Shorter sperm telomere length in association with exposure to polycyclic aromatic hydrocarbons: Results from the MARHCS cohort study in Chongqing, China and in vivo animal experiments

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ARTICLE INFO

Article history:
Received 15 April 2016
Received in revised form 23 June 2016
Accepted 5 August 2016
Available online 10 August 2016

Keywords:
Polycyclic aromatic hydrocarbon
Urinary metabolites
Sperm telomere length
Telomerase
Semen quality

ABSTRACT

It has been well demonstrated that polycyclic aromatic hydrocarbons (PAHs) can cause reproductive toxicity, and shorter telomere length in sperm may be one of the factors causing male infertility. However, whether exposure to PAHs is associated with sperm telomere length (STL) has never been evaluated. The present study aimed to assess the potential association between PAHs exposure and STL, and to explore potential biomarkers that may predict the effects of low-level exposure to PAHs on human sperm. Questionnaires and biological samples were collected from 666 volunteers participating in the Male Reproductive Health in Chongqing College Students (MARHCS) cohort study in 2014. Semen parameters were measured for 656 participants, while urinary PAH metabolites, STL and sperm apoptosis were successfully measured for 492, 444 and 628 participants, respectively. The linear regression analysis revealed that increased levels of urinary 1-hydroxypyrene (1-OHPyr) and 1-hydroxynaphthalene (1-OHNap) were associated with decreased STL (−0.385; 95% CI, −0.749, −0.021 for 1-OHPyr; and −0.079; 95% CI, −0.146, −0.011 for 1-OHNap). The significant negative associations remained after adjusting for potential confounders. However, no significant associations were observed between urinary PAH metabolites and semen quality or sperm apoptosis. We also administrated rats with benzo[a]pyrene (B[a]P; 0, 1, 5, and 10 mg/kg) for 4 weeks and found shorter STL and decreased telomerase expression in germ cells in a dose-dependent manner. In conclusion, environmental exposure to some PAHs may be associated with decreased human STL, and the in vivo animal results also demonstrate the adverse effects of B[a]P on telomere of male germ cells.

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1. Introduction

Male semen quality has declined at an average rate of 1% per year over the past 50 years, with human sperm concentrations decreasing dramatically from 113 to 61 million/ml (Carlson et al., 1992). Approximately 10% of couples worldwide suffer from infertility (Nachtigall, 2006), and a male contributing factor is involved in approximately 50% of these cases (Smith et al., 2014). Although it has been confirmed that genetic and pathological factors can result in male infertility in the general population, accumulating evidence also suggests that male reproductive health can be damaged by environmental factors, especially endocrine disrupting chemicals. Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants characterized by their hazardous carcinogenic and mutagenic potentials (Bansal and Kim, 2015). These chemicals have been observed to contribute to male reproductive damage in animal and in vitro studies. Benzo[a]pyrene (B[a]P), an important member of the PAHs group, has been detected by the presence of its metabolites in the reproductive organs of rats after acute oral exposure (Ramesh et al., 2001). Benzo[b]fluoranthene (B[b]FA) and B[a]P have been reported to induce apoptosis of Sertoli cells in vitro, may potentially result in toxic effects on germ cells development (Raychoudhury and Kubinski, 2003). Our preliminary study has suggested that in vivo exposure to B[a]P can disturb the secretion of testosterone, influence the production and morphology of sperm and damage seminiferous tubules (Chen et al., 2011). Further, a large body of data indicates that the direct adverse effects of PAH compounds on germ cells may result from the formation of PAH-DNA adducts and the associated induction of mutation and infertility (Revel et al., 2001).

Abbreviations: MARHCS, the Male Reproductive Health in Chongqing College Students; PAHs, polycyclic aromatic hydrocarbons; B[a]P, benzo[a]pyrene; STL, sperm telomere length; 1-OHNap, 1-hydroxynaphthalene; 2-OHPhe, 2-hydroxynaphthalene; 2-OHFla, 2-hydroxyfluorene; 1-OHPhe, 1-hydroxyphenanthrene; 2-OHPhe, 2-hydroxyphenanthrene; 3-OHPhe, 3-hydroxyphenanthrene; 4-OHPhe, 4-hydroxyphenanthrene; 1-OHPyr, 1-hydroxypyrene.

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http://dx.doi.org/10.1016/j.envint.2016.08.001
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A limited number of human studies have suggested a potential association between exposure to PAHs and male reproductive toxicity. In an occupational group exposed to PAHs in a coke oven plant, urinary 1-hydroxypyrene (1-OHPyr), a biomarker of PAH exposure, has been shown to be positively associated with DNA denaturation and abnormal sperm morphology, and topside-oven workers with higher PAHs levels have been shown to exhibit a higher frequency of oligospermia than side-oven workers (Hsu et al., 2006). Xia et al. (2009) have reported that decreased semen quality, including the sperm concentration and number per ejaculation, in men with higher 1-OHPyr levels. However, the detrimental impact of PAHs exposure on human sperm parameters remains controversial. A recent study of coke oven workers has shown that PAHs do not affect the sperm concentration or vitality but increased morphological abnormalities and bulky DNA adducts in sperm (Jeng et al., 2013). Further, our previous study of a general male population has indicated that the environmental level of PAHs exposure is associated with increased sperm DNA damage but not with semen quality (Han et al., 2011). Although the ultimately affected parameters have varied among different studies, the published data are in agreement that DNA damage is consistently present in human sperm and that it involves the formation of PAH-DNA adducts (Jeng et al., 2013), morphological changes (Hsu et al., 2006) and DNA strand breaks (Han et al., 2014). Moreover, the extent and characteristics of sperm damage have been suggested to be generally determined by the levels of PAHs exposure. Hence, more sensitive biomarkers related to sperm DNA damage should be explored to evaluate the reproductive toxicity of environmental exposure to PAHs in humans, especially among individuals in the general population who have likely been exposed to low PAHs levels.

Telomeres, located at the end of eukaryotic chromosomes, are composed of hexameric tandem repeats of the sequence TTAGGG. Telomeric DNA is a rich source of guanine bases, and this structural characteristic indicates that telomeres are more susceptible to internal/external factors, especially oxidative stress injury, which can cause telomeric DNA damage and ultimately, the shortening of telomeres (Kawanishi and Oikawa, 2004). Accumulating evidence suggests that telomere length in blood cells may be affected by environmental chemicals, including benzene (Hoxha et al., 2009), particulate matter (PM) (Hou et al., 2012) and cigarette smoke (Valdes et al., 2005). Occupational exposure to PAHs can also lead to shortened telomere lengths in blood leukocytes (Pavanello et al., 2010), indicating that telomeric DNA may be one of the genetic targets damaged by PAHs. However, no epidemiological or experimental studies have examined the potential effects of PAHs exposure on sperm telomere length (STL).

Telomeres play a critical role in maintaining chromosome stability and genomic integrity. Sperm with shortened telomere length may not respond to oocyte signals to form a pronucleus, ultimately resulting in impaired cleavage, a poor-quality blastocyst, increased apoptosis, or failed implantation (Hemann et al., 2001). These data indicate that shorter telomere lengths in sperm may be one of the factors causing male infertility (Thilagavathi et al., 2013a). Therefore, the effects of PAHs on reproductive viability may be associated with telomeres. The objective of the current study was to determine whether urinary PAH metabolite levels in the non-occupational population were associated with STL semen quality and sperm apoptosis. Based on our epidemiological findings, we performed in vivo animal experiments to detect the potential effects of BitP on STL to further explore the testicular expression of telomerase, a reverse transcriptase involved in the maintenance of telomere length, and to elucidate the mechanisms underlying the effects of PAHs on sperm telomere shortening.

2. Materials and methods

2.1. Study population

The participants included in this study were Chongqing college students who were subjects in a prospective cohort study, the Male Reproductive Health in Chongqing College Students (MARHCS) study, which was established in June 2013 and first followed up in June 2014. The MARHCS study aimed to investigate factors affecting male reproductive health in young adults that were associated with socio-psychological-behavioral factors and changes in environmental exposure. A total of 666 eligible subjects participated in the follow-up study in 2014. Additional detailed information about the study had been previously described (Yang et al., 2015). Briefly, second-grade college students studying in university town who were over 18 years of age were eligible for inclusion in the study. The exclusion criteria were as follows: abstinence of <2 days or >7 days and a history of reproductive or urologic diseases. All eligible participants were required to complete a unified questionnaire that included demographic characteristics, familial history, medical history, sexual behavior, sleeping type and behavior/lifestyle. Physical examinations were performed, and biological samples (semen, blood, and urine) were collected in the school infirmary. All of the men were made aware of the study purpose and were asked to sign informed consent forms to agree to take part in the study. The study protocol and consent forms were approved by the Ethical Committee of The Third Military Medical University.

2.2. Urinary PAH metabolites measurement

Urine samples were collected, frozen at −20 °C, and stored in the dark until analyses were performed to determine the levels of PAH metabolites. The urinary concentrations of PAH metabolites were measured using a sensitive and selective liquid chromatography tandem mass spectrometry (LC-MS/MS) method (Xu et al., 2004). A total of 492 individuals completed the test. Briefly, the frozen urine samples were ultrasonically dissolved for 5 min and then aspirated into β-glucuronidase and arylsulfatase (Sigma-Aldrich, Inc., St Louis, MO, USA) until they were fully mixed. Next, the urine samples were incubated at 37 °C for 16 h of enzyolysis in the dark. Subsequently, they were transferred into C18 solid phase extraction (SPE) cartridges at a flow rate of <1 ml/min. The extracts were concentrated and dried under a stream of nitrogen gas at 35 °C, and then methanol was added to dissolve the residue. They were then fully mixed and centrifuged for 15 min at 12,000 rpm/min, then aspirated to remove the supernatant using an Agilent 1260 high-performance liquid chromatograph, and finally detected using an Agilent G6420 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The limit of detection (LOD) for the eight PAH metabolites was 0.012–0.058 ng/ml. The relative standard deviation (RSD) of the within-series imprecision was between 2.9 and 10.1%, and the recovery was between 85.6% and 118.3%. The urinary creatinine (CR) concentrations were used to adjust the PAH concentrations. These were measured in all samples using an automated chemistry analyzer (Shimazu CL-8000; Shimazu, Inc., Tokyo, Japan). Samples containing CR concentrations of >300 or <300 mg/dl were excluded because these sample concentrations were too high or too low to obtain valid results (Xia et al., 2009).

2.3. Semen analysis

The semen samples were each collected into a sterile, wide-mouth, plastic container by masturbation in a private room after a self-reported period of abstinence. The samples were then immediately placed in a 37 °C incubator, and almost all samples (out of 656 available samples) were liquefied within 1 h. Semen quality was evaluated according to the WHO 2010 guidelines by an experienced urologist to reduce the variation among assessments of sperm characteristics. The appearance of the semen was recorded by observation, and semen volume was assessed by measurement of weight, assuming that a weight of 1 g was equivalent to a volume of 1 ml. The sperm concentration, total sperm counts and sperm motility were determined by computer-aided sperm analysis (SCA CASA System; Microptic S.L., Barcelona,
Spain). Sperm motility was graded as progressive (PR) motility, non-progressive (NP) motility, or immotility (IM) (Yang et al., 2015).

2.4. STL analysis

Of the 666 subjects that were included in our first follow-up study in June 2014, qualified sperm DNA was extracted and PCR analysis was successfully completed for a total of 444 participants. The DNA used in this assay was extracted from semen using an E.Z.N.A.™ DNA Isolation Kit (Omega Bio-Tek Inc., Norcross, GA, USA) according to the manufacturer’s recommendations. STL was determined by real-time quantitative PCR (Q-PCR) as previously described (Thilagavathi et al., 2013a), based on the method developed by Cawthon (2002). This method measures the relative STL by calculating the T/S ratio, which consists of the telomere repeat copy number (T) and the single copy gene copy number (S). Then, the T/S ratio of the experimental samples was compared to the T/S the ratio of a reference pooled sample. The standard curve and the reference DNA were obtained using the same 50 DNA samples that were randomly selected from the participants in the present study. For the standard curve, the pooled DNA sample was serially diluted by 4-fold per dilution to produce 5 solutions with concentrations ranging from 116 to 0.45 ng/μl for use in assessing inter-plate variations. The R² value for each standard curve was ≥0.99. Each PCR was performed using 2 μl of a DNA sample (20 ng of DNA per μl) in a final reaction volume of 20 μl. All samples, standard curves, the reference DNA and the no-template control were run in 96-well plates using a Bio-Rad CFX (Bio-Rad, Hercules, CA, USA). The following forward and reverse telomeric primers were used: 5′-CGGTGTTGTTTGTCCCGGTGTTTGGG TTTGCGTGGTT-3′ and 5′-GGTTGCTTACCCCTATCCCTACCTTT ACCCTTATACT-3′. The following forward and reverse primers were used for the single copy gene 36B4: 5′-CAGCAAGTTGGG AAGGTGTAACTC-3′ and 5′-CCCATTCTATCATACGAAGGTGATAA-3′. The program used for both the telomere and the 36B4 PCR reactions was one cycle of 95 °C for 10 min and 40 cycles of 95 °C for 30 s and 58.5 °C for 30 s. The mean Ct values were used to compute the relative telomere length according to the T/S ratio with the following formula: 

$$
\Delta C = \Delta C_{\text{telomere}} - \Delta C_{\text{control}}; \Delta C = C_{\text{sample}} - C_{\text{control}}; \text{T/S} = 2^{-\Delta C_{\text{telomere}}} - 2^{-\Delta C_{\text{control}}},
$$

All samples were run in triplicate, and the mean of the three measurements was used in statistical analyses.

2.5. Sperm apoptosis assay

In apoptotic cells, membrane phosphatidylserine (PS) is translocated from the inner leaflet of the plasma membrane to the outer leaflet (Vermes et al., 1995). A phospholipid-binding protein, Annexin V, has a high affinity for PS, and fluorochrome-labeled Annexin V can be used to detect exposed PS using flow cytometry. In the present study, sperm apoptosis was measured using an Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, San Jose, CA) according to the manufacturer’s instructions. The test was completed for a total of 628 subjects’ semen samples. Briefly, 500 μl of each semen sample was centrifuged, and the seminal plasma was separated and stored at −20 °C. The remainder of the sperm cells were washed with phosphate-buffered saline (PBS), the sperm density was adjusted to approximately 1 × 10⁶, and the sperm cells were then resuspended in 1 × buffer containing 3 μl of Annexin V-FITC and 3 μl of propidium iodide (PI). The cells were gently vortexed and then incubated at room temperature for 15 min in the dark. They were immediately analyzed using a flow cytometry (Accuri C6, BD Biosciences, San Jose, CA). A total of 15,000 cells were counted for each sample. Data acquisition and analysis were performed using FlowJo Version 7.6 software (Tree Star, Stanford, CA, USA).

2.6. Animal experiments

The study was approved by The Third Military Medical University Institutional Animal Care and Use Committee. Male Sprague Dawley rats (n = 32; 200–220 g) were provided by the Laboratory Animal Center of The Third Military Medical University and acclimatized for 7 days. The animals were housed in a room maintained at a temperature of 20–22 °C and at 50–70% humidity with a 12 h light/dark cycle. Rats were randomly assigned to four groups (n = 8 per group). The rats in each treatment group were dosed with corn oil (control) or B[a]P (1, 5, and 10 mg/kg) every other day at the same time (9:00–11:00 a.m.) via gavage for 4 weeks. It had been reported in our previous study that exposure to B[a]P at the dose of 5 mg/kg for up to 8 weeks was not found to cause abnormal sperm morphology or significant changes in testicular histology or sperm parameters (Chen et al., 2011). Therefore, in the current study, the rats were treated with 1 and 5 mg/kg B[a]P for 4 weeks to observe the potential effects on sperm telomeres under the condition that do not cause obvious testicular toxicity. In addition, we exposed the rats to a higher dose of B[a]P (10 mg/kg). The B[a]P was dissolved in corn oil. The volume that was orally administered was adjusted to 5 ml/kg body weight. The rats were deeply anesthetized using 20% urethane at 24 h after the last treatment, and their testes and epididymides were quickly removed and weighed. Germ cells were isolated from the testes as previously described (Daxian et al., 2005). The epididymides were separated to prepare sperm suspensions for telomere length analyses. The assay used to determine telomere length was the same as that described above.

2.7. Western blot assay

Spermatogenic cells were lysed in RIPA lysis buffer (Beyotime, China) for 30 min on ice. Protein samples (80 μg) were separated on 10% SDS–PAGE gels and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked for 1 h using 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween-20 and then incubated over-night at 4 °C with an anti-TERT antibody (Abcam, Cambridge, UK). Subsequently, the membranes were washed and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescence kit (Millipore, Billerica, MA, USA).

2.8. Statistical analysis

Demographic data were reported as the mean ± standard deviation (SD) for continuous variables and as counts and percentages for categorical variables. The urinary PAH metabolites, sperm apoptosis and STL are presented as geometric means or percentiles. Univariate linear regression analysis was performed to assess the influences of the internal doses of PAHs (as an independent variable) on STL, semen quality and sperm apoptosis (as a dependent variable) of the participants. To eliminate the effects of potential confounders, including age, body mass index (BMI), parental age, duration of abstinence (as continuous), smoking status, and the consumption of alcohol, tea and baked food, were included in the multiple linear regression analysis. In the animal experiments, the values are expressed as the mean ± SD. Differences between the groups were evaluated by one-way ANOVA. The Statistical Package for the Social Sciences (SPSS) version 13.0 (SPSS Inc., Chicago, IL, USA) was used to perform the statistical analyses. In all cases, p < 0.05 was considered to indicate significance.

3. Results

3.1. Population study

The general characteristics of the 666 eligible participants are summarized in Table 1. The mean age of the men was 21 years, and
their average BMI was 21.6 kg/m². The mean duration of abstinence was 4.2 days. The average paternal and maternal ages were 48.3 and 46.7, respectively. Approximately one-fifth of the subjects (22.1%) were current smokers. The majority of the subjects (74.3%) were current drinkers. Almost one-fifth of the subjects (22.4%) were current tea drinkers, including all types of tea, such as green tea, red tea, black tea, and other teas. A large majority of the subjects (81.8%) ate baked foods less than one time per week.

### 3.1.2. PAH metabolites

We measured eight urinary PAH metabolites derived from four types of parent PAHs (fluorene, pyrene, naphthalene, and phenanthrene), including 2-hydroxyfluorene (2-OHFlu, a metabolite of fluorene), 1-hydroxypyrene (1-OHPyr, a metabolite of pyrene), 1-hydroxynaphthalene and 2-hydroxynaphthalene (1-OHNap and 2-OHNap, metabolites of naphthalene), and 1-hydroxyphenanthrene, 2-hydroxyphenanthrene, 3-hydroxyphenanthrene and 4-hydroxyphenanthrene (1-OHPhe, 2-OHPhe, 3-OHPhe and 4-OHPhe, metabolites of phenanthrene). The concentrations of the eight PAH metabolites were adjusted to creatinine (CR) and are shown in Table 2. Among the eight PAH metabolites, 2-OHNap had the highest geometric mean (0.717 μg/g CR), followed by 2-OHFlu, 3-OHPhe, 2-OHPhe, 1-OHPhe, 1-OHNap and 1-OHPyr, while 4-OHPhe displayed the lowest geometric mean (0.008 μg/g CR).

### 3.1.3. PAH exposure and STL

The results of the univariate regression analyses are summarized in Table 3. No significant correlations were detected between the total PAH metabolite levels and STL in analysis of all eight PAH metabolites together. We then analyzed the individual concentrations of the eight metabolites and found that 1-OHPyr and 1-OHNap were negatively associated with STL (p < 0.385; 95% CI, −0.749, −0.021; and −0.079; 95% CI, −0.146, −0.011, respectively). The associations remained significant following adjustments for subject age, paternal age, BMI, duration of abstinence, smoking status, the consumption of alcohol and baked food, which have been previously demonstrated to be associated with STL (Table 4). There were no statistically significant associations between 2-OHFlu, 2-OHNap, 1-OHPhe, 2-OHPhe, 3-OHPhe, 4-OHPhe and STL. Because heavy drinking behavior (>4 drink-units/day) has been shown to be an important factor for reducing leukocyte telomere length (Pavanello et al., 2011), we classified the current drinkers as light drinkers (<4 drink-units/day) or heavy drinkers (>4 drink-units/day). After adjusting the drinking behavior in the linear regression model, 1-OHPyr and 1-OHNap were still found to be negatively associated with STL (p < 0.05) (data not shown).

### 3.1.4. PAH exposure and semen parameters

In the univariate regression analyses, 2-OHFlu was slightly positively correlated with the total sperm count (p = 0.045), and 1-OHPhe was positively correlated with the sperm concentration (p = 0.043) (data not shown). However, when these results were adjusted for age, duration of abstinence, BMI, smoking status, the consumption of alcohol, tea and baked food, the associations were no longer significant (p > 0.05). No significant associations were found between 2-OHFlu,

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**Table 1.** Characteristics of the study population (n = 666).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Descriptive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>21.4 ± 1.2</td>
</tr>
<tr>
<td>Paternal age (years)*</td>
<td>48.3 ± 4.5</td>
</tr>
<tr>
<td>Maternal age (years)*</td>
<td>46.7 ± 4.1</td>
</tr>
<tr>
<td>BMI (kg/m²)*</td>
<td>21.6 ± 2.8</td>
</tr>
<tr>
<td>Abstinence time (days)*</td>
<td>4.2 ± 1.4</td>
</tr>
<tr>
<td>Smoking status**</td>
<td></td>
</tr>
<tr>
<td>Never smoker</td>
<td>483 (72.6)</td>
</tr>
<tr>
<td>Ever smoker</td>
<td>182 (27.4)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>147 (22.1)</td>
</tr>
<tr>
<td>Former smoker</td>
<td>35 (5.3)</td>
</tr>
<tr>
<td>Alcohol drinking***</td>
<td></td>
</tr>
<tr>
<td>Never drinker</td>
<td>130 (19.5)</td>
</tr>
<tr>
<td>Ever drinker</td>
<td>536 (80.5)</td>
</tr>
<tr>
<td>Current drinker</td>
<td>495 (74.3)</td>
</tr>
<tr>
<td>Former drinker</td>
<td>41 (6.2)</td>
</tr>
<tr>
<td>Tea drinking**</td>
<td></td>
</tr>
<tr>
<td>Never drinker</td>
<td>350 (53.9)</td>
</tr>
<tr>
<td>Current drinker</td>
<td>149 (22.4)</td>
</tr>
<tr>
<td>Former drinker</td>
<td>158 (23.7)</td>
</tr>
<tr>
<td>Baked food consumption**</td>
<td></td>
</tr>
<tr>
<td>&lt;1 time/week</td>
<td>545 (81.8)</td>
</tr>
<tr>
<td>1–2 times/week</td>
<td>107 (16.1)</td>
</tr>
<tr>
<td>≥3 times/week</td>
<td>14 (2.1)</td>
</tr>
</tbody>
</table>

BMI = body mass index.

- * The results are presented as the mean ± SD.
- ** The results are presented as the no. (%).
- ** One subject failed to report BMI.
- ** One subject failed to report smoking status.

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**Table 2.** Distributions of urinary PAH metabolites, sperm apoptosis and sperm telomere length.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Geometric mean (SD)</th>
<th>Selected percentiles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5th</td>
<td>50th</td>
</tr>
<tr>
<td>Metabolites (μg/g CR) (n = 492)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorene metabolite 2-OHFlu</td>
<td>0.413 (1.838)</td>
<td>0.149</td>
</tr>
<tr>
<td>Pyrene metabolite 1-OHPyr</td>
<td>0.020 (4.065)</td>
<td>0.003</td>
</tr>
<tr>
<td>Naphthalene metabolites 1-OHNap</td>
<td>0.068 (7.195)</td>
<td>0.003</td>
</tr>
<tr>
<td>Phenanthrene metabolites 2-OHPhe</td>
<td>0.650 (2.099)</td>
<td>0.216</td>
</tr>
<tr>
<td>Sperm apoptosis (n = 628)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annexin V+/PI− (%)</td>
<td>18.084 (1.472)</td>
<td>0.951</td>
</tr>
<tr>
<td>Sperm telomere length (n = 444)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Of the 666 subjects, semen parameters were measured for 656 participants, while urinary PAH metabolites, STL and sperm apoptosis were successfully measured for 492, 444 and 628 participants, respectively.


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**Table 3.** Unadjusted regression coefficients of urinary PAH metabolites and sperm telomere length using a linear regression analysis.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>β-Coefficient</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm telomere length</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorene metabolite 2-OHFlu</td>
<td>−0.037</td>
<td>−0.125 to 0.051</td>
<td>0.408</td>
</tr>
<tr>
<td>Pyrene metabolite 1-OHPyr</td>
<td>−0.385</td>
<td>−0.749 to −0.021</td>
<td>0.038*</td>
</tr>
<tr>
<td>Naphthalene metabolites 1-OHNap</td>
<td>−0.079</td>
<td>−0.146 to −0.011</td>
<td>0.023*</td>
</tr>
<tr>
<td>Phenanthrene metabolites 1-OHPhe</td>
<td>−0.168</td>
<td>−0.395 to 0.059</td>
<td>0.147</td>
</tr>
<tr>
<td>2-OHPhe</td>
<td>−0.002</td>
<td>−0.197 to 0.193</td>
<td>0.985</td>
</tr>
<tr>
<td>3-OHPhe</td>
<td>−0.070</td>
<td>−0.238 to 0.099</td>
<td>0.417</td>
</tr>
<tr>
<td>4-OHPhe</td>
<td>−0.502</td>
<td>−1.155 to 0.151</td>
<td>0.131</td>
</tr>
<tr>
<td>∑ PAH metabolites*</td>
<td>−0.011</td>
<td>−0.026 to 0.005</td>
<td>0.187</td>
</tr>
</tbody>
</table>

* p < 0.05.

▲ ∑ PAH metabolites: combined sum of the eight examined PAH metabolites.
Table 4
Adjusted estimates (95% CIs) of urinary 1-OHPyr and 1-OHNap and sperm telomere length using a multiple linear regression analysis.

<table>
<thead>
<tr>
<th>Sperm telomere length</th>
<th>β-coefficient (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-OHPyr</td>
<td>−0.414 (−0.774, −0.054)</td>
<td>0.024*</td>
</tr>
<tr>
<td>1-OHNap</td>
<td>−0.081 (−0.149, −0.014)</td>
<td>0.018*</td>
</tr>
</tbody>
</table>

* p < 0.05.

PAHs are a large family containing the most widespread environmental contaminants in the world. Environmental exposure is usually assessed by detecting the presence of several PAHs, including B[a]P, in air, dust, food, water or other products. Exposure to PAHs generally occurs as mixtures that can be absorbed through the skin, respiratory tract, and gastrointestinal tract. Although it is difficult to examine external exposure levels via multiple routes, measuring of PAH metabolites in biological samples provides a means to assess internal exposure and can be utilized as a sensitive biomarker of exposure (Boogaard, 2008). Because PAHs are rapidly metabolized, PAHs concentrations in serum are considerably lower than those in urine. Therefore, biomonitoring of the levels of urinary PAH metabolites is an important approach to measure human exposure and the burden of PAHs in the body (Jeng et al., 2013). Some studies have reported that the consistent detection of urinary PAH metabolites not only in occupationally exposed workers but also in general population who usually exposed to low environmental PAH levels (Tuntawiroon et al., 2007). Considering the limited detection rates of some PAH metabolites and previous studies of prevalent PAHs exposure in China (Han et al., 2011; Xia et al., 2009), we measured eight urinary PAH metabolites, including 2-OHflu, 1-OHPyr, 1-OHNap, 2-OHNap, 1-OHPhe, 2-OHPhe, 3-OHPhe and 4-OHPhe. Among various urinary PAH metabolites, 1-OHPyr is widely used as a biological indicator of internal doses of mixed PAHs exposure, and many studies have confirmed that a correlation exists between 1-OHPyr and the toxic effects of environmental exposure to PAHs (Bouchard et al., 2002; Marie et al., 2009). Our data indicate that the levels of 1-OHPyr and 1-OHNap are correlated with telomeric DNA damage in sperm, further confirming that PAH metabolites are favorable biomarkers that can be used to assess the internal doses of activated PAHs.

None of the eight urinary PAH metabolites detected in present college students was correlated with semen quality or apoptosis. We suggest that these results are attributed to the lower levels of PAHs exposure in this study. For example, the median concentration of 1-OHPyr dose-dependently shortened STL and decreased telomerase expression in germ cells.

### 3.1.5. PAH exposure and sperm apoptosis

Using a linear regression model, we analyzed the relationships between urinary PAH metabolites and markers of cell apoptosis, including Annexin V+/PI−, Annexin V− and Annexin V+/PI+. No statistically significant associations were detected between the urinary PAH metabolites and sperm apoptosis (Supplemental Table 2).

### 3.2. Animal experiments

The present study confirmed that treatment with B[a]P at the doses of 1 and 5 mg/kg did not decrease sperm parameters, in agreement with the previous results (Chen et al., 2011). However, the sperm number and concentration were significantly decreased at the doses of 10 mg/kg compared with the control (p = 0.001) (data not shown). A dose-dependent decrease in telomere length was detected in the sperm samples collected from the epididymides of the B[a]P-treated rats, and significant decreases were observed in the samples from the rats administered a 5 mg/kg or higher dose compared with those observed in the control rats’ samples (Fig. 1A). In addition, Western blot analysis showed that the TERT expression in germ cells was significantly decreased in a dose-dependent manner with B[a]P administration (Fig. 1B). The results of the in vivo animal experiments thus confirmed that at a dose that did not result in significant changes in sperm parameters, B[a]P dose-dependently shortened STL and decreased telomerase expression in germ cells.

#### 4. Discussion

PAHs are a large family containing the most widespread environmental contaminants in the world. Environmental exposure is usually assessed by detecting the presence of several PAHs, including B[a]P, in air, dust, food, water or other products. Exposure to PAHs generally occurs as mixtures that can be absorbed through the skin, respiratory tract, and gastrointestinal tract. Although it is difficult to examine external exposure levels via multiple routes, measuring of PAH metabolites in biological samples provides a means to assess internal exposure and can be utilized as a sensitive biomarker of exposure (Boogaard, 2008). Because PAHs are rapidly metabolized, PAHs concentrations in serum are considerably lower than those in urine. Therefore, biomonitoring of the levels of urinary PAH metabolites is an important approach to measure human exposure and the burden of PAHs in the body (Jeng et al., 2013). Some studies have reported that the consistent detection of urinary PAH metabolites not only in occupationally exposed workers but also in general population who usually exposed to low environmental PAH levels (Tuntawiroon et al., 2007). Considering the limited detection rates of some PAH metabolites and previous studies of prevalent PAHs exposure in China (Han et al., 2011; Xia et al., 2009), we measured eight urinary PAH metabolites, including 2-OHflu, 1-OHPyr, 1-OHNap, 2-OHNap, 1-OHPhe, 2-OHPhe, 3-OHPhe and 4-OHPhe. Among various urinary PAH metabolites, 1-OHPyr is widely used as a biological indicator of internal doses of mixed PAHs exposure, and many studies have confirmed that a correlation exists between 1-OHPyr and the toxic effects of environmental exposure to PAHs (Bouchard et al., 2002; Marie et al., 2009). Our data indicate that the levels of 1-OHPyr and 1-OHNap are correlated with telomeric DNA damage in sperm, further confirming that PAH metabolites are favorable biomarkers that can be used to assess the internal doses of activated PAHs.

None of the eight urinary PAH metabolites detected in present college students was correlated with semen quality or apoptosis. We suggest that these results are attributed to the lower levels of PAHs exposure in this study. For example, the median concentration of 1-
OHPy was 0.021 μg/g CR in our study, which is much lower than those measured in other population, Jeng et al. (2013) reported that the level of 1-OHPyr was 12.8 μg/g CR in coke oven workers compared to 0.3 μg/g CR in controls. In the general population, 1-OHPyr metabolite level was 0.046 μg/g CR in the report from America (Li et al., 2008) and 1.11 μg/g CR from Nanjing in eastern China (Xia et al., 2009). In our previous study of a general population aged 20–40 years old in Chongqing in 2007, the PAH levels were also higher than those in the current study (1-OHPyr, 0.66 μg/g CR) (Han et al., 2011). We speculate that these differences among results are due to the lower pollution levels in Chongqing as a result of strengthened environmental management, which has decreased environmental pollutant levels and has improved living standards. In addition, they may be attributed to differences in physical conditions, education levels and other factors.

However, negative relationships between STL and some PAH metabolites were observed in the present study even at the lower level of PAHs exposure. Telomeres play a critical role in meiosis, with short telomeres resulting in meiotic arrest, segregation abnormalities and chromosomal disjunction, leading to an increased incidence of aneuploid germ cells and apoptosis (Thilagavathi et al., 2013b). Critically short telomeres in sperm have been shown to be predictive of an increased risk of male infertility. Studies in India (Thilagavathi et al., 2013a) and China (Shuyuan et al., 2015) have analyzed STL in men with idiopathic infertility and have found that the STL was relatively shorter in infertile men than in controls (p < 0.005). These findings suggest that short STL is a possible contributor to male infertility. Therefore, we propose that shorter telomere length in sperm chromosomes impairs spermatogenesis and eventually alters fertility. It has been previously demonstrated that PAHs cause reproductive toxicity, however, whether shortened STL in the college students exposed to low levels of PAHs may affect male fertility warrants further study.

Rapidly accumulating evidence indicates that telomere length may be affected by environmental chemicals that have been frequently associated with chronic diseases (Hou et al., 2012; Hoxha et al., 2009; Li et al., 2011). With regard to PAHs exposure, two human studies have reported that leukocyte telomere length is shorter in Chinese (Bin et al., 2010) and Italian coke-oven workers (Pavanello et al., 2010). It is also well known that PAHs are present in cigarette smoking. A study of women has shown that smoking results in shorter telomere length in white blood cells (Valdes et al., 2005). Thus, telomeric DNA may be one of the important targets of environmental pollutants. Here, we found the negative associations between PAH metabolites and STL in population study, further confirmed that shortened STL induced by PAH compound in animal experiments. These findings suggest for the first time that sperm telomeric DNA also might be a sensitive target of PAHs’ genotoxicity. There are two possible mechanisms of telomere shortening induced by PAHs: on the one hand, PAHs form, through a series of metabolic process, active metabolites that can react with DNA to produce DNA adducts. PAH metabolites may also trigger an elevation in reactive oxygen species (ROS). The ROS produced by normal cellular metabolism and in response to exogenous genotoxic insults can shorten telomeres by oxidizing the guanine-rich telomeric DNA and triggering a DNA damage response, leading to the excision of telomere repeats (Kalmbach et al., 2013). On the other hand, telomeres in germ cells differ from those in somatic cells, in which telomere gradually shorten with growth and age, whereas longer STL is maintained to protect the integrity and stability of sperm chromosomes (Thilagavathi et al., 2013b). The mechanism of telomere maintenance relies on a complex system including many components, such as telomerase and telomere-associated proteins, and the system can be disturbed by a variety of environmental factors. These disturbances can lead to abnormal sperm telomere structures.

Telomerase is a ribonucleoprotein that extends telomeres at the ends of eukaryotic chromosomes, which contains two essential components: the telomerase reverse transcriptase (TERT), and the telomerase RNA (Counter, 1996). TERT is a major determinant of telomerase activity and telomere length maintenance (Bodnar et al., 1998). During spermatogenesis, it is necessary for telomeric DNA to be consistently maintained (Fujisawa et al., 1998). Telomerase, which is involved in the elongation of telomeres, thus enables germ cells to divide continuously. Unlike somatic cells, telomerase activity is known to decrease as germ cell differentiation increases, and mature spermatids do not exhibit telomerase activity (Eisenhauer et al., 1997). Although the roles of telomerase in spermatogenesis and/or the fertility potential of sperm are unknown, a large body of evidence indicates that telomerase activity and human TERT (hTERT) expression are lower in infertile males, suggesting a close association between telomerase regulation and male fertility (Fujisawa et al., 1998; Schrader et al., 2000). No studies published to date have described the relationships between PAHs and STL or telomerase. One recent study has shown that polychlorinated biphenyls (PCBs) significantly reduce telomerase activity, resulting in shortened telomere lengths and the inhibition of cell proliferation (Senthilkumar et al., 2012). Therefore, exogenous harmful factors may more easily cause their damaging effects by impairing telomerase. Although we did not detect telomerase activity in human germ cells because of population-based study limitations, the results of our in vivo animal experiments indicated that B[a]P induced a decrease in telomerase expression in germ cells and STL.

Our study has several strengths. First, it is based on a healthy and relatively large population and not on infertile men. Second, our environmental exposure surveillance methods covered the full spermatogenesis cycle, and internal exposure to PAHs was evaluated instead of external exposure. Third, we used high-performance liquid chromatography with an electrospray triple-quadrupole tandem mass spectrometry system, which is a highly advanced method, to detect urinary PAH metabolites. We also recognize that there are several limitations to our study. First, results obtained from evaluation of a population with such a narrow age range may not be representative of other age groups. Second, due to the limited detection rates of B[a]P metabolites, we did not include some hydroxylated B[a]P metabolites in the epidemiologic study to make a better link with the results of the in vivo animal study. Third, due to the population-based study limitations, we could not measure telomerase activity in human germ cells, thus further in-depth in vivo and in vitro studies will be needed to analyze the change of telomerase activity and to explore the potential underlying mechanisms contributing to the influence of PAHs on shortened STL.

5. Conclusion

Our study is the first to demonstrate that urinary levels of PAH metabolites, including 1-OHNap and 1-OHPyr were negatively associated with human STL, and that rats exposed to B[a]P may cause telomere dysfunction of germ cells, including shortened STL and decreased telomerase expression, even at doses that do not cause alterations in sperm parameters. Further studies should be performed to obtain more information regarding how the reduced STL affects male infertility.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

We thank all of the fieldworkers for their help and the interviewees for their cooperation. This work was supported by the National Natural Science Foundation of China (grant number 81130051) and the National Scientific and Technological Support Program (grant number 2013BAI12B02).
Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.envint.2016.08.001.

References


