	0.73 (0.08)	0.73	0.62	0.69	0.80	0.83	0.52		
Blood LINE_1 methylation										
By occupational exposure status										
BPA-exposed	64	0.88 (0.04)	0.88	0.81	0.86	0.91	0.93	0.74	0.95	0.324
Unexposed	59	0.88 (0.03)	0.89	0.82	0.86	0.91	0.94	0.81	0.96	
By urine BPA level										
Low tertile	50	0.89 (0.03)	0.89	0.84	0.86	0.91	0.95	0.83	0.96	0.257
Middle tertile	50	0.88 (0.04)	0.88	0.80	0.85	0.90	0.93	0.79	0.94	
Top tertile	49	0.88 (0.04)	0.88	0.82	0.86	0.91	0.94	0.81	0.95	

[a]Adjusted for: age, education, history of disease, smoking and alcohol consumption.

Table 4 Linear regression of urine BPA and methylation in LINE-1

	Sperm LINE_1		Blood LINE_1	
	Beta coefficient	*p*-value[a]	Beta coefficient	*p*-value[a]
All subjects	−0.011	<0.001	−0.002	0.176
Among subjects without occupational BPA exposure	−0.012	0.026	−0.003	0.250
Stratified analysis				
Smoking				
Yes	−0.012	0.000	−0.002	0.347
No	−0.010	0.031	−0.002	0.308
Alcohol consumption				
Yes	−0.019	0.040	−0.003	0.210
No	−0.011	<0.001	−0.002	0.143
Age group				
22–29	−0.007	0.192	−0.001	0.653
30–35	−0.012	0.019	−0.003	0.197
36–50	−0.016	0.006	0.001	0.723

[a]Adjusted for: age, education, history of disease, smoking and alcohol consumption. Smoking was not adjusted in the stratified analysis by smoking and drinking was not adjusted in the stratified analysis by alcohol consumption.

Rusiecki *et al.*, 2008; Pilsner *et al.*, 2009; Lambrou *et al.*, 2012; Tajuddin *et al.*, 2013), which supports underlying biological plausibility of the observed association.

Almost all of the previous studies were based on analysis of blood samples (Baccarelli *et al.*, 2009; Pilsner *et al.*, 2009; Hossain *et al.*, 2012; Lambrou *et al.*, 2012; Peluso *et al.*, 2012; Doehring *et al.*, 2013; Haggarty *et al.*, 2013; Tajuddin *et al.*, 2013), which have shown LINE-1 methylation is vulnerable to different environmental factors. This study demonstrated that the relationship between BPA exposure and LINE-1 hypomethylation was only present in semen samples but not in peripheral blood samples. Specimen-dependent relationship between environmental exposures and LINE-1 methylation has not been reported previously. Different types of normal human tissues have distinct epigenetic profiles (Christensen *et al.*, 2009), spermatozoa has one of the highest levels of LINE-1 methylation among other human cells tested (Chalitchagorn *et al.*, 2004). Measuring LINE-1 methylation status in spermatozoa could be explored as a more sensitive marker than in peripheral blood for global methylation status.

In contrast to other studies evaluating the effects of endocrine disruptors, the wide variation in BPA concentrations observed has enabled us to investigate global DNA methylation across a wide BPA range. We did not observe a more profound difference in mean methylation level between the second and the third tertile than the one between the first and the second tertile.

Table 5 Associations between urine BPA & semen quality adjusted and stratified by sperm LINE-1 methylation

	Sperm motility (% moving forward)	Concentration (10^6 spermatozoa/mL)	Sperm vitality (% alive)
No sperm LINE-1 methylation adjusted			
Beta coefficient[a]	−1.091	−5.966	−1.919
p-value	0.075	0.010	0.017
Sperm LINE-1 methylation adjusted			
Beta coefficient[b]	−1.570	−6.316	−2.290
p-value	0.017	0.012	0.008
LINE-1 methylation > median			
Beta coefficient[a]	−2.015	−8.808	−2.808
p-value	0.053	0.028	0.045
LINE-1 methylation ≤ median			
Beta coefficient[b]	−0.962	−3.632	−1.543
p-value	0.224	0.206	0.122

[a]Adjusted for: age, history of disease, smoking, drinking and abstinence days. [b]Adjusted for: age, history of disease, smoking, drinking, abstinence days and methylation.

Moreover, similar regression coefficients were found among the unexposed group. This non-monotonic effect was consistent with previous reports (Vom Saal *et al.*, 1997; Newbold *et al.*, 2009), in which a quantitative change in endpoints as the dose increases is not always observed.

Abnormal epigenetic programming has been proposed as a possible mechanism compromising male fertility and abnormal methylome existed in spermatozoa of infertile men (Houshdaran *et al.*, 2007; Kobayashi *et al.*, 2007; Filipponi & Feil, 2009). Recently, two endocrine disruptors, methoxychlor and vinclozolin, were shown to alter spermatogenic capacity of male germ cells and sperm viability via their effects on DNA methylation (Anway and Skinner 2006; Tabb and Blumberg 2006). Moreover, BPA exposure has been found to cause DNA damage in human spermatozoa (Meeker *et al.*, 2011) and disrupt spermatogenesis in animal models (Chapin *et al.*, 2008; Vandenberg *et al.*, 2009; Wong & Cheng, 2011). The sperm DNA methylation regions provide potential epigenetic biomarkers for environmental BPA exposure (Manikkam *et al.*, 2013).

Our previous studies showed that exposure to BPA was associated with poor semen quality in humans (Li *et al.*, 2011). Our current results show that sperm LINE-1 methylation is associated with BPA exposure. However, other studies did not detect a relationship between LINE-1 methylation level and abnormal human spermatogenesis (Houshdaran *et al.*, 2007; Marques *et al.*, 2008). We examined whether the observed hypomethylation played an intermediate role in the effect of BPA on semen quality. Our results show that additional adjusting LINE-1 methylation in the linear regression model did not attenuate the association between BPA and semen quality. However, stronger association between BPA and semen quality parameters was observed among those with higher LINE-1 methylation. This may be partly because of the less disturbance from other confounding chemicals in this group, as lowered methylation has been found to be associated with exposure to environmental hazardous chemicals (Bollati *et al.*, 2007; Hanna *et al.*, 2012; Hossain *et al.*, 2012; Tajuddin *et al.*, 2013). It is possible that abnormal DNA methylation of individual genes may still be involved in BPA-related impact on sperm quality.

Hypomethylation of LINE-1 is correlated with higher LINE-1 expression and retrotransposition (Hata & Sakaki, 1997; Yu *et al.*, 2001). If the regulation by methylation is eliminated, the increase in the mobile element expression leads cells to have an increased genetic instability with adverse consequences (Han *et al.*, 2004). Particularly, some environmental heavy metals, such as cadmium, caused LINE-1 hypomethylation (Hossain *et al.*, 2012) and stimulated human LINE-1 retrotransposition in cell culture (Kale *et al.*, 2005). There are several reported examples of diseases caused by LINE-1 insertions, including muscular dystrophy (Narita *et al.*, 1993) and haemophilia A (Kazazian *et al.*, 1988) and male boar infertility (Sironen *et al.*, 2007). Interestingly, the transcripts for ORF2 of LINE 1 have been reported to be present in ejaculate spermatozoa (Miller, 2000). In animal studies, abnormal DNA methylation in spermatozoa seemed involved in environmental factors (including BPA)-induced transgenerational disruptive spermatogenesis (Anway *et al.*, 2005; Manikkam *et al.*, 2013). In addition, low doses of BPA caused paternal birth defects in male mouse offspring (Salian *et al.*, 2009; Manikkam *et al.*, 2013).

This study has several limitations. First, LINE-1 methylation is a global methylation marker and the effect of BPA on individual promoter DNA has not been explored. Second, the high occupational exposure in the BPA-exposed group and small sample size restricted the generalizability of this study. Third, single BPA measurement in the unexposed group may subject to non-differential misclassification, thus attenuating the observed association although a spot sample has been suggested to be representative of a 3 months period of exposure (Braun *et al.*, 2011). Lastly, similar to other sperm studies (Juhler *et al.*, 1999), a proportion of participants declined to provide biological samples (urine and semen) leading to potential participation bias (not representative of the whole population).

In summary, BPA exposure is associated with LINE-1 hypomethylation in spermatozoa, but not in peripheral blood of the participating male factory workers. However, LINE-1 hypomethylation does not appear to play a major role for the reported association between BPA exposure and poor semen quality. The implication of observed BPA-LINE-1 hypomethylation association is to be elucidated. Although the associated difference is small, this observation may have important public health implications as BPA is a commonly encountered chemical.

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COMPETING FINANCIAL INTEREST DECLARATION

All authors declare no competing financial interests.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Semen characteristics in BPA-exposed and unexposed groups.

