Polycyclic aromatic hydrocarbons (PAHs) are a large family of environmental pollutants generated from the incomplete combustion of coal, wood, oil, gas, garbage, or other organic substances. PAH exposure is a major public health concern worldwide, because the active metabolites of PAHs are believed to act as mutagens and carcinogens and because PAHs are associated with an increased risk for developing many types of cancers, including lung, prostate, skin, lymphatic, and hematopoietic malignancies [Armstrong et al. 2004; Agency for Toxic Substances and Disease Registry (ATSDR) 1995; Rybicki et al. 2008].

Accumulating evidence has also suggested that PAH exposure has harmful effects on reproductive health. Early studies demonstrate that intraperitoneal injection of benzo(a)pyrene into adult rats resulted in the atrophy of seminiferous tubules and a lack of spermatids and spermatozoa (Payne 1958). In utero exposure to benzo(a)pyrene (10 mg/kg body weight) markedly impairs the fertility of F1 male mice (MacKenzie and Angevine 1981). Moreover, subacute exposure of male rats to inhaled benzo(a)pyrene leads to reduced testosterone concentrations and impaired epididymal function (Inyang et al. 2003; Ramesh et al. 2008). More recent reports have also suggested that certain PAHs or their metabolites can interact with the estrogen receptor (ER) and initiate ER signaling pathways in vitro and in vivo (Kummera et al. 2008; Vinggaard et al. 2000).

Similar effects for PAHs on the male reproductive system have been reported among infertility clinic clients or occupational populations. For example, a recent study (Xia et al. 2009) reported that exposure to PAHs at environmental levels is associated with an increased risk of male idiopathic infertility. Hsu et al. (2006) reported that the ambient PAH levels of occupationally exposed individuals are associated with decreased spermatozoa quality. However, the biological effects of PAHs on the reproductive system have not been confirmed in the general population.

Our study was designed to investigate the relationships between PAH exposure and male reproductive function in a general population in the city of Chongqing, China. Chongqing is one of the largest cities with heavy industries along the Yangtze River. In our earlier study, we found that PAHs were some of the most ubiquitous environmental contaminants in the Chongqing of southwestern China and that the levels of PAHs in this city were higher than those in other cities (Guo et al. 2006; Shu et al. 2002; Tian et al. 2003). In another study, Li et al. (2009) found that 61.1% of male subjects from the general population in Chongqing had one or more semen parameters below those recommended by the World Health Organization (WHO 1999). Based on these observations, we employed the hydroxylated metabolites of PAH present in human urine samples, including 2-hydroxynaphthalene (2-OHNa), 9-hydroxypyrenanthrene (9-OHP), 2-hydroxyfluorene (2-OHFlu), and 1-hydroxypyrene (1-OHP) as biomarkers of PAH exposures, and we evaluated the potential association between exposures to PAH and semen quality and sperm genetic integrity.
A questionnaire, physical examination, and semen collection were carried out at the reproductive unit at Chongqing Family Planning Research Institute. All the men were required to be permanent residents of the Chongqing area, 20–40 years of age, and without reproductive or urological diseases or occupational exposure to PAHs (e.g., road paving, coke oven work, aluminum industry, and bitumen manufacturing). The participants were instructed to abstain from ejaculation for 2–7 days before producing the semen samples. Written informed consent was obtained from all participants. The study proposal was reviewed and approved by the Ethical Committee of the Third Military Medical University.

Of the 1,346 subjects included in our semen quality study during the period from June to December 2007, we only included those who participated during the winter (i.e., December) in the present study (n = 232), because the winter weather may have a slight impact on semen quality. Levine et al. (1990) noted that the level of PAH pollution is relatively heavy and stable during this time [Chongqing Environmental Protection Bureau (CEPB) 2007]. There were no statistical differences between the subjects included in this study and the rest of the study population with regard to demographic characteristics and socioeconomic status.

**PAH metabolites analysis.** Urine specimens and semen samples were collected on the same day. Urine samples were stored at −20°C and kept away from light until analysis. Four hydroxylated PAH metabolites (2-OHNa, 9-OHPh, 2-OHFlu, and 1-OHP) in the urine samples were analyzed by an experienced analytical chemistry technician in a blind fashion.

The details of the analytical procedure have been described previously (Wang et al. 2005). In brief, urine samples (8.0 mL) were combined with hydrochloric acid (HCl) to adjust the pH to 5.0, followed by hydrolysis with β-glucuronidase and arylsulfatase (HP-2; Sigma-Aldrich, Inc., St Louis, MO, USA) in sodium acetate buffer. The mixture was shaken overnight at 37°C. After centrifugation, the supernatant was lyophilized to dryness. The residue was dissolved in 1 mL of 0.1 M sodium phosphate buffer and separated by solid-phase extraction (SPE) cartridges (500 mg/mL; Supelco, Inc., Bellefonte, PA, USA) at a flow rate of < 1 mL/min. The extracts were concentrated under a stream of nitrogen gas, then analyzed by high-performance liquid chromatography with a fluorescence detector (HPLC-FD, Agilent 1100 series and Agilent Technologies 1200 series; Agilent, Inc., Palo Alto, CA, USA). Creatinine, which was used to adjust PAH concentrations, was measured in all samples by an automated chemistry analyzer (Shimazu CL-8000; Shimazu, Inc., Tokyo, Japan).

Samples containing creatinine concentrations > 3.0 or < 0.3 g/L were excluded, as these sample concentrations were too high or too low to get valid results (Xia et al. 2009). The lower limit of detection was 0.12 µg/L for 1-OHP, 0.37 µg/L for 2-OHNa, 0.64 µg/L for 9-OHPh, and 0.68 µg/L for 2-OHFlu. The intraday variations ranged from 1.92% for 2-OHNa to 3.68% for 1-OHP, and the interday variations ranged from 4.43% for 1-OHP to 9.87% for 2-OHFlu. The SPE recoveries ranged from 79.8% to 106.3%.

**Semen analysis.** Semen samples were donated by masturbation after a self-reported abstinence period of 2–7 days. Samples were allowed to liquefy by incubation at 37°C in a water bath; almost all samples were liquefied within 1 hr. We used 1-milliliter samples to assess apoptosis and seminal parameters; the rest of the sample was kept for other semen parameters.

**Annexin V assay.** One of the earliest apoptotic events occurs when the membrane phospholipid phosphatidylserine (PS) translocates to the outer leaflet of the plasma membrane (Vermes et al. 1995). The membrane PS translocation of sperm was determined by Annexin V staining in combination with the propidium iodide (PI), which stains dead cells, according to the manufacturer’s instructions (Bender MedSystems, Vienna, Austria). Briefly, 0.5–M semen samples were washed with phosphate-buffered saline (PBS), and the sperm were then resuspended in the binding buffer to obtain a cell density of approximately 5 × 10^6. A 50-µL semen specimen plus 5 µL Annexin V–FITC were added to 145 µL buffer, and the mixture was then incubated at room temperature for 10–15 min. Samples were then washed once with buffer and resuspended in 200 µL buffer containing 1 µg/mL of PI. Samples were immediately analyzed by flow cytometry (Becton Dickinson, San José, CA, USA). All tests were run in duplicate. Sperm were classified as Annexin V–/PI– spermatozoa (living cells without PS translocations), Annexin V+/PI– spermatozoa (living cells with PS translocations), or PI– spermatozoa (necrotic cells), and the results were expressed as the percentage of total sperm.

**Comet assay.** The DNA integrity of individual spermatozoa was determined using the alkaline single-cell gel electrophoresis assay (i.e., comet assay). The procedure was based on existing methods (Irvine et al. 2000) with slight modifications. Briefly, 10-µL sperm samples were embedded in low melting point agarose (Sigma-Aldrich) and added to a slide covered with normal agarose. Slides were then immersed in cold lysis buffer solution [2.5 M sodium chloride (NaCl), 100 mM EDTA, 10 mM Tris (hydroxymethyl) aminomethane hydrochloride, 10% dimethylsulfoxide (DMSO), and 1% Triton X-100, pH 10.0] for 1 hr to dissolve the membranes and break down the protein matrices. After lysis, slides were incubated overnight at 37°C in a solution of proteinase K (100 mg/mL; proteinase K in 2.5 M NaCl, 100 mM EDTA, 10% DMSO, pH 7.4). Slides were then transferred to an electrophoretic system with alkaline buffer (300 mM sodium hydroxide, 1 mM EDTA, pH 12.0) for 1 hr to allow DNA unwinding. The slides were washed twice with neutralization buffer, followed by staining with 20 µg/mL ethidium bromide, and observed under a fluorescence microscope (Eclipse E2000-S; Nikon, Inc., Tokyo, Japan). For each sample, two duplicate slides were prepared, and 100 randomly selected cells were scored for each slide. The percentage of tail DNA (tail%), tail length, and the tail distributed moment (TDM) were evaluated with the Comet Assay Software Project Lab (CaspLab 2004) image analysis system.

**Statistical analysis.** Bivariate associations between each of the metabolites and each of the semen parameters were evaluated by Spearman correlation coefficient analyses. According to the previously reported method (Duty et al. 2003a), semen parameters were dichotomized based on the WHO reference values for sperm number (≥ 40 × 10^6), sperm motility (grade A + B ≥ 50% or grade A ≥ 25%), and normal morphology (≥ 15%) (Luben et al. 2007). The men with all three semen parameters greater than or equal to the reference values were defined as...
the comparison group; a man could contribute data to any or all of the below-referenced value groups. The relationships between the dichotomized semen parameters and categorized (tertiles and quartiles) PAH metabolite concentrations were analyzed using nonconditional logistic regression models. Linear regression analysis was also used to explore the association between continuous measures of semen quality and urinary PAH metabolites.

The associations between spermatozoa damage (comet assay and apoptosis parameters) and continuous measures of urinary PAH metabolites were analyzed using multivariate linear regression analysis. Because the distributions of PAH metabolite levels and the percentages of Annexin V+/PI− were skewed, log transformation was applied for the analyses. The percentage of Annexin V+/PI− spermatozoa, tail%, tail length, and TDM were close to normal distribution and therefore used without transformation in the analyses.

As potential confounders, age, body mass index (BMI), duration of abstinence (as continuous), smoking status (no smoking, ≤ 10 and > 10 cigarettes/day), alcohol consumption (no drinking, ≤ 120 g standard drinks/month, and > 120 g standard drinks/month), and grilled and smoked foods ingestion (grams per week) were included in the analyses. We used the change-in-estimate method (Greenland 1989) to decide which of the potential confounders to adjust for in the multivariate models. Potential confounders were included if the regression coefficient was changed by more than 10% when they were included one by one in the multivariate models. The Statistical Package for the Social Sciences (SPSS) version 13.0 (SPSS Inc. Chicago, Illinois, USA) was used for statistical analysis.

Results

Participants. All of the 232 participants were Han Chinese with an average age of 32 years, mean BMI of 22.55 kg/m², and an average abstinence period of 4.63 days (range from 2 to 7 days). The participants were asked to report their smoking and alcohol intake during the 6 months prior to sample collection. As shown in Table 1, more than half of the participants used tobacco (62.5%) and alcohol (67.7%). The Annexin V+/PI− assay was not performed on the samples from 10 men, and the sperm samples from 11 men were not archived for the comet assay because of limited semen volume (< 1.2 mL). In addition, eight semen samples were lost during the comet assay. The percentage of Annexin V−/PI− spermatozoa, tail%, tail length, and TDM were close to normal distribution and therefore used with log transformation for the analyses.

As shown in Table 3 and Figure 1, the tail% increased, on average, by 13.26% [95% confidence interval (CI), 7.97–18.51] per 1-μm increase in the log₁₀ of urinary 2-OHNa (micrograms per gram creatinine). Log₁₀-transformed 2-OHNa was also significantly associated with tail length and TDM (12.25; 95% CI, 0.01–24.52 and 7.55; 95% CI, 1.28–18.83, respectively). Additionally, a positive relationship was found between 1-OHP and tail% (5.39; 95% CI, 0.47–10.17). Log₁₀-transformed 2-OHFlu was weakly associated with tail% (5.04; 95% CI, −0.99 to 11.07, p = 0.07).

We also summed the total of four PAH metabolites (2-OHNa, 2-OHFlu, 9-OHPh, and 1-OHP) and found that total PAH associated with the increased tail% (15.96; 95% CI, 8.86–23.07). However, there were still no associations between the total PAH metabolites and semen parameters (data not shown).

Discussion

Chongqing is a heavily industrialized city and suffers from significant air pollution. Although air conditions have improved recently, air

Table 1. Characteristics of the study participants (n = 232).

| Characteristic | Value           |
|---------------|----------------|
| Age (years)   | 31.89 ± 5.53   |
| BMI (kg/m²)   | 22.55 ± 2.76   |
| Abstinence duration (days) | 4.63 ± 1.77   |
| Tobacco use (cigarettes/day) | None ≤ 10 > 10 |
| Alcohol use (grams/month) | None ≤ 120 > 120 |
| Grilled/smoked food consumption (grams/week) | 183.78 ± 137.73 |

Values are mean ± SD or no. (%).

Table 2. Distribution of PAH metabolites and sperm quality parameters.

| Characteristic | Geometric mean (µg/g creatinine) | 5th | 50th | 95th |
|---------------|----------------------------------|-----|------|------|
| PAH metabolite |                                 |     |      |      |
| 2-OHNa        | 7.72                            | 1.16| 8.77 | 20.94|
| 2-OHFlu       | 2.95                            | 1.14| 3.25 | 9.43 |
| 9-OHPh        | 1.92                            | 0.64| 2.15 | 6.57 |
| 1-OHP         | 0.66                            | 0.13| 0.64 | 2.94 |
| Apoptotic marker |                              |     |      |      |
| Annexin V−/PI− spermatozoa (%) | 67.46                   | 40.22| 73.22| 88.55|
| Annexin V+/PI− spermatozoa (%) | 6.25                     | 2.49| 7.11 | 16.58|
| PI− spermatozoa (%)                 | 17.20                | 5.75| 8.11 | 53.36|
| Comet parameter |                              |     |      |      |
| Tail% | 32.24                           | 1.67| 35.91| 61.08|
| Tail length (µm) | 77.11                       | 18.48| 90.60| 128.61|
| TDM (µm) | 29.24                           | 11.51| 30.76| 60.21|

Abbreviations: V−/PI−, living cells without PS translocation; V+/PI−, living cells with PS translocation; PI−, necrotic cells.
quality is reportedly worse, and the PAH levels are higher in December in this city (CEPB 2007). Additionally, several reports have indicated that sperm quality is subject to seasonal changes because of temperature changes, the length of daylight, or ejaculatory frequency (Carlsen et al. 2004; Chia et al. 2001; Hansen et al. 2010). The winter season in Chongqing sees mild weather with overcast conditions and average temperatures around 10°C. Our previous study of semen quality, which included several months representative of summer, autumn, and winter, also found that season significant affected most of the semen parameters (Li et al. 2009). Therefore, we chose December as our sample time, when levels of PAH pollution are heavy and steady and the climate may have a slight influence on semen quality.

PAHs are a large family of environmental pollutants, and different PAHs may have different toxicological properties (Li et al. 2008). Although 1-OHP is the most commonly used indicator of PAH exposure in many previously reported studies, it may not represent the numerous PAH metabolites. To obtain a more accurate measure of exposure to different compounds, we selected 2-OHNa, 2-OHFlu, 9-OHPh, and 1-OHP as exposure biomarkers to represent different parent compounds. Our results indicated that only 2-OHNa and 2-OHFlu, not 9-OHPh, were associated with sperm DNA damage in the study population in Chongqing.

In this study, detection rates of the four metabolites were similar to another study from Nanjing, one of the biggest cities in eastern China (Xia et al. 2009), but our rates were slightly higher than those found in an American population (Li et al. 2008). We speculate that these variations result from the increase in pollution in China due to the rapid expansion of industry and increase in automobile use. Compared with the Nanjing study, the level of urinary 2-OHNa found in our study was 1.6 times higher, the level of 2-OHFlu was comparable, and the level of 1-OHP was 1.7 times lower. Differences in PAH quantities and composition may result from the different industrial structures and the variance in geography and climate between the two cities. Although there were some differences in the absolute levels of PAH metabolites detected in our study and those of others, there was a similar trend with regard to the relative metabolite levels: 2-OHNa was the PAH metabolite with the highest concentration in all three studies, followed by 2-OHFlu and 1-OHP (Xia et al. 2009; Li et al. 2008).

Experimental studies in rats indicate that PAH exposure is negatively correlated with daily sperm production and sperm motility (Ramesh et al. 2008). Human studies in fertile populations (Xia et al. 2009) suggest a negative association between abnormal semen quality and 1-OHP levels. However, our study found no associations between urinary PAH metabolites and semen parameters. Similarly, in a study in the general population, Rubes et al. (2005) reported that PAH exposures do not change semen quality. One potential explanation for these differences may be the variations in the PAH background levels (the level of urinary 1-OHP in our study was 1.7 times lower than that of the men from the study by Xia et al. 2009). Another reason may be that our study and that by Rubes et al. (2005) were based on general populations compared with the infertile population in the study by Xia et al. (2009).

Apoptotic markers have been considered useful indicators of male fertility (Varum et al. 2007). Several studies have shown that the Annexin V–negative sperm have superior quality compared with vital Annexin V–positive sperm (Hoogendijk et al. 2009; Sion et al. 2004). PAHs may reach the epididymis and then interfere with epidyphidial function, leading to apoptosis of spermatozoa (Inyang et al. 2003; Ramesh et al. 2008). We noted that the levels of urinary PAH metabolites were correlated only with PI– cells, not Annexin V+/PI+ spermatozoa. This phenomenon may occur because mature sperm lack normal apoptotic machinery. The sperm with positive staining for Annexin V may have originated from the apoptotic process that occurs during spermatogenesis. Those sperm whose apoptosis has started in spermatogenesis may have an increased sensitivity to external damaging agents (Lachaud et al. 2004). The active metabolites of PAH may lead to defects that indirectly induce cell death in fragile and damaged mature sperm.

Our data indicated that exposure to PAHs was associated with sperm DNA damage. Consistent with an earlier study (Meeker et al. 2007), we observed that increased urinary 2-OHNa levels were associated with

Table 3. Adjusted regression coefficients (95% CI) of urinary PAH metabolites and annexin V/PI and comet assay parameters.

| Outcome | 2-OHNa (µM) | 2-OHFlu (µM) | 9-OHPh (µM) | 1-OHP (µM) | ΣPAH metabolites (µM) |
|---------|-------------|-------------|-------------|-----------|---------------------|
| Annexin V/PI parameter | | | | | |
| Annexin V+/PI– (%) | −9.59 (−15.78 to −3.41)** | −11.10 (−17.31 to −4.89)** | −7.37 (−13.04 to −1.69)** | −5.78 (−11.17 to 0.37)* | −13.02 (−21.55 to −4.50)** |
| Annexin V+/PI+ (%) | −0.02 (−0.12 to 0.08) | −0.01 (−0.11 to 0.09) | −0.01 (−0.10 to −0.08) | 0.02 (−0.07 to 0.10) | 0.03 (0.11 to 0.17) |
| PI– (%) | 7.68 (3.58 to 15.72)** | 8.91 (2.99 to 14.84)** | 6.69 (1.32 to 12.06)** | 4.56 (−0.56 to 9.68)* | 12.59 (4.15 to 21.02)** |
| Comet parameter | | | | | |
| Tail% | 13.26 (7.97 to 18.55)** | 5.04 (−0.99 to 11.07)* | 3.92 (1.9 to 5.96)* | 5.32 (0.47 to 10.17)* | 15.56 (8.68 to 23.07)** |
| Tail length | 12.25 (0.01 to 24.52)** | 6.48 (−6.30 to 19.26) | 5.23 (−5.98 to 16.43) | 2.60 (−8.05 to 13.24) | 16.56 (−0.39 to 33.52)** |
| TDM (µm) | 7.55 (1.28 to 18.83)** | 3.39 (−3.08 to 9.86) | 3.58 (−2.00 to 9.14) | 1.75 (−3.55 to 7.05) | 6.29 (−2.36 to 14.95) |

aRegression coefficients were adjusted for age, abstinence, and smoking status. cLog10-transformed. dΣPAH metabolites: combination of four PAH metabolites. **p < 0.05. *p < 0.1.

Figure 1. The crude association between the log of the urinary concentration of 2-OHNa and the comet assay parameters. (A) Tail% (r = 0.294, p = 0.00). (B) Tail length (r = 0.194, p = 0.005). (C) TDM (r = 0.204, p = 0.00).
increased tail%. Reactive metabolites of PAHs might reach the testes and epididymis and then react with sperm DNA to form adducts, causing DNA damage (Gaspari et al. 2003). Additionally, compounds resulting from the oxidation of PAHs have the ability to enter redox cycles, which increased the formation of reactive oxygen species (ROS) (Farmer et al. 2003) and thus caused sperm DNA damage (Barroso et al. 2000). Rather than using the neutral comet assay, which measures only double-strand DNA breaks (Duty et al. 2003b; Meeker et al. 2008), we used the alkaline comet assay in this study to determine the various sperm DNA damages caused by PAHs, that is, base-free sites, single- and double-stranded DNA damages (Sakkas and Alvarez 2010).

DNA damage occurring in the absence of other changes in semen quality is also possible. The presence of defective spermatozoa containing DNA damage does not affect the sperm count and morphology (Agarwal and Said 2003; Sakkas and Alvarez 2010). A growing body of evidence also supports the notion that sperm DNA damage is an objective and independent marker of sperm function. Sperm DNA damage in the male germ line is a major contributor to infertility and is linked to an increased incidence of miscarriage and the appearance of various kinds of birth defects in the offspring (Aitken et al. 2009; Fernández-Gonzalez et al. 2008; Morris et al. 2002).

Smoking is reported as a risk factor for male reproductive function and may confound the analyses for the relationships between environmental pollution and sperm quality (Calogero et al. 2009). In our study, after adjusting for smoking status, we found that the coefficients differed < 12% [see Supplemental Material, Tables 1 and 2 (doi:10.1289/ehp.1002340)]. That result seemingly indicates that cigarette smoking changed the association between environmental PAH exposures and sperm DNA damage slightly, which appears to support the findings of other studies (Gammon et al. 2002). Additionally, the time interval for spermatogenesis is about 90 days, longer than the half-life of PAH metabolites (several hours to days). Using only a single urine sample to predict metabolite concentrations over longer periods may be a potential limitation of our study. Although chronic repeated exposure to exogenous material may result in a steady-state level of PAH metabolites and thus improved accuracy in estimating exposure using urinary metabolites, the temporal variability in metabolites from environmental exposure might have led to a bias during our study. It is possible that participants may have had different exposures on different days based on their occupation, travel, or other factors. Thus, although we believe that urinary metabolites provide an initial estimate of exposure, the use of additional biomarkers (e.g., DNA adduct formation) that can accurately reflect chronic exposure should be considered in future studies.

**Conclusion**

The environmental exposure levels of some PAHs affect human spermatozoa quality and male fertility. Our study indicates that PAHs may disrupt male reproduction by damaging sperm DNA, rather than disrupting semen quality and triggering apoptosis of mature sperm. We speculate that the potential impact of exposure to environmental pollutants on human sperm DNA should be considered. Future large-scale studies should incorporate different markers and different seasons to generate a more accurate and full assessment of the adverse effects of PAH exposure on male fertility.

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