FINAL PROGRESS REPORT

PROJECT TITLE

Polycyclic Aromatic Hydrocarbons and Male Reproductive Health
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ABBREVIATIONS

PAHs	polycyclic aromatic hydrocarbons
PPE	personal protection equipment
NIOSH	National Institute for Occupational Safety and Health
1-OHP	1-hydroxypyrene
BMI	body mass index
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling
SCSA	sperm chromatin structure assay

ABSTRACT

The objective of this study was to examine whether polycyclic aromatic hydrocarbons (PAHs) from occupational exposure was associated with alterations of coke-oven workers' semen quality and sperm DNA integrity. The long term goals of the project were: i) to develop field data collection methods to monitor human occupational exposure to PAHs; ii) to evaluate the reproductive altering potential of the exposure; and iii) to ultimately distribute the information to protect workers' health and safety. The longitudinal study included repeated measurements to account for PAH exposure as it relates to their effect over the course of spermatogenesis. Features of this design included efficient control for confounding factors, accurate exposure ascertainment, and sufficient power to detect exposure related changes in reproductive indicators. A total of 85 human subjects (29 topside-oven workers for the high exposure group, 35 side-oven workers for the low exposure group, and 21 administrators and rolling-steel workers) voluntarily participated in this project. Semen quality (volume, pH, sperm concentration, morphology, motility, and vitality) was examined according to the World Health Organization guidelines. Sperm DNA integrity was examined by measuring DNA fragmentation and bulky DNA adducts. Exposure assessment was conducted to quantify PAH levels in the personal breathing zones and urinary 1-hydroxypyrene to depict PAH intake levels. Statistical analysis was conducted to assess correlations between PAH levels and semen quality, while controlling for age, smoking status, alcohol consumption, and metals. Research results show that coke-oven workers have been exposed to significant PAH levels despite the use of personal protection equipment. Exposure to PAHs at the levels detected in this study did not significantly change sperm concentration. Coke-oven workers, however, had a significant decrease in normal sperm morphology as compared with the control subjects. Topside-oven workers had a 16.6% and 22.5% reduction in mortality and vitality, respectively, as compared to the control. Regarding sperm DNA integrity, exposure to PAHs did not associate with decreased DNA fragmentation, but did associate with increased bulky DNA adduct levels. The findings of the study expand our understanding about the role of PAH exposure from coke oven emissions and male reproductive health. This information has proved useful in promoting improvement of workplace practices to protect the reproductive health of workers.

SECTION 1 FINAL PROGRESS REPORT

Significant Findings. The research project aimed to assess whether exposure to polycyclic aromatic hydrocarbons (PAHs) was associated with changes in semen quality and sperm DNA integrity of coke-oven workers. Research results showed:

- Nighty percent of the coke-oven workers remained throughout the course study.
- Semen quality did not significantly change during the cycle of spermatogenesis in men exposed to PAHs from coke oven emissions.
- Readings of semen quality endpoints in the two sampling events suggest that one semen sample is sufficient to represent the status of semen quality for assessment of insults by environmental toxins.
- The total level of 16 PAH compounds in the personal breathing zones ranged from 19,887 ng/m^3 to 41,620 ng/m^3.
- Coke-oven workers had been exposed to significant PAH levels despite the use of personal protection equipment (PPE).
- Topside-oven workers were exposed to the highest levels of PAHs followed by side-oven workers and the control.
- Exposure to PAHs at the levels detected in this study did not significantly change sperm concentration, while exposure to PAHs could impact on other semen quality endpoints:
    - Coke-oven workers had a significant decrease in normal sperm morphology as compared to the control subjects.
    - Topside-oven workers had a 16.6% and 22.5% reduction in motility and vitality, respectively, as compared to the control. However, the reduction did not reach a significant level.
- Exposure to PAHs at the levels detected in this study increased the levels of bulky DNA adducts in sperm.
- PAH compounds with high molecular weights correlated with normal morphology and motility.

Translation of Findings. The research results showed that i) the coke-oven workers were continuously exposed to significantly high concentrations of PAHs, and ii) semen quality was associated with exposure to PAHs at the levels detected in this study. Based on the research results, there exists a need to reduce workers' PAH exposure from the coke oven emissions and communicate with workers regarding environmental exposure and reproductive health. Certain recommendations have been made to plant managers:

- Examine whether the workers have used PPE properly and consistently, when they are in the coke-processing zones, and remind workers not to remove their respirators while they engage in coke processing, due to discomfort from heat or other factors.
- Consider relocating rest areas away from the coke-processing area.
- Implement rotation between high-PAH exposure workers (topside-oven) and low-PAH exposure workers (side-oven) at each coke-processing site to reduce PAH exposure.

- Communicate to workers about reproductive health effects and issues related to PAH exposure. [The PI has worked with the plant managers on developing non-technical language to communicate this to the coke-oven workers.]

It was a longitudinal study that included a sampling design, which considered the subjects' working schedule and the cycle of spermatogenesis. The study design provided an example for future epidemiologic research of reproductive health in relation to occupational exposure. The key to the success of this study was to establish trust and a collegial working relationship with the plant managers and the workers. The project identified reproductive hazards related to PAH exposure. Based on the findings of this study, the plant managers have considered re-examining worker protection procedures and workers' behavior related to PPE use. Finally, the workers have gained an interest in knowing about PAH exposure in relation to male reproductive health.

The study has thoroughly examined PAH exposure and semen quality and DNA integrity. The detection of bulky DNA lesions in sperm raised concerns about whether PAHs and/or their metabolites may enter testicular cells. Further investigation should address the questions of i) whether DNA lesions come from PAH metabolites, ii) whether the DNA lesions are repairable or can be passed along to offspring, and iii) specific polymorphisms in relation to formation and repair of DNA lesions, which could yield useful results in developing stringent intervention strategies to prevent PAH-related occupational health effects in special populations at risk

Outcomes/Impact. The activities and outcome of this project have addressed the long term goals: i) development of field data collection methods to monitor human occupational exposure to PAHs; ii) development of field data collection methods to monitor human occupational exposure to PAHs and distribution of the information to protect workers' health and safety; iii) evaluation of the reproductive altering potential for individuals exposed to PAHs; and iv) distribution of the information to protect workers' health and safety. The project closely relates to occupational safety and health regarding practice, prevention and intervention.

- Potential outcomes: Research findings suggest that coke-oven workers under the current practice in the plant have been exposed to significantly high PAH levels. PAH levels detected in this study were linked to a significant change in sperm morphology and DNA integrity.
- Intermediate outcomes: The findings of the study expand our understanding about the role of PAH exposure from coke oven emissions and male reproductive health. Research findings have been used by the plant managers to examine best practices and their safety management program to determine how to reduce workers' exposure to coke-oven emissions.
- End outcomes: At the completion of the study, there was no documented reduction in work-related exposure to coke-oven emissions, which could be attributed to the findings of this study. However, the study's findings have prompted the plant managers to consider developing strategies to reduce workers' exposure to coke-oven emissions.

SECTION 2 SCIENTIFIC REPORT

Background

Male reproductive health. Several reports have shown trends of declining male reproductive health.[1,2,13] Almost all cancer registries in the Western world have noted remarkable increases in testicular cancer incidence. Also, other reproductive dysfunctions have progressively increased.[15,16,17] Chemical exposure was found linked with these observed changes in male reproductive health and fertility.[16,18] In its research agenda, the National Institute for Occupational Safety and Health (NIOSH) underscores the importance of studying reproductive hazards of occupational exposure and recognizes reproductive health as a priority research area in need of further studies.[19]

PAHs and biomarkers for exposure assessment. PAHs constitute a group of toxic and lipophilic chemicals that are generated from incomplete combustion of organic matter and fossil fuels.[20,33] PAHs can undergo metabolic activation by phase I enzymes (members of cytochrome P450) and form diol epoxides.[22] The reactive intermediates are capable of covalently binding to DNA, potentially initiating a carcinogenic process. Also, the intermediates are subject to biotransformation by phase II enzymes [glutathione S-transferase (GST)] into metabolites that can be easily excreted from the body.[21,22] Several studies have suggested that bulky DNA adducts are a reliable biomarker for PAH exposure.[23,34,57] In addition, bulky DNA adducts are directly related to the biological changes that are causal factors for carcinogenesis and other diseases.[24] A wealth of information has shown that the levels of bulky DNA adducts correlate with PAH exposure in humans; coke-oven workers and foundry workers, particularly, represent a group of subjects highly exposed to PAHs.[9,25]

1-hydroxypyrene (1-OHP), a metabolite of pyrene in urine, has also been used as a biological marker of PAH exposure and as an internal dose of activated PAHs.[27] Elevated levels of 1-OHP have been found in coke-oven workers (before and after their shifts) and smokers (versus nonsmokers).[28] The general advantages of the urinary marker include non-invasiveness, ready accessibility, and identification of groups and/or individuals with recent exposure.[27]

PAHs and the male reproductive function. The PAH exposure responsible for male reproductive health effects is a matter of some controversy.[29,55] Also, although a wealth of evidence has shown PAH biomarkers detected in humans after PAH exposure, very few epidemiologic studies have been conducted to assess PAH biomarkers related to reproductive health. Sram et al. (1999) examined the connection between air pollution and sperm quality,[50] and Gaspari et al. (2003) studied PAH-DNA adduct levels in infertile versus fertile men.[10] Paracchini et al. (2005) detected PAH-DNA adducts in sperm due to occupational exposure.[31]

Animal studies have shown that PAHs cause negative effects on the male reproductive function and trigger increased risks for developing lung and skin cancers.[6,32,33] Benzo[a]pyrene and benzo[*b*]fluoranthene were observed to induce apoptosis in Sertolic cells *in vitro*. That in turn could affect germ cell development and maintain spermatogenesis.[6] PAH-DNA adducts were found to inhibit meiotic division during spermatogenesis in rats and could be associated with infertility.[7,35] Adult male Fisher rats,

which inhaled benzo[a]pyrene at 25 to 100 μg/m^3 for 10 days, experienced reduced sperm motility and declining plasma testosterone concentrations.[36] Currently, epidemiologic information on the link between PAH exposure and male reproductive dysfunction is still limited and inconclusive. Researchers have examined PAH exposure on plasma testosterone concentrations in young male Koreans,[37] and have studied semen quality of Czech Men exposed to seasonal air pollution.[30]

Study significance. Animal studies on the toxic effects of PAH exposure on reproductive health are available, however, epidemiologic data relevant to humans is limited. Taken together, the evidence for the effect of PAHs on reproductive health is still inconclusive. Also, their subsequent influence on sperm quality is largely unknown. This study evaluated the effect of PAH exposure on sperm quality of coke-oven workers. The results have enhanced our understanding about the relationship of PAH exposure and its adverse effects on human male reproduction. Furthermore, these findings provide data on the detection of risk levels for exposed workers. Such information is critical in establishing workplace practices and policies to reduce reproductive risks attributable to PAH exposure.

A positive association of exposure and altered reproductive capacity should trigger an alarm for plant managers and coke-oven workers leading to altered policies and practices in the interest of safety and health. This study holds promise to generate the type of hazard information that could be directly communicated to individuals as an innovative behavior change strategy for reproductive disease prevention.

Specific Aims

The long term goals of the project were: i) to develop field data collection methods to monitor human occupational exposure to PAHs; ii) to evaluate the reproductive altering potential of the exposure, ultimately, and iii) to distribute and promote the use of the information to protect workers' health and safety. The objective of this study was to examine whether PAHs from occupational exposure is associated with the alteration of coke-oven workers' semen quality and sperm DNA integrity. We proposed to conduct a longitudinal study with repeated measurements to account for PAH exposure as it relates to their effect over the course of spermatogenesis. The specific aims were to:

1. Assess sperm quality by examining total sperm count, concentration, motility, morphology, and sperm chromatin integrity of coke-oven workers and the control group.

   Hypothesis 1: The sperm quality among topside-oven and side-oven workers exposed to PAHs will significantly differ from the sperm quality of rolling-steel workers with no PAH exposure.

   Hypothesis 2: The sperm chromatin integrity among coke-oven workers exposed to PAHs will significantly differ from the sperm chromatin integrity of rolling-steel workers with no PAH exposure.

2. Determine the correlation between PAH-DNA adducts in sperm and urinary 1-OHP levels in the human subjects.

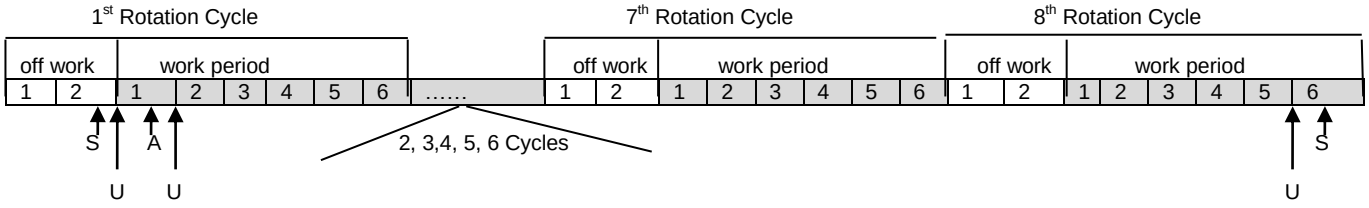
Hypothesis 3: Bulky DNA adducts in sperm of the coke-oven workers (topside and side-oven) will positively correlate with urinary 1-OHP levels.

Research Design and Methods

The proposed investigation was a longitudinal study on the effects of PAH exposure among coke-oven workers.[56] Sixty-four coke-oven workers (29 topside-oven workers and 35 side-oven workers as an exposed group) and 21 rolling-steel workers and administrative staff as a comparison group) were randomly selected from the complete roster of a steel company in south central Taiwan. Selected human subjects met the eligibility criteria described herein.

The selection of the sampling scheme was based on i) the regular schedule of the coke-oven workers who work for 6 days straight, then take 2 days off; ii) the cycle of spermatogenesis (65-75 days), which covers approximately 8 rotation cycles of coke-oven workers [8* (2 rest days + 6 work days) = 64 days]. Below is the detailed sampling scheme (Figure 1). The personal breathing-zone air samples were collected at two different times: during the 1st work day of the 1st rotation cycle when they returned from work after two days off and at the 6th work day of the 8th rotation cycle. The sperm samples were collected in the evening of the 2nd rest day of the 1st rotation cycle and in the evening of the 6th work day (end-of-shift) of the 8th rotation cycle. The four urine spot samples were collected in the morning (pre-shift) and evening of the 1st work day of the 1st rotation cycle and in the morning and evening (end-of-shift) of the 6th work day of the 8th rotation cycle. Features of this design include control for confounding factors, the accurate ascertainment of exposure, and sufficient power to detect changes in biomarker results from PAH exposure. Main confounders to be considered are: age, years of employment, alcohol consumption, metals, ambient temperature, and other PAH exposure.

Figure 1. Study Sampling Scheme*



*S: sperm; A: air; U: urine

Setting and Participants

Setting: A steel plant in southern Taiwan was selected for the study. In this steel plant, increased concentrations of PAHs had been found in several work areas, particularly near coke ovens and blast furnaces.[45,58] A total of 250 male coke-oven workers had been employed for at least one year. Eighty-four of them were topside-oven workers and 166 were side-oven workers. We selected this plant because i) The coking process in the plant has remained standard for over a decade; ii) Plant management offers annual check-ups for the coke-oven workers at the clinic, which operates under an agreement with the company, and iii) We had an established a working relationship with the steel plant and clinic.[8,45]

Participants: A total of 334 human subjects were contacted to participate in screening to determine eligibility. After screening, we randomly selected coke-oven workers who were exposed to PAHs and rolling-steel workers who had no or minimal PAH exposure as a comparison group. Our previous studies showed that topside-oven workers, who work in the areas above the ovens where fumes are more concentrated, have higher PAH exposure levels (502 ± 3 ng/m^3) than those at the side-oven (63 ± 2 ng/m^3).[42,45] Thus, the participants in the exposed group were classified into two sub-groups: topside-oven workers (lidmen, tar chasers, and larry car operators) as high exposure, and side-oven workers (wharfmen, benchmen, coke side machine operators, quenchers, pushers, body repairmen, and temperature controllers) as low exposure.[45] Rolling-steel workers inside the steel company were used as a comparison group. We selected this group because i) they were all blue-collar workers; ii) the rolling-steel plant was about 1,000 feet away from the coke-oven plant. The PAH exposure in the rolling-steel plant was minimal; and iii) the rolling-steel workers likely came from the same geographic location as coke-oven workers.

Sample Size and Power for Sperm Quality Analyses

We used the maximum allowed differences and their standard deviation for these variables at the high, low, and no exposure levels for the power analysis.[43] Table 1 shows the required sample sizes needed to establish significant results for the selected variables in the study to yield a power of 0.95 or more. So, sample size of 16-20 for each variable satisfies the desired power analysis to study a meaningful effect size.

Table 1. Sampling size estimation

Variables	Max diff	Std dev	Sampling size
Sperm concentration	2.4	1.4	12
Total number of sperm	223	104	8
Morphological abnormality	.568	.19	5
Motility	.519	.24	8

Eligibility and Sample Selection

Participants were first contacted through a mailed letter introducing the study and requesting their participation in an interview. This letter was followed by a telephone call to assess their eligibility and, if eligible, to obtain their consent for a telephone interview during the week prior to specimen collection and the workers' physical examination visit. The collection of urine and semen specimens was performed in the medical clinic at a municipal hospital nearby the steel company.

Eligibility: General eligibility was based on the criteria that participants had been employed at the steel company at least one year, had personally worked in coke-oven processing, were between 25 to 50 years old, and had no reproductive dysfunction that precluded a sperm sample. The first criterion helped to assure occupational comparability, particularly with regard to prior cumulative PAH exposure. In addition, the period of PAH exposure should cover the entire process of spermatogenesis that takes about 65-75 days in the human male.[26] The second eligibility criterion enabled us to distinguish between job classifications of coke-oven workers. The age requirement helped to minimize the sampling

bias in that older men are unlikely to provide semen samples.[39] Upon workers' meeting eligibility and expressing consent, we sent them a timetable for specimen collection coordinated with the regular schedule of their physical examination. The mailing contained written guidelines related to the specimen collection. For example, abstaining from ejaculation for at least three days prior to the annual physical examination visit was a designated requirement for participation.

Interview and Health Survey

The questionnaire instrument was used to collect information pertaining to demographic information and potential confounding exposures. We conducted interviews by telephone a week before the collection of semen. Interviewers were trained before conducting the interviews by staff from the Human Studies Facility at the Kaohsiung Medical University, in Kaohsiung, Taiwan. The contents of the questionnaire instrument consisted of six categories: socio-demographics, smoking, alcohol and food consumption, health history, and employment history and working conditions, and avocational activities. Demographic information included age, education, marital status and body mass index (BMI). Questions on employment history emphasized current occupational duties, production processes, respirator usage and job classification. Information pertaining to avocational activities asked about time spent in hot environments: baths, showers, hot tubs, and saunas. Additionally, smoking and alcohol consumption were assessed. While diet history is a potential confounding factor, we particularly inquired about consumption of charbroiled food. The information in all categories covered the 90-day period before the semen sample to encompass the entire process of spermatogenesis.[30]

Exposure Assessment

Air sampling**:** We collected personal breathing-zone air samples from selected human subjects, who were randomly selected from the pool of eligible human subjects, meeting the criteria described above. The air samples were collected by battery-operated personal air sampling pumps, with an average flow rate of 2.0 l/min. PAHs were collected by XAD-2 tubes in sorbent tubes and glass fiber filters to collect the vapor phase and particulate phase, respectively (Figure 3). The personal breathing-zone air samples were collected during the 1st work day of the 1st rotation cycle when they returned to work after two days off. The average sampling time ranged from five to seven hours during the standard work schedule.[43, 45] Sixteen PAHs were detected using a gas chromatogram/quadrupole mass spectrometer. Details of the analyses followed those described in our previous study.[42, 45] The lowest detection limit of the 16 PAHs is 0.01 ppm.[45] The relative standard deviation ranged from 2.32% for chrysene to 19.2% for benzo(a)pyrene. Mean concentration of these 16 PAHs from the two days of sampling were used in calculations for this study.[45] After collecting biological samples, we stored our samples using a sealer bag in an ice chest at 4^{o}C to minimize temperature effect on the sperm number and viability.

Urine collection**.** The two urine spot samples were collected in the morning (pre-shift) and evening of the 1st work day of the 1st rotation cycle. The sampling selection could assess the fluctuation of urinary 1-OHP between the pre-shift and end-of-shift, observed in our previous study.[42,45] Average urinary 1-OHP levels from the pre-shift and end-of-shift were used for statistical analyses. Urine samples were collected in a sterilized 50 ml polypropylene cup during the annual physical examination. The workers were asked to

wash their hands prior to urine collection to avoid environmental contamination. The minimum acceptable volume of urine specimens was ~20 cc, which is sufficient for the analysis of 1-OHP. Immediately after collection, samples were stored at -80^{o}C until analysis.

Semen collection. A week prior to semen collection, we instructed the participants by phone to abstain from ejaculation for at least three days before the clinic visit. The medical clinic consisted of two examination rooms and a lobby (Figure 2). Each exam room was about 200 square feet with a 100-foot distance between each one. The space and distance provided the necessary client privacy while collecting semen. This addressed a major weakness of low participation rates in occupational and population-based studies due to privacy considerations. A volume of 10 µL of semen was held in a Makler chamber. Semen volumes and sperm concentrations were measured immediately. Within one hour of semen collection, sperm counts, motility, and morphology were analyzed. For the morphology assessment, we evaluated 300 sperms per sample. The remaining semen was aliquoted into small tubes and snap frozen on dry ice at -70^{o}C for further analysis.

Parameter Analysis Methods

The following section describes analysis methods for selected parameters of the study. We have sufficient experience in measuring PAH biomarkers and general sperm quality examination.

Specific Aim 1: To assess semen quality, DNA fragmentation and denaturation of participants

Semen quality. Conventional semen analyses included semen volume, sperm concentration, motility, morphology, and vitality according to guidelines provided by WHO.[51] Within one hour of semen collection, sperm counts, motility, and morphology were analyzed. Samples were allowed to liquefy at room temperature for not more than an hour. All laboratory tests were done in a blind fashion. For the morphology assessment, we evaluated 300 sperm per sample from air-dried Papanicolaou-stained preparations and classified them as either normal or abnormal according to strict criteria.[51]

We have assessed sperm DNA fragmentation and DNA denaturation by using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay and sperm chromatin structure assay (SCSA), respectively.[11,54] DNA fragmentation, both single and double DNA strand breaks, was detected using the TUNEL assay, which can label free 3'OH ends in genomic DNA with fluorescein-dUTP. The sperm chromatin structure assay (SCSA), a flow cytometric test, can detect sperm nuclei with increased susceptibility to denaturation, a feature that is associated with DNA damage.[52,53] Detailed procedures are described in the Methods for Other Investigators section.

Specific Aim 2: To assess PAH biomarkers in participants. We analyzed bulky DNA adducts in sperm and 1-OPH in urine. For bulky DNA adducts, we employed the ^{32}P-postlabeling method for analysis.[47,60] In brief, DNA samples (6 µg) was digested by a mixture of micrococcal endonuclease and spleen phosphodiesterase. The nuclease P1 was used for adduct enrichment. The mixture was then phosphorylated using polynucleotide kinase. The adduct levels were calculated from radioactivity counts. A detailed description is included in the Materials Available for Other Investigators section.

For urinary 1-OH-pyrene level measurement, we used HPLC with a fluorescence detector to analyze urinary 1-OHP.[8,45,58] In brief, a 10-ml urine specimen was first adjusted to pH 5.0 with 1.0 N acetic acid. Then, the sample was incubated for 24 hrs with 15 µl of ß-glucuronidase/sulfatase at 37^{o}C. A sample purification and enrichment cartridge, packed with C18 reverse-phase liquid chromatograph material, was used to extract the PAH metabolites in urine. Twenty µl of extract was injected into a column of the HPLC system with an auto-injector and a fluorescence detector. For quality control and assurance, we prepared seven different calibration solutions by serial dilutions of 1-OHP concentrations ranging from 0.39 - 290 ng/ml. The standard curves should be linear with a correlation coefficient of ≥0.99 and an intercept not significantly different from zero. The recovery of the analyte was determined in spiked urine samples at three concentrations (3.50, 10.67, and 96.67 ng/ml). Reproducibility of the analysis of 1-OHP in urine was established by a repeated analysis of three samples. The concentration of urinary 1-OHP was normalized as µg/g creatinine to control for variation in urinary output.[38] The urinary creatinine was determined spectrophotometrically at a wavelength of 520 nm.

In addition, we measured metal concentrations in seminal fluid by using flame atomic absorption spectrometry and electrothermal atomic absorption spectrometry. Considering that workers may be exposed to metals during the manufacturing processes, we assessed cadmium, lead, zinc, copper, and arsenic, which commonly occur in coke-oven emissions and have been found to have potential effects on sperm quality.[40,41] By analyzing the metal concentrations, we controlled for the effect of cofounders by using multiple regression analyses described in the statistical analysis section. A detailed description is included in the Materials Available for Other Investigators section.

Statistical analysis and data interpretation

In Specific Aim 1, we hypothesize that the sperm quality among coke-oven workers exposed to PAH will be significantly different from the sperm quality of the rolling-steel workers with minimal PAH exposure. The distribution of the sperm quality was examined for skewness to determine if a normal distribution can be assumed. After this step, the following statistical analysis was employed to test for any significant difference in sperm quality of coke-oven workers exposed to PAHs from the rolling-steel workers with minimal PAH exposure, and also to control confounders:

a) The Pearson correlation coefficient was used to characterize the linear relationship between sperm quality parameters and PAH concentration, such as the mean α_{ij} and PAH, where i= 1,...,a; j = 1,…,b; a and b represent different levels of sperm quality and PAH concentration, respectively.
b) For each response variable, the sperm volume, sperm concentrations, motility, mean α_t, differences in the high exposure, low exposure and the comparison groups were compared by the one-way ANOVA test, followed by the Scheffe's test.
c) Adjusted multiple linear regression controlling for significant confounders was estimated using the linear relationship between PAH exposure level and sperm quality. Using the mean α_t level as an example, such a model may take the form:

$$(\alpha_t) = ß + ß_{(1)}E + ß_{(2)}S + ß_{(3)}A + ß_{(4)}AL + ß_{(5)}M + ß_{(6)}T + ß_{(7)}O + e$$

where, E = exposure; S = smoking; A = age; AL = alcohol; M = Metals, T = ambient temperature, O = other PAH exposure, e =error term

The information on possible confounders, including alcohol, food consumption, and exposure to other PAH sources was obtained from the questionnaire, and metals in the seminal plasma.

In Specific Aim 2, we hypothesized that PAH biomarker levels among exposed coke-oven workers are significantly different from those in the rolling-steel workers as a comparison group. In addition, PAH biomarker levels in both groups should correlate with sperm quality. The following statistical analyses were employed to test the hypothesis:

a) For each response variable, bulky DNA adduct levels and 1-OHP levels in the high exposure, low exposure, and the comparison groups were compared using the one-way ANOVA test, followed by the Scheffe's test.
b) The Pearson correlation coefficient was used to characterize the linear relationship between PAH biomarkers and sperm quality parameters, such as bulky DNA adducts and the means α_t, and 1-OHP and the means α_{ij}.

Study limitations

Of particular concern in this study is the identification of other exposure that may alter sperm quality. Coke-oven workers may be exposed to PAHs from other sources, such as ambient air, food consumption, and smoking. To address this limitation, we inquired about human subjects' food consumption and smoking. These factors were adjusted in the statistical analysis. All subjects came from the same geographical location, where ambient air quality is the same according to continued air quality monitoring by the Taiwan Environmental Protection Administration. Thus, the effect of such extraneous exposure should be the same in both groups.

Results

Our research results addressed all specific aims of this study, with data presented accordingly.

Overall status of the project

Table 2 charts the human subjects in the cohort of the male reproductive health project. A total of 85 human subjects (29 topside oven workers for the high exposure group, 35 side-oven workers for the low exposure group, and 21 administrators and rolling-steel workers for the control) voluntarily participated in this project. A total of 89 human subjects who reported no reproductive diseases and dysfunction voluntarily donated their biological samples. Four subjects (three coke-oven workers and one control subjects) were removed from the project since their semen samples contained no sperm. All of the subjects met the health status criteria and were randomly selected from a total of 200 coke-oven workers, 100 rolling-steel workers, and 34 administrators. Retention rates for the coke-oven workers were greater than 88%, which was similar with our previous study.[42] As for the control subjects, seventy percent of rolling steel workers participated in the second sampling, while only 20% of administrative staff members took part in the second sampling.

Table 3 tabulates sample sizes corresponding to endpoints of the project. The sampling took place from May-August 2010 and November 2011-January 2012 with a final visit to the

sites in August 2012 to complete the project. Questionnaires were collected from all of the human subjects on the 1st sampling. A total of 150 semen samples were collected. However, four samples contained no sperm, which were excluded. Thus, a total of 146 semen quality samples (85 at 1st sampling + 61 at 2nd sampling) including semen volume, pH, sperm concentration, motility, morphology, and vitality were assessed. Along with semen quality analysis, DNA fragmentation for the 149 samples was analyzed. A total of 51 personal breathing zone samples were collected from non-smoking human subjects. All 16 PAH compounds were quantified. Five metals (As, Pd, Cd, Zn, and Cu) in seminal plasma and urine from the 85 human subjects on the first sampling were measured. Due to budget constraints, only 51 sperm DNA samples from the first sampling were analyzed for bulky DNA adducts.

Specific aim

Specific aim 1. Assess coke-oven workers' sperm quality by examining total sperm count, sperm concentration, motility, morphology, and sperm chromatin integrity

Table 4 indicates demographic characteristics of the coke-oven workers. The mean ages of the study population ranged from 35 to 44 years old. The educational level of the majority of the workers was high school. We did not find any significant differences in BMI and abstinence time among the three groups. No significant difference in age and BMI existed between the high exposure group and low exposure group. Greater than 55% of the human subjects never drank alcohol. 20% to 38% of the human subjects drank more than three times a week. 35% to 45% of human subjects smoked. There was a significant difference in the percentage of smokers in the PAH exposed groups as compared with the control subjects ($P = 0.042$). There was a significant difference in alcohol consumption between the PAH exposed groups and the control ($p=0.048$). Age, smoking status and alcohol consumption were controlled in the multivariate regression analysis.

Table 5 summarizes longitudinal changes in semen parameters of the human subjects in the two sampling events, which took place in the evening (end-of-shift) of the first day of the 1st rotation cycle and at the 6th work day of the 8th rotation cycle. Sperm concentration, motility, vitality, and morphology had slightly changed and indicated a stable pattern as measured in the cycle of spermatogenesis. Due to the low number of human subjects in the control group, a comparison of the measurements between the exposed groups and the control was not conducted. However, a statistical comparison of semen quality endpoints (motility, morphology, and vitality) from the high exposure and the low exposure groups was conducted. There was no significant difference in the endpoints of the two exposed groups.

Table 6 summarizes semen quality of the human subjects from the 1st sampling event. Mean sperm concentrations of the human subjects ranged from 104.8×10^6 to 122.6×10^6 /ml. 52.4% of sperm from the high exposure group were motile as compared to 69.6% of the control. Sperm with normal morphology were 13.4%, 13.9%, and 32.9% for the high exposure group, the low exposure group, and the control, respectively. Both the high exposure group and the low exposure group had a lower percentage of normal morphology than the control group ($p<0.01$). Regarding vitality of sperm, an average of 61.0%, 71.1%, and 83.8% of sperm were viable from the high exposure group, low exposure group, and control group, respectively. Regarding smoking status, there was no significant difference in semen quality endpoints between the smokers and nonsmokers in the three groups.

Table 7 shows changes by percent in conventional semen quality of the human subjects. As a comparison between the high exposure group and the control, the high exposure group had a reduction of 16.6% on motility, 20% on normal morphology, and 22.5% on vitality. The changes by percent were less than in the comparisons between the low exposure group vs. the control, and the high exposure group vs. the low exposure group.

Table 8 tabulates semen quality parameters where measurements failed to meet the WHO reference values. The high exposure group consistently had the highest percentage reduction in motility, normal morphology, and vitality as compared to the control.

Figures 4 and 5 show DNA fragmentation and denaturation, which reflect sperm chromatin integrity, were measured by the TUNLE assay and the SCSA assay, respectively. For sperm DNA fragmentation, the topside-oven workers had 39.7% DNA fragmentation in sperm as compared with 34.8 % and 30.1% in the side-oven workers and the control, respectively (Figure 4). There was no significant difference in DNA fragmentation among the three groups (p=0.059). Also, there was no significant difference in DNA fragmentation between smokers and nonsmokers. For sperm DNA denaturation, the topside-oven workers had the highest percentage of DNA denaturation (12.9%) following the side-oven workers (10.5%) and the control (9.3%) (Figure 5). There was no significant difference in DNA denaturation among the exposed groups and the control.

Specific Aim 2. Assess PAH-DNA adducts in sperm and urinary 1-OHP levels in the human subjects

Exposure assessment was conducted to assess intake levels of PAHs and metals, shown in Table 9 and Figure 6. **Table 9** tabulates PAH concentrations from the personal breathing zone samples of nonsmoking individuals to accurately reflect coke processing as the source of PAH levels. Both vapor and particulate phases of 16 compounds of PAHs were quantified. Sample sizes for the high exposure, the low exposure, and the control were 16, 20, and 15, respectively. An average of the measurements for both phases was recorded at each PAH compound concentration. The 16 PAH compounds include acenaphthene, acenaphthylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(g,h,i)perylene, benzo(k)fluoranthene, chrysene, diben(a,h)anthracene, fluoranthene, fluorene, indeno(1,2,3-cd)pyrene, naphthalene, phenanthrene, and pyrene. Total PAH concentrations in the high exposure group were 2.1 times higher than the low exposure group (p=0.001). The control group had minimal PAH exposure with 57.0 ng/m^3 total PAH concentration. The means of all targeted PAH compound concentrations in the high exposure group were significantly greater than those in the low exposure group except acenaphthene (p=0.098), anthracene (p=0.613), chrysene (p=0.252), benzo(g,h,i)perylene (p=0.067), benzo(k)fluoranthene, and dibenzo(a,h)anthracene (p=0.370). The means of all targeted PAH compound concentrations in both the high exposure and low exposure groups were significantly higher than those in the control group (p<0.0001).

Figure 6 also includes results for exposure assessment on PAH intake levels in the coke-oven workers and the control subjects by measuring 1-OHP, a PAH metabolite, in urine from the study subjects. The averages of 1-OHP levels in the urine specimens collected on the shifts before and after the work were quantified. Urinary 1-OHP levels were consistent with the PAH levels from the personal breathing zone samples, showing that topside-oven workers had the highest 1-OHP level of 12.76 µg/g creatinine followed by the low exposure

group (4.39 µg/g creatinine) and the control (0.24 µg/g creatinine); 1-OHP levels in the exposed group were significantly higher than those in the control (p=0.02). Urinary 1-OHP levels positively correlated with the levels of all 16 PAH species (p<0.0001) (Table 10). Coke-oven workers who smoke had increased urinary 1-OHP levels as compared with those who did not smoke; however, the increased urinary 1-OHP levels did not reach a significant level.

Table 11 tabulates the correlation between PAH compounds and semen quality endpoints, and DNA fragmentation, while controlling for age, alcohol consumption, and metal concentrations shown in Table 12. The sample sizes for the comparison were based on those related to PAH compounds, where a total of 51 human subjects participated in the air sampling. Several PAH compounds significantly correlated with normal morphology: Benzo(b)fluoranthene, benzo(g,h,i)pyrene, benzo(k)fluoranthene, dibenzo(a,h)anthracene, indeno(1,2,3-cd)pyrene, and naphthalene significantly correlated with motility (p=0.045, 0.045, 0.044, 0.050, 0.038, and 0.021, respectively). All 16 PAH compounds and 1-OHP did not significantly correlate with DNA fragmentation.

Figure 7 shows a pattern of bulky DNA adducts in sperm from the coke-oven workers exposed to PAHs. There were 8 spots of DNA adducts detected in sperm from the coke-oven workers. **Figure 8** shows that the high exposure group and the low exposure group had 70.45 in 10^9 nucleotides and 68.31 in 10^9 nucleotides of bulky DNA adducts in sperm, respectively, which were significantly higher than those of the control (26.01 in10^9 nucleotides, p=0.03).

Discussion

The research team gained support for the project from the plant managers, Director of Environmental Health and Safety at the plant, and the Taiwan Council of Labor Affairs. Such support and collaboration was a key to the success of the project. The personal breathing zone samples were collected in the steel plant. Figure 2 demonstrates the setup of the air samplers as attached to the workers. Biological samples were collected at a clinic in a municipal hospital near the steel plant (Figure 1). All research was carried out according to the plan stated in the proposal except for two items: sample size and control subjects. Also, the results and activities were aimed to address the long term goals stated in the proposal.

The total sample size of human subjects was 85, which was lower than the proposed number of 110. However, the sample size for each of the three studied groups was sufficient to generate over 90% power to detect a 1.5 fold difference between the mean levels in the exposed and unexposed for sperm quality parameters and PAH biomarker parameters, using a two-sided ANOVA test (= 0.05). The main reason for the lower sample size was budget. The approved budget was only sufficient to provide compensation for a limited number of individuals to participate in this project. Also, the funds had to cover analyses of conventional semen quality analysis, 16 PAH compounds from 51 personal breathing zone samples, urinary 1-OHP, and DNA chromatic integrity analyses on the semen, and DNA adducts on 51 sperm DNA samples. The School of Community and Environmental Health at Old Dominion University provided matching funds that supported a research assistant to work on this project. In addition, the Taiwan Council of Labor Affairs

provided field equipment and a technician to assist in personal breathing zone sampling without any charge. Finally, a colleague from the Kaohsiung Medical University, an affiliated institute on the project, analyzed metals at a minimal cost.

For the control subjects, we originally proposed to use rolling steel workers to serve as the control. Due to a conflict with work schedules, only a small number of those workers was available during the time that the first sampling took place. After communicating with the Director of the Environmental Health and Safety Office and plant managers at the steel plant, we recruited administrators, technicians, and security personnel instead to serve as the control. Prior to biological sample collection, we monitored PAH levels in ambient air to ensure those subjects had minimal PAH exposure. Also, the plant managers provided ongoing bio-monitoring data confirming minimal PAH exposure of the control subjects from the coke processing. Urinary 1-OHP results also confirmed that the selected control subjects had minimal PAH exposure. Thus, we were confident that the selected control subjects met the study design and criteria.

In the second round of sampling, the sample size decreased from 85 to 63. In general, the workers in the steel plant were very cooperative, since the plant manager encouraged them to participate in this study. This is evident by approximately 90% of the coke-oven workers who remained throughout the course of the study. 70% percent of the rolling steel workers returned to donate their biological samples. However, administrative staff members with a 20% return rate were less enthusiastic to take part in the second sampling. The main reasons for the low return rate include 1) the plant managers did not communicate with them regarding the second sampling; 2) the subjects knew their semen quality results after the first sampling and had less incentive for a second measurement. Since a significant number of coke oven workers, including topside oven and side-oven workers, stayed in the cohort, any trend in semen quality changes could still be examined within the cycle of spermatogenesis.

The readings of the semen quality endpoints (motility, vitality, and normal morphology) from all studied subjects as a whole did not significantly change during the cycle of spermatogenesis. Also, DNA fragmentation measured by the TUNLE assay remained stable in the two sampling events. Our results support the position that one semen sample in the spermatogenesis cycle could be representative for examining the impact of insults on semen quality and DNA damage by environmental toxin exposure. However, we observed that 40% of individuals had more than a 20% difference in DNA fragmentation. This may simply reflect the DNA repair process during spermatogenesis, whereby sperm DNA damage can be repaired in the germ cell stage, but the repair doesn't occur in condensed spermatids and mature sperm where protamine has replaced the somatic histone.

One of the strengths of this study was the accurate ascertainment of PAH exposure to the human subjects by analyzing PAH levels in the personal breathing zone areas and detecting urinary 1-OHP levels. The exposure assessment revealed that both topside-oven and side-oven workers were exposed to significantly higher PAH concentrations than the control. More than 95% of the coke oven workers reported use of PPE during working times around the ovens. However, our results suggested that, despite wearing respirators, they were still exposed to high PAH concentrations as indicated by urinary 1-OHP concentrations from the coke oven emissions, particularly the topside-oven workers. This

raised concerns about whether the workers had used PPE properly or that the PPE in use was appropriate to protect the workers from the PAH exposure.

We shared a summary of the results with the plant managers under the protection of workers' privacy and discussed how to identify underlying issues related to the findings. Plant managers identified possible contributing factors: i) workers may remove PPE from time to time during work, ii) workers did not wear PPE during their breaks, which take place close to the coke-oven processing, and iii) the break room may require better ventilation. The plant managers indicated that they would consider implementing a voluntary job rotation option and strengthening the training program on PPE use. Furthermore, they agreed to encourage workers to use PPE all the time in the coke-processing zone. In addition, the principal investigator and physician Dr. Wen-Yi Lin hosted an educational session for workers to address their questions regarding male reproductive health and environmental exposure. The purpose of this activity was to increase their awareness of how to protect their male reproductive health and safety.

PAH exposure did affect sperm concentration; although, motility and vitality were not significantly impacted. Normal morphology was the only semen quality endpoint that showed a significant difference between the exposure groups and the control. When semen quality parameters were compared with the WHO reference values, the PAH high exposure group consistently had a higher percentage of individuals who did not meet the WHO reference values. For example, 20.7 % of the high exposure group had <40% motility as compared with 4.6% of the control. Our results concisely showed that among the three studied groups, the PAH high exposure group had the lowest percentage of motility vitality, and the highest percentage of individuals who did meet one of the WHO reference values. Taken together with the results of the comparisons of semen quality endpoints and the WHO reference values, our results suggested that exposure to PAHs at the levels detected in this study may take a toll on semen quality.

Increasingly, published studies have agreed that exposure to PAHs is associated with altering human semen quality. However, inconsistencies appear in the parameters that were ultimately affected. Rubes et al. investigated the impact of PAHs on semen quality in a group of city policemen in Prague and reported that PAHs did not cause significant differences in any of the standard semen parameters except for viability of sperm.[12] Hsu et al. reported that PAHs did not affect sperm concentration and viability, but increased abnormal sperm morphology.[43] Xia et al. found that men with higher 1-OHP have lower sperm concentrations and number per ejaculation.[64]The variations in outcomes among studies may be attributed to the differences in race of the subjects and exposure levels.

DNA integrity associated with exposure to PAHs was examined by assessing DNA fragmentation and bulky DNA adducts. The percentage of DNA fragmentation in the human subjects' sperm as measured by the TUNEL assay was comparable with the finding for men living in high air pollution areas containing PAHs.[59] Also, it tracked with those working around high sulphur coal fired furnaces used in industry and for home heating.[3] However, the DNA fragmentation percentages in the present study were higher than in males from fertility clinics (5-10%) and the general population (2-5%).[4,5] The study did observe that exposure to PAHs was associated with increased DNA fragmentation. Such DNA fragmentation could occur in the absence of other changes in semen quality. Our study

showed that DNA fragmentation had a weak negative correlation with motility and vitality (p=0.08 and 0.09, respectively), and no correlation with normal morphology (p=0.53), while semen parameters, including motility, morphology, and vitality, were significantly correlated (Table 13). The presence of DNA defective sperm did not affect the sperm concentration, morphology and motility. Our results suggest that DNA fragmentation may be an independent indicator for semen quality. The findings are in accordance with certain studies[23,24] and in disagreement with others.[34,46] A growing body of evidence also supports the theory that sperm DNA damage may be an objective and independent marker of sperm function.[48,49,61]

Our study is one of a few examining the correlation between individual PAH compounds and semen quality endpoints. PAH compounds with heavier molecular weights in the range of 252-278, including benzo(b)fluoranthene, benzo(k)fluoranthene, dibenzo(a,h)anthracene, and indeno(1,2,3-cd)pyrene, were found to negatively correlate with decreased sperm morphology and/or motility. PAH species with molecular weights greater than 252 generally have higher redox activities than those with low molecular weights.[62] The higher redox activities indicated a potential for initiating the formation of reactive oxygen species (ROS), consequently leading to oxidative stress in biological systems. A total of 51 samples was used to determine the correlations, since that was the number of personal breathing zone samples collected from nonsmokers. Due to the small sample size, future studies with larger sample sizes are necessary to confirm the findings.

Urinary 1-OHP has been recommended as a reliable biomarker for assessing intake of PAHs. Urinary 1-OHP positively correlated with all 16 PAH species. The results were similar with other findings. Regarding its relationship with semen quality endpoints, only urinary 1-OHP negatively correlated with normal morphology and did not correlate with other semen quality endpoints. Urinary 1-OHP was suitable to predict biological response doses of PAHs. However, such a relationship may be insufficient to conclude that urinary 1-OHP could serve as a biomarker to assess the relationship between PAHs and semen quality.

In addition, urinary 1-OHP did not correlate with DNA fragmentation, but positively correlated with bulky DNA adducts. DNA fragmentation has been commonly used in the clinical setting to monitor genetic integrity of sperm. Urinary 1-OHP levels alone may not be sufficient to reflect multiple factors that contribute to DNA strand breakages, e.g. apoptosis or remodeling of sperm chromatin.[63] Such findings showed the limitation of the urinary metabolite to serve as a biomarker for assessing cellular and molecular changes induced by the metabolic pathways of PAHs, which are a large family of compounds with different toxicological properties.

Conclusion

In summary, our research results and project activities have met the long term goals of the project, including 1) development of field data collection methods to monitor human occupational exposure to PAHs, 2) development of field data collection methods to monitor human occupational exposure to PAHs; and 3) distribution of the information to protect workers' health and safety.

Table 2. Human subjects in the cohort of the male reproductive health project

	High exposure group (n)	Low exposure group (n)	Control (n)	Total sample (n)
Sampling I	29	35	21	85
Sampling II	27	31	9	64
Retention (%)	93	88	43*	

*70% retention rate for the rolling workers and 20% retention rate for the administrators and security staff.

Table 3. Sampling size for analysis of endpoint parameters of samples collected during the entire cohort period

	Collected samples from Sampling I & II	Measured samples
	n	n
Questionnaire	89	85
Semen quality	150	146
PAH analysis*	51	51
Urinary 1-OHP**	250	250
Sperm DNA chromatin integrity***	146	146
Bulky DNA adduct	51	51
Metals in urine****	85	85
Metals in seminal plasma	85	85

*PAH analysis included 16 compounds of PAHs: acenaphthene, acenaphthylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(g,h,i)perylene, benzo(k)fluoranthene, chrysene, diben(a,h)anthracene, fluoranthene, fluorene, indeno(1,2,3-cd)pyrene, naphthalene, phenanthrene, and pyrene.
**Two urine samples were collected from each individual.
***Sperm DNA fragmentation was measured by the TUNLE assay and SCSA assay
****Metals included As, Pb, Cd, Zn, and Cu.

Table 4. Demographic characteristics of human subjects

	High exposure group n = 29	Low exposure group n = 35	Control n = 21	p value
Age (years, mean ± SD)	43.9 ± 8.9	38.3 ± 9.4	35.6 ± 9.2	0.23
BMI (kg/m^2, mean ± SD)	22.3 ± 2.5	23.9 ± 3.5	22.7 ± 3.0	0.67
Education (%)				
Elementary school	7.2	5.1	0	
Junior high school	40.4	45.5	8.1	
High school	47.6	46.2	37.2	
College	4.8	3.2	52.3	
Post-college	0	0	2.4	
Drinking status [n(%)]				
Never drank	9 (57)	11 (55)	12 (80)	0.048[a.b]
Ever drink	7 (43)	9 (47)	3 (20)	
Current drinker	6 (38)	8 (42)	3 (20)	
Ex-drinker	1 (6)	1 (5)	0 (0)	
Smoking				
Yes (%)	45	35	28	0.049[a]
No (%)	55	65	72	
Abstinence time [n(%)]				
≤3 days	2 (12)	4 (19)	3 (18)	0.78
4-6 days	10 (62)	12 (60)	9 (59)	0.58
≥7 days	4 (26)	4 (21)	3 (23)	0.86

P<0.05 as statistically significant
[a]significant difference between the high exposed group and the control group
[b]significant difference between the low exposed group and the control group

Table 5. Semen quality parameters from semen samples collected in the two samplings within the cycle of spermatogenesis

Semen parameters	Sampling I	Sampling II	*P*
Concentration (10^6/mml)	116.4 ± 102.1	112.4 ± 101.2	0.76
Motility (%)	53.08 ± 17.8	56.6 ± 18.4	0.65
Normal morphology (%)	21.3 ± 3.6	24.3 ± 2.9	0.51
Vitality (%)	70.6 ± 20.3	74.2 ± 19.7	0.83
DNA fragmentation (%)	22.6	26.6	

Note: Sampling I took place in the evening (end-of-shift) of the first day of the 1st rotation cycle when worker returned from work after two days off. Sampling II took place in the evening (end-of-shift) of the 6th work day of the 8th rotation cycle. Semen samples (n=64) from coke-oven workers were included in the comparison.

Table 6. Semen quality and sperm DNA integrity from the samples collected during Sampling I

	High exposure group n = 29		Low exposure group n = 35		Control n = 21		p value
	Nonsmoker n = 16	Smoker n = 13	Nonsmoker n = 20	Smoker n = 15	Nonsmoker n = 15	Smokers n = 6	
Concentration (x 10^6/ml)	122.6 ± 114.4	126.2 ± 101.9	104.8 ± 77.9	102.4 ± 91.2	121.9 ± 102.7	122.9 ± 103.2	0.79
Volume (ml)	2.1 ± 1.3	2.1 ± 1.5	2.0 ± 1.1	2.1 ± 1.0	2.2 ± 1.3	2.1 ± 1.2	0.95
pH	8.4 ± 0.2	8.3 ± 0.4	8.3 ± 0.3	8.2 ± 0.3	8.4 ± 0.3	8.2 ± 0.2	0.39
Motility (%)	53.3 ± 20.5	51.4 ± 20.9	60.8 ± 24.5	57.1 ± 20.5	70.7 ± 15.7	68.5 ± 13.9	0.059
Progressive (%)	6.7	6.1	7.2	8.5	10.3	10.1	
Non-linear (%)	33.3	35.2	27.3	23.5	38.7	34.2	
Non-progressive (%)	13.3	10.1	36.3	25.1	22.7	24.2	
Immotile (%)	46.7	48.6	39.2	42.9	29.3	31.5	
Morphology							
Normal morphology (%)[e]	14.5 ± 3.4	12.2 ± 3.1	15.0 ± 3.1	12.8 ± 3.0	34.5 ± 2.6	31.2 ± 2.1	<0.01[*a,b]
Total head defects (%)	79.2 ± 7.8	79.9 ± 9.9	79.6 ± 5.5	80.4 ± 7.9	61.3 ± 5.5	64.8 ± 4.6	0.348
Total coiled tail (%)	6.3 ± 6.3	7.9 ± 6.5	5.6 ± 6.2	6.8 ± 5.6	4.2 ± 4.2	9.6 ± 6.2	0.23
Vitality (%)	61.9 ± 19.2	60.1 ± 17.8	71.0 ± 24.1	69.1 ± 20.1	84.4 ± 14.3	83.1 ± 22.1	0.078

Mean ± SD
*P<0.05
[a] Statistical significance between the high exposed group and the control
[b] Statistical significance between the low exposed group and the control
[c] Oligospermia was defined as sperm concentration <20 x 10^6/ml.
[d] Asthenospermia was defined as the percentage of motile sperm at less than 50.
[e] Strict method

Table 7. Change in conventional semen quality among three groups*

	Motility	Normal morphology	Vitality
	%	%	%
High exposure group vs. Control	16.8	20.0	22.5
Low exposure group vs. Control	7.6	19.5	13.4
High exposure group vs. Low exposure group	7.5	1.5	10.0

***Based on the endpoint measurements from the nonsmokers

Table 8. Semen quality compared with WHO reference values

	High exposure group	Low exposure group	Control
Concentration < 15 x 10^6/ml (%)	0	2.8	0
Motility < 40% (%)	20.7	17.5	4.6
Vitality < 58% (%)	24.1	12.8	13.6
Normal morphology < 4% (%)	44.8	38.9	5

Table 9. Mean concentrations of 16 species of PAHs in the personal breathing zone of human subjects

PAH compounds (ng/m^3)	High Exposure Group n = 16	Low Exposure Group n = 20	Control n = 15	P1 value	P2 value	P3 value
Acenaphthene	341.05 ± 191.96	598.53 ± 656.06	4.89 ± 2.16	<0.0001	<0.0001	0.098
Acenaphthylene	315.24 ± 218.96	193.09 ± 145.98	9.64 ± 5.67	<0.0001	<0.0001	0.003
Anthracene	353.54 ± 93.33	340.98 ± 66.58	2.31 ± 2.07	<0.0001	<0.0001	0.613
Benzo(a)anthracene	2062.98 ± 1147.33	918.42 ± 1035.69	1.98 ± 0.73	<0.0001	<0.0001	<0.0001
Benzo(a)pyrene	2114.09 ± 352.18	1517.97 ± 571.15	0.71 ± 0.93	<0.0001	<0.0001	<0.0001
Benzo(b)fluoranthene	1464.27 ± 354.40	913.00 ± 393.14	1.38 ± 0.32	<0.0001	<0.0001	<0.0001
Benzo(g,h,i)perylene	3553.93 ± 1250.74	3001.95 ± 1367.16	2.03 ± 1.50	<0.0001	<0.0001	0.067
Benzo(k)fluoranthene	436.16 ± 112.40	666.65 ± 719.16	0.71 ± 0.22	<0.0001	<0.0001	0.059
Chrysene	920.50 ± 132.16	1036.86 ± 461.60	0.91 ± 0.40	<0.0001	<0.0001	0.252
Diben(a,h)anthracene	326.72 ± 124.28	358.64 ± 108.07	0.49 ± 0.93	<0.0001	<0.0001	0.370
Fluoranthene	12336.00 ± 11444.69	2374.69 ± 2488.87	1.54 ± 0.04	<0.0001	<0.0001	<0.0001
Fluorene	5043.05 ± 2281.81	3368.96 ± 1836.26	5.11 ± 4.91	<0.0001	<0.0001	0.004
Indeno(1,2,3-cd)pyrene	746.87 ± 136.61	617.95 ± 231.16	1.36 ± 0.36	<0.0001	<0.0001	0.007
Naphthalene	47.71 ± 55.14	7.61 ± 7.22	0.46 ± 0.04	<0.0001	<0.0001	0.0006
Phenanthrene	10651.44 ± 7908.62	3182.06 ± 2356.71	22.41 ± 14.00	<0.0001	<0.0001	<0.0001
Pyrene	906.74 ± 95.09	790.23 ± 139.79	1.05 ± 0.11	<0.0001	<0.0001	0.002
Total PAHs	41620.32 ± 17697.6	19887.61 ± 1378.1	57.00 ± 18.10	<0.0001	<0.0001	<0.001

Note: An average of PAH levels from the personal breathing-zone air samples collected at two different times: in the 1st work day of 1st rotation cycle when they returned from work after two days off and at the 6th work day of the 8th rotation cycle.
Data represented as mean ± standard deviation
Significance level: $P<0.05$
P1 value between high exposure group and control group; P2 value between low exposure group and control; P3 value between high exposure group and low exposure group

Table 10. Correlations of urinary 1-OHP levels and 16 targeted PAHs in the personal breathing zone of coke oven workers

PAH compounds (ng/m^3)	Mean ± SD	Correlation[a]	
		β	*p*
Acenaphthene	496.5 ± 423.6	0.568	<0.0001
Acenaphthylene	254.2 ± 182.4	0.601	<0.0001
Anthracene	347.3 ± 79.9	0.598	<0.0001
Benzo(a)anthracene	1490.6 ± 1091.5	0.595	<0.0001
Benzo(a)pyrene	1815.9 ± 698.3	0.602	<0.0001
Benzo(b)fluoranthene	1188 ± 373.5	0.598	<0.0001
Benzo(g,h,i)perylene	3277.4 ± 1308.5	0.577	<0.0001
Benzo(k)fluoranthene	551.2 C 415.5	0.578	<.0.0001
Chrysene	978.5 ± 584.3	0.578	<.0.0001
Diben(a,h)anthracene	342.7 ± 116.1	0.579	<.0.0001
Fluoranthene	4205.5 ± 2341.4	0.629	<.0.0001
Fluorene	4205.5 ± 2085.5	0.591	<.0.0001
Indeno(1,2,3-cd)pyrene	681.5 ± 138.9	0.567	<.0.0001
Naphthalene	27.7 ± 10.5	0.479	0.0013
Phenanthrene	6916.5 ± 5132.4	0.670	<.0.0001
Pyrene	848.7 ± 118.4	0.596	<0.0001
Total PAHs	3075.3 ± 2537.5	0.623	<0.0001

[a]Correlations between 1-OHP and 16 PAH compounds; β Pearson coefficient; p values <0.05 as significant

Table 11. Correlation between PAH species, semen quality, and DNA fragmentation

PAH species and metabolites	Concentration (x 10^6/ml)		Normal morphology* (%)		Motility (%)		Vitality (%)		DNA fragmentation (%)	
	β	p	β	p	β	p	β	p	β	p
Acenaphthene	0.047	0.755	0.543	0.113	0.159	0.295	0.140	0.191	0.042	0.783
Acenaphthylene	0.016	0.916	-0.490	0.115	-0.058	0.704	0.138	0.365	0.020	0.894
Anthracene	0.011	0.938	0.503	0.014*	-0.194	0.201	0.207	0.171	0.388	0.799
Benzo(a)anthracene	-0.00	0.997	-0.501	0.003*	-0.172	0.064	-0.249	0.072	0.146	0.337
Benzo(a)pyrene	0.047	0.757	-0.516	0.002*	-0.170	0.075	-0.188	0.076	0.058	0.700
Benzo(b)fluoranthene	-0.004	0.977	-0.516	<0.001*	-0.211	0.045*	-0.229	0.053	0.089	0.560
Benzo(g,h,i)pyrene	-0.061	0.690	-0.557	0.020*	-0.215	0.045*	-0.209	0.055*	0.029	0.847
Benzo(k)fluoranthene	-0.052	0.733	-0.528	0.002*	-0.231	0.044*	-0.204	0.051*	0.039	0.798
Chrysene	0.038	0.802	0.536	0.003*	0.231	0.106	0.192	0.205	0.032	0.830
Dibenzo(a,h)anthracene	-0.018	0.903	-0.532	0.014*	-0.200	0.050	-0.199	0.091	0.034	0.824
Fluoranthene	-0.010	0.947	0.454	0.013*	-0.117	0.108	0.179	0.238	0.058	0.705
Fluorene	0.018	0.905	0.486	0.042*	-0.171	0.260	0.170	0.261	0.024	0.873
Indeno(1,2,3-cd)pyrene	-0.005	0.970	-0.524	0.002*	-0.197	0.038*	-0.202	0.101	0.076	0.615
Naphthalene	0.018	0.904	-0.276	0.024*	-0.216	0.021*	-0.270	0.072	0.001	0.989
Phenanthrene	-0.033	0.829	-0.416	0.051	0.121	0.426	0.157	0.302	0.052	0.731
Pyrene	-0.005	0.972	-0.528	0.007*	0.180	0.234	0.189	0.213	0.049	0.744
1-OHP	-0.002	0.981	-0.280	0.022*	-0.067	0.065	-0.108	0.113	0.005	0.964

*P <0.05; **0.05P<0.1
Log-transformation on all data
Controlled for age, smoking, and alcohol consumption

Table 12. Metal levels in seminal plasma and urine from coke-oven workers and the control

	High exposure group Mean ± SD	Low exposure group Mean ± SD	Control Mean ± SD
Cd (µg/l)	0.57 ± 0.24	0.55 ± 0.25	0.67 ± 0.29
Cu (µg/l)	159.34 ± 218.27	189.74 ± 234.99	152.93 ± 123.76
Pb (µg/l)	2.24 ± 0.071	7.35 ± 8.19	12.62 ± 6.34
Zn (µg/l)	175.22 ± 94.72	174.67 ± 113.71	174.67 ± 113.71
As (µg/l)	12.38 ± 7.86	9.91 ± 4.64	6.32 ± 3.45

Table 13. Correlations between semen quality and DNA fragmentation

	Concentration	Morphology	Motility	Vitality	DNA fragmentation
Concentration	1	0.175 (0.11)	0.015 (0.98)	0.077 (0.48)	0.028 (0.79)
Morphology		1	0.375 (0.0004)*	0.334 (0.002)*	-0.069 (0.53)
Motility			1	0.879 (<0.0001)*	-0.188 (0.08)
Vitality				1	-0.184 (0.09)
DNA fragmentation					1

β(p): Pearson coefficient (p value)
*****p <0.05

Figure 2. Locations for collecting biological samples. A) Kaohsiung Municipal Hsiaokang Hospital where the health examination clinic was located; B) The entrance to the health examination clinic where biological samples were collected. The entire clinic was available for the research project at 4:30 pm from Monday to Friday; C) A certified nurse at the health examination clinic took a biological sample from a coke-oven worker; D) An examination room where semen samples were analyzed within one hour after collection.

Figure 3. Personal breathing zone sampling. A) Each human subject carried two air sampling pumps attached onto their belts; B) A glass fiber filter cascade and a XAD-2 sampling tube for collecting particulate and vapor phases of PAHs, respectively, were attached onto the collar of the shirt.

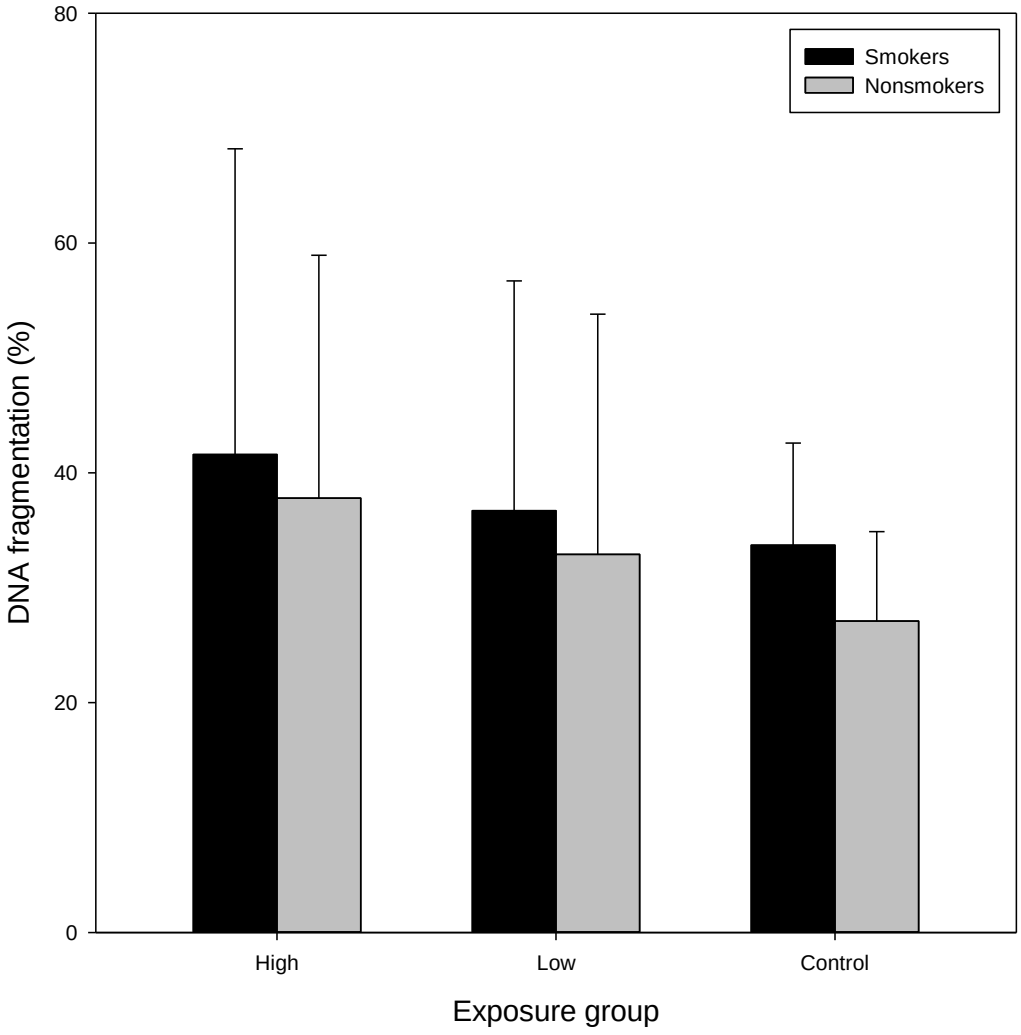


Figure 4. Percentages of DNA fragmentation for the exposure groups and the control. There was no significant difference among the three groups.

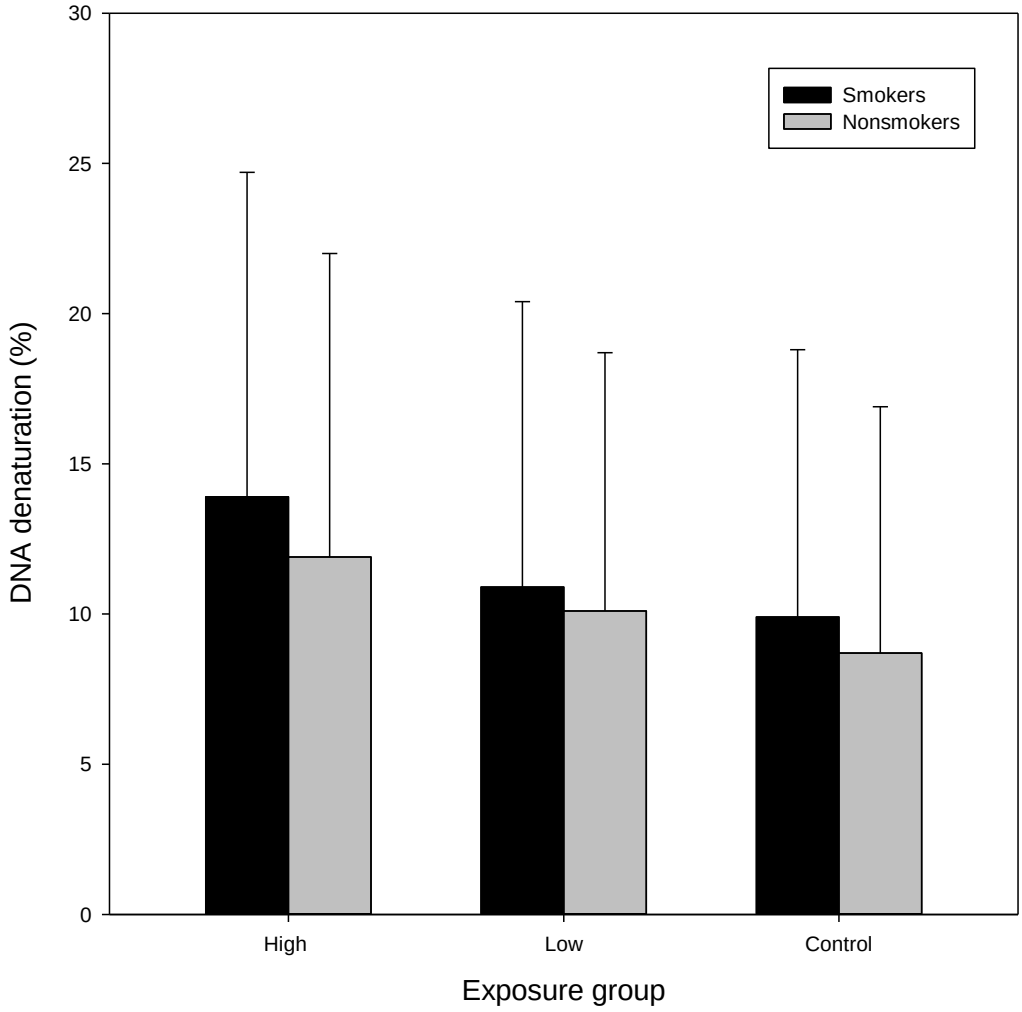


Figure 5. Percent of DNA denaturation of sperm from the exposure groups and the control. There was no significant difference among the three groups.

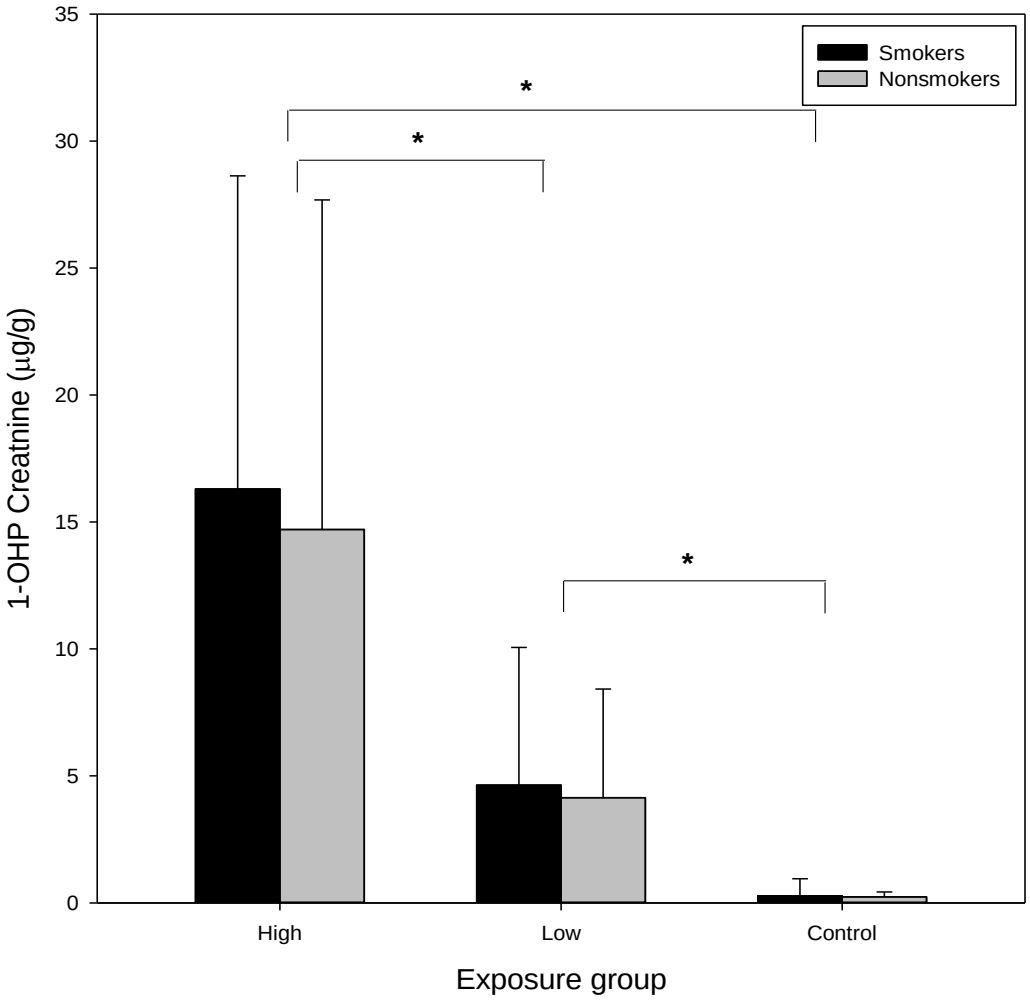


Figure 6. The levels of urinary 1-OHP from the coke oven workers and the control subjects. The data represented an average of urinary 1-OHP levels from two urine samples. There was a significant difference between the high exposure group and the control, the low exposure group and the control. $P<0.05$.

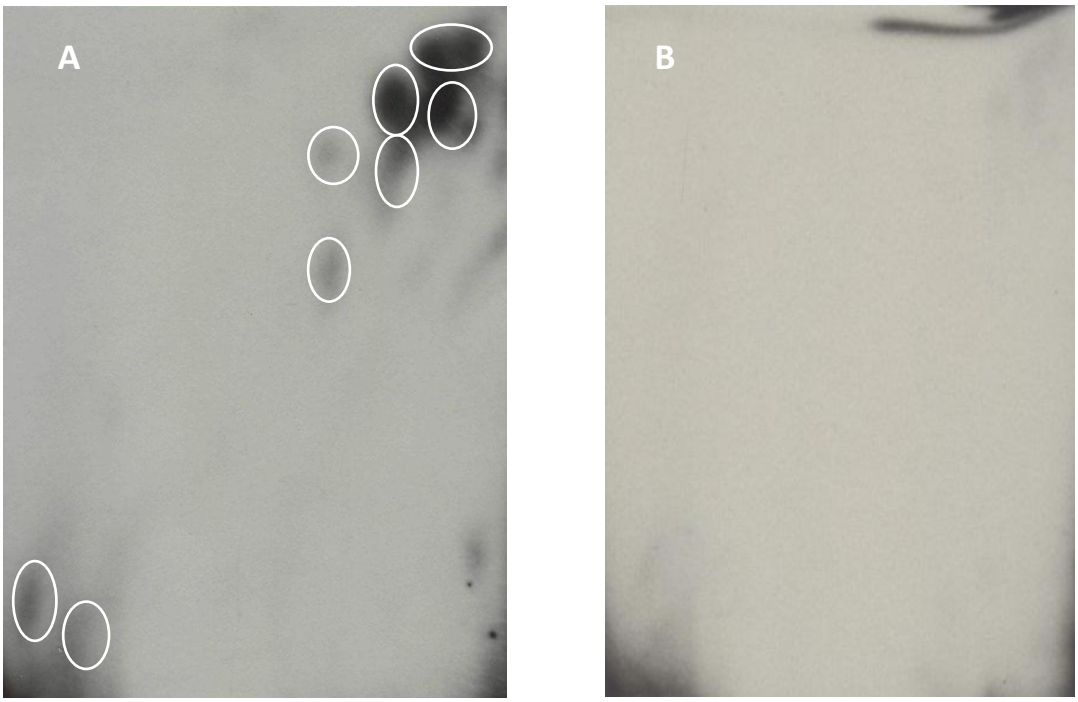


Figure 7. Thin-layer chromatograms of DNA samples analyzed by ^{32}P-postlabeling method. Patterns of bulky PAH-DNA adduct spots representative of DNA from human sperm of the coke-oven workers (A) and the control group (B). Panel A: For PAH-exposed subjects, the numbers 1-8 indicate the detected bulky PAH-DNA adducts. Panel B: For the control group, no detectable bulky PAH-DNA adducts detected.

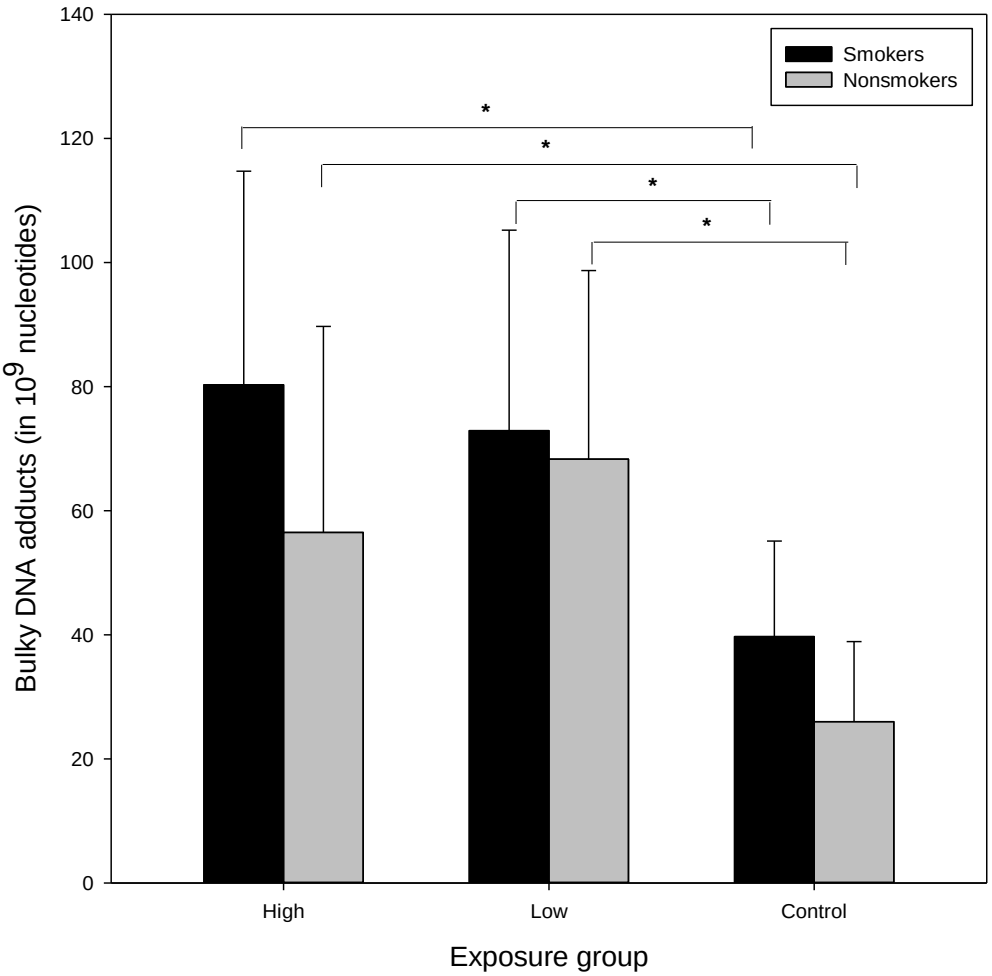


Figure 8. Bulky PAH-DNA adducts of the coke-oven workers and the control. The high exposure group had significantly higher bulky PAH-DNA adduct levels than the control for both smokers and nonsmokers. The low exposure group had significantly higher bulky PAH-DNA adduct levels than the control for both smokers and nonsmokers.

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Publications

Peer-reviewed articles

These articles are based on the results from the supported grant. NIOSH support is acknowledged in each article. In addition, two manuscripts are under review and preparation, which are not included in this report:

Jeng HA, Pan CH, Chang-Chien GP, Chao MR: [2012] 1-Hydroxypyrene as a biomarker for assessing the influence of exposure to polycyclic aromatic hydrocarbons on semen quality and sperm DNA Integrity. *Journal of Environmental Health and Sciences*, Part A.

Jeng HA, Pan CH, Chang-Chien GP, MT Wu, Diawara N, Lin WY: [2012] Polycyclic aromatic hydrocarbons from coke oven emissions alter sperm quality and DNA fragmentation of nonsmoking workers. *Journal of Hazardous Materials (in press)*

Jeng HA, Pan CH, Chao MR, Zhou G, Chiu CC, Lin WY: [2012] Semen quality and sperm DNA integrity of coke oven workers exposed to polycyclic aromatic hydrocarbons. *International Journal of Occupational Medicine and Environmental Health (revised)*

Presentations

Jeng HA, Pan CH. Polycyclic aromatic hydrocarbons and sperm quality, Society of Toxicology, the 51st Annual Meeting of the Society of Toxicology, March 11-15, 2012, San Francisco, CA

Jeng HA, Pan CH. Exposure to polycyclic aromatic hydrocarbons in relation to DNA integrity of coke-oven workers. The 23rd International Conference on Epidemiology in Occupational Health, International Commission on Occupational Health. June 18-21, 2013, Utrecht, NL (accepted)

Inclusion Enrollment Table

Total enrollment: 89

TARGED ENROLLEMNT: Number of Subjects			
Ethnic Category	**Females**	**Males**	**Total**
Hispanic or Latino	0	0	0
Not Hispanic or Latino	89	89	89
Ethnic Category: Total of All Subjects*	0	0	0
Racial Categories			
American Indian/Alaska Native	0	0	0
Asian	89	89	89
Native Hawaiian or Other Pacific Islander	0	0	0
Black or African American	0	0	0
White	0	0	0
Racial Categories: total of All Subjects*	89	89	89

*The “Ethnic Category: Total of All Subjects” must be equal to the “Racial Categories: Total of All Subjects

Inclusion of Gender and Minority Human Subjects

No subjects were excluded from the study based on gender, race, or ethnicity. We selected male workers for this study because the objective of this study was to assess male reproductive health and PAH exposure. Thus, women were not included in this study.

This study targets Asians in Taiwan where the steel plant is located. The reasons for selecting the population are stated in the proposal. Thus, ethnic minorities constituted 100% of our population group.

Inclusion of Children

The objective of this study was to assess PAH exposure and its impact on male reproductive health. Male coke-oven workers from 25 to 50 years old were selected to meet the proposed objective. Also, persons under 18 are not eligible for the study due to employment restrictions related to the inherent occupational hazards related to coke-ovens. Thus, children, defined by NIH as younger than 21 years of age, were not included in this study.

Materials Available for Other Investigators

TUNLE Assay and SCSA Assay

DNA fragmentation, both single and double DNA strand breaks, was detected using the TUNEL assay, which can label free 3'OH ends in genomic DNA with fluorescein-dUTP. A sperm pellet was obtained after semen was centrifuged at 250g for 5 min. The pellet was resuspended, washed with PBS, and spread onto slides. Then, cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate at 4°C for 2 min. A TUNEL mixture (Roche Diagnostic, Manheim, Germany) was deployed onto sperm cells according to the manufacturer's instruction. Each test included both positive and negative controls to ensure the performance of the assay. Cells in the positive control were treated with 50 µL of DNase solution, while cells in the negative control did not get treated with the TUNEL mixture. After the cells were incubated one hour at 37^{o}C, the cells were washed twice with 1% HAS in PBS. The sperm cells were visualized using an Olympus BX61 fluorescence microscope. Fluorescence detected in sperm was recorded as a positive for DNA fragmentation. At least 300 sperm cells from each sample were accounted for in this manner, and percentage of sperm with DNA fragmentation was calculated by dividing the number of fluorescent sperm by the total number of counted sperm.

We employed SCSA to measure sperm nuclear integrity. SCSA assesses flow cytogram-generated staining patterns, measuring a shift from green (native DNA) to red (denatured DNA) fluorescence in properly stained sperm chromatin. This shift is seen under conditions of stress to the sperm caused by PAH exposure. We used flow cytometry to detect green (515-530 nm band pass filter) and red (630 nm long pass filter) fluorescence in 4,000 individual sperm per sample. The extent of DNA denaturation per cell were quantified as alpha-t (α_t), expressed as a ratio or percent of the quantity of [red/(red + green)] fluorescence. Also, we used the "cells outside the main population" (COMP α_t) variable, which represents the percentage of cells containing denatured DNA. High COMP α_t values have been associated with spermatogenic disorders and infertility. To ensure reliable data collection on semen in this study, research assistants and technicians working on our previous studies participated in an external quality assurance program to assess analysis variation. The coefficient of variation between assays fluctuated from 5% to 10.2%. For the mean αT, the coefficients of variation between and within assays ranged from 5.1% to 7.5% and 1.0% to 8.4%, respectively.

Sperm DNA extraction

Sperm DNA were extracted using the referenced method with some modifications.[38] Briefly, sperm samples (15-100 × 10^6 cells) were washed with 1% HSA in PBS and centrifuged at 3000 g for 5 min. Added to the resulting pellet was 600 µl of ice-cold extraction buffer, 70 µl of 10% (w/v) sodium lauryl sulfate and 30 µl of dithiothreitol (1M). After 30 µl of proteinase K was added, the samples were incubated at 55 °C for 1 h. Then, 30 µl of RNase A (10 mg/ml) and 8 µl of RNase T1 (1 U/µl) were added. The mixture was incubated at 37 °C for 1 h and then cooled to 4 °C for 5 min. Subsequently, 1.2 ml of NaI solution and two ml of 2-propanol were added. After centrifugation at 5000 g for 5 min, the DNA pellet was washed with 1 ml of ice-cold 40% (v/v) 2-propanol. Finally, the DNA pellet was collected by centrifugation (5000 g for 5 min) and dissolved in 200 µl of 0.1 mM deferoxamine mesylate overnight. DNA concentration was measured by the absorbance method at 260 nm.

DNA Adduct Measurement

DNA adducts were analyzed by the nuclease P1-enhanced ^{32}P-postlabeling assay. DNA (10 µg) was enzymatically degraded to normal (Np) and adducted with (Xp) deoxyribonucleoside 3'-monophosphates. After treatment of the sample with nuclease P1 to convert Nps to nucleosides, adducted nucleotides (Xp) were converted to 5'-^{32}P-labeled deoxyribonucleoside 3',5'-bisphosphates (pXp) by incubation with carrier-free [5-^{32}P]ATP and polynucleotide kinase. Radioactively labeled products were purified by one-dimensional development overnight with solvent 2.3 M sodium phosphate, pH 5.7 (D1). Labeled DNA adducts retained in the lower (2.8 x 1.0 cm) part of the D1 chromatogram were contact-transferred to fresh thin-layer sheets and resolved by two-dimensional thin-layer chromatography (TLC). The DNA adducts were separated with 3.82 M lithium formate, 6.75 M urea, pH 3.35 and 0.72 M sodium phosphate, 0.45 M Tris–HCl, 7.65 M urea, and pH 8.2 in the first and second dimensions, respectively. Radioactivity of TLC fractions from individual samples was determined with the aid of an Instant Imager. The extent of covalent DNA adducts was estimated by calculating relative adduct labeling (RAL) values from sample count rates, the amount of DNA assayed (expressed as pmol DNA monomer units or DNA-P), and the specific activity of [γ-^{32}P]ATP

$$RAL = \frac{\text{DNA adduct(s) [cpm]}}{\text{DNA-P [pmol]} \times \text{Spec. act.}_{ATP} \text{ [cpm/pmol]}}$$

Metal Analysis

The semen samples were prepared as follows: aliquot of semen sample was transferred into a pre-cleaned 1.5ml microtube, and treated with nitric acid (1:1, v/v) at room temperature for 20min. After homogenization, the mixture was diluted with deionized water. After vigorous shaking, the sample solution was centrifuged at 12,000 rpm for 5 min. The obtained supernatant was decanted to microtube and directly analyzed by flame atomic absorption spectrometry (FAAS) for selenium and zinc, electrothermal atomic absorption spectrometry (ETAAS) for copper, cadmium and lead, and hydride generation atomic absorption spectrometry (HGAAS) for arsenic. The accuracy was validated by standard spiked method, the As, Cd, Cu, Pb, Se and Zn concentrations in spiked semen samples obtained by similarly analytical methods. The recoveries for the target analytes in spiked semen ranged from 95.10 to 105.97%. The calibration curves were obtained after the standard series were analyzed by FAAS, ETAAS, HGAAS as described, respectively. A good linearity was obtained at the concentration range of 5-30 (r^2=0.9973), 0.5-2.5 (r^2=0.9999), 5-50 (r^2=0.9990), 5-60 (r^2=0.9997), 1-6 (r^2=0.9991) and 250-1000 (r^2=0.9989) µg/L for As, Cd, Cu, Pb, Se and Zn respectively.