2015

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Abstract

The objective of this study was to investigate DNA damage and adducts in sperm from coke oven workers who have been exposed to polycyclic aromatic hydrocarbons. A longitudinal study was conducted with repeated measurements during spermatogenesis. Coke-oven workers (n=112) from a coke-oven plant served the PAH-exposed group, while administrators and security personnel (n=67) served the control. Routine semen parameters (concentration, motility, vitality, and morphology) were analyzed simultaneously; the assessment of sperm DNA integrity endpoints included DNA fragmentation, bulky DNA adducts, and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dGuo). The degree of sperm DNA fragmentation was measured using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay and sperm chromatin structure assay (SCSA). The PAH-exposed group had a significant increase in bulky DNA adducts and 8-oxo-dGuo compared to the control subjects (Ps = 0.002 and 0.045, respectively). Coke oven workers' percentages of DNA fragmentation and denaturation from the PAH-exposed group were not significantly different from those of the control subjects (Ps = 0.232 and 0.245, respectively). Routine semen parameters and DNA integrity endpoints were not correlated. Concentrations of 8-oxo-dGuo were positively correlated with percentages of DNA fragmentation measured by both TUNEL and SCSA (Ps = 0.045 and 0.034, respectively). However, the concentrations of 8-oxo-dGuo and percentages of DNA fragmentation did not correlate with concentrations of bulky DNA adducts. In summary, coke oven workers with chronic exposure to PAHs experienced decreased sperm DNA integrity. Oxidative stress could contribute

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Conflict of interests: The authors declare that they do not have any competing financial interests.

to the degree of DNA fragmentation. Bulky DNA adducts may be independent of the formation of DNA fragmentation and oxidative adducts in sperm. Monitoring sperm DNA integrity is recommended as a part of the process of assessing the impact of occupational and environmental toxins on sperm.

Keywords

DNA integrity; DNA fragmentation; bulky DNA adducts; polycyclic aromatic hydrocarbons; semen quality

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are formed during the incomplete combustion of organic materials and are widely distributed into the environment in complex mixtures, such as vehicle exhaust, tobacco smoke, cooked food, and water and urban air. PAHs can undergo CYP 450 Phase I metabolism and form active intermediates, which have been reported to attach covalently to DNA in lymphocytes and tissues of organs including gonads [1]. These reactive intermediates are then capable of covalent binding to DNA, potentially initiating a carcinogenic process [2]. PAHs can form free-radical cations via the one-electron oxidation process and, thus, have the potential to cause oxidative damage to DNA [3-5]. When unrepaired, DNA adducts can cause mutations, including mutational hotspots in the p53 tumor suppressor gene and other genes, which ultimately may induce cancer [6].

Several PAH compounds have been reported to impair the reproductive capacity of males adversely, including decreased semen quality and DNA integrity [7-9]. Animal studies have reported significant levels of PAH metabolites in the testis and epididymis of exposed rats [10]. The substantial presence of PAH metabolites in the male gonads even after 8 h (oral), or 4 h (inhalation) post-exposure, suggests that PAHs can pass through a blood-testis barrier and then be incorporate into the Leydig lipogenic tissue [10,11]. Also, PAH exposure has been linked to oxidative DNA damage and possible single and double-DNA strand breaks [7,8]. Animal studies detected PAH-DNA adducts in testicular tissues and raised the possibility that PAHs can compromise the function of the barrier and affect spermatogenesis [12]. Limited epidemiological studies have also detected PAH-DNA adducts in human sperm [9,12,13]. Sperm DNA repair commonly occurs during the early stages of spermatogenesis (spermatocytes and early spermatids), but not in mature spermatids and spermatozoa [14]. This limited window of repair indicates the possible accumulation of non-repaired DNA damage.

Sperm quality has been used as the most convenient way to assess impact from environmental toxin exposure. However, recent reports showed semen parameters may not address the integrity of the male genome in the sperm head [15]. Common methods used to test sperm DNA fragmentation and denaturation in the clinic setting include the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and the sperm chromatin structure assay (SCSA) [16]. Emerging evidence has suggested that sperm DNA integrity may be a better predictor of male fertility potential than routine semen parameters

[17, 18]. However, DNA fragmentation has been criticized for providing little specific information on the nature, mechanism, and severity of the DNA damage detected [19].

Urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG), generated by the interaction of the hydroxyl radical with guanine, is one of the most abundant products of oxidative damage to DNA; it has been used as a biomarker to depict systemic oxidative stress [18, 22] and to estimate oxidative stress linking occupational and environmental exposures [22, 23]. The detection of a lesion has been considered important because of its abundance and mutagenic potential through the G to T transversion mutation upon replication of DNA [21]. Park et al. demonstrated that metabolic activation of PAHs by aldo-keto reductases leads to the formation of redox active o-quinones and reactive oxygen species [5], which oxidize the C-8 position of the guanine base of DNA and form 8-oxo-dG [24, 25]. Recent evidence has detected 8-oxo-dGuo in human sperm [19]. However, very limited data are available to support the notion that urinary 8-oxo-dGuo could be a reliable biomarker for assessing oxidative stress and damage of sperm. Also, measurements of oxidative damage in sperm have not been fully standardized and examined due to the interference of DNA isolation from the compact nature of sperm chromatin. The use of DNA isolation and extraction methods developed and recommended by the European Committee for Standardization, could minimize oxidation during DNA extraction from tissues and cells. Such method is particularly useful for DNA extraction from sperm cells, which could facilitate the process of measuring oxidative damage to sperm more accurately and the possibility of identifying a reliable biomarker.

The objective of this study was to assess sperm DNA integrity of coke oven workers chronically exposed to PAHs as compared to the control subjects. DNA fragmentation, denaturation, 8-oxo-dGuo, and bulky adducts were assessed to determine nuclear DNA integrity of sperm. The study used well established ^{32}P-labeling for DNA adduct detection, while employing the newly established liquid chromatography-mass spectrometry/mass spectrometry with an on-line solid phase extraction procedure for 8-oxo-dGuo analysis [27,28,36].

2. Materials and methods

2. 1. Human subjects and sampling scheme

Human subjects included coke oven workers and administrative staff who worked at a steel plant in southern Taiwan. Participants were recruited during their annual health examination at the Kaohsiung Municipal Hsiao-Kang Hospital, a main municipal hospital system that provides health care for occupational workers in the southern region of Taiwan.

Coke oven workers (n = 112) served as the PAH-exposed group who had chronically exposed to PAHs, while administrative staff, including administrators and security personnel (n = 67) served as the control group with minimal exposure to PAHs. Our preliminary data showed that PAH concentrations around the coke oven processing area ranged from 15,000 ng m$^{3 -1}$ to 40,000 ng m$^{3 -1}$, while PAH concentrations in offices were less than 50 ng m$^{3 -1}$ [29]. Criteria for human subject selection included being a male between 25 and 50 years old, having no reproductive dysfunction, and being employed at the plant more than one

year. We recruited non-smokers only because tobacco smoke contains PAHs and other chemicals that could induce oxidative damage.

We repeatedly collect biological samples (Sampling I and Sampling II) throughout the entire process of spermatogenesis, which takes 74 days, including the transport on duct system. The selection of the sampling scheme is based on: (i) the regular schedule of the workers who work for 6 continuous days, followed by 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]. Furthermore, the sampling scheme won't create any significant interference with operational processes based on our discussion with the plant manager. Semen samples were collected in the evening of the 2nd rest day of 1st rotation cycle, and in the evening of the 5th and 6th work days (end-of-shift) of the 8th and 12th rotation cycles. Because PAH metabolites in urine fluctuated during the rotation cycle [29], 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 and 12th rotation cycles. One blood sample was collected in the evening of the 1st work day of the 1st rotation cycle. We collected a questionnaire from each participant in the evening of the 1 work day of the 1st rotation cycle to ascertain basic demographic data, which had the potential to covary with or to confound our main measures. These factors included age, body mass index (BMI), education, marital status, smoking, drinking habits, and employment history. The study was undertaken after and with ongoing institutional review board approval. All participants were fully informed about the objective of this study and signed the consent form. Human subject information for this study remains confidential and within the institution.

2.2. 1-Hydroxybenzene (I-OHP) measurement—Urine samples were collected in sterilized 50 ml polypropylene cups right before sperm sample collection. Immediately after collection, samples were stored at -80°C until analysis. Urinary 1-OHP was analyzed using a high performance liquid chromatography (HPLC) with a fluorescent detector. A 10-ml urine specimen was adjusted first to pH 5.0 with 1.0 N acetic acid. The sample was incubated for 24 hrs with 15 μl of β-glucuronidase/sulfatase at 37°C. A sample purification and enrichment cartridge, packed with C18 reverse-phase liquid chromatograph material, was used to extract the PAH metabolites from urine. Twenty μl of extract was injected into a column of the HPLC system with an auto-injector and a fluorescence detector. Normalized concentrations of urinary 1-OHP were expressed as ng/g of creatinine. The detection limit was determined to be 0.1 μg/l based on seven repeated measures of 1-OHP at 1.0 μ/1. The variation in the coefficients of repeated measures for 1-OHP standard solutions with concentrations was less than 10% and ranged from 1 mg/l to 100 mg/l was less than 10%. The limit of qualification for this method was 1 μg/1 [30].

2.3. Semen collection and analysis

Semen samples were produced by masturbation after 3 to 5 days of sexual abstinence and allowed to liquefy at room temperature. After liquefaction of semen, standard semen quality analysis was conducted according to World Health Organization (WHO) recommendations [31]. Sperm concentration, motility, and viability were assessed within one hour after

ejaculation. Manual evaluation of sperm concentration and motility were conducted using a Makler chamber. The eosin stained method was used to assess sperm vitality. At least 300 sperm per sample were assessed for vitality analysis. For the morphology assessment, two slide smears were prepared from each semen sample. 300 sperm per slide were evaluated from air-dried Papanicolaoustained preparations and classified as either normal or abnormal according to the criteria recommended by the WHO [31]. None of the semen samples had significant leukocytospermia per the WHO guidelines [31].

2.4. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay

The TUNEL assay was used to detect sperm DNA fragmentation [16]. A sperm pellet was obtained after 200 µl of semen was centrifuged at $250 \times g$ for 5 min. The pellet was resuspended, washed with 1% human serum albumin (HAS) in 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 nucleotide labeling mixture prepared according to the Roche Diagnostic manufacturer's instruction was deployed onto sperm cells. After the cells were incubated for one hour at 37°C, the cells were washed twice with 1% HSA in phosphate buffer saline. Each test included both positive and negative controls. Cells in the positive control were treated with 50 µl of DNase solution, while cells in the negative control were not treated with the nucleotide labeling mixture. Fluorescence in sperm cells recorded as a positive for the TUNEL assay was assessed using an Olympus BX61 fluorescence microscope. At least 300 sperm cells from each sample were accounted for, and the percentage of TUNEL positive cells was calculated as the outcome of interest.

2.5. Sperm chromatin structure assay (SCSA)

The SCSA was used to detect DNA denaturation as described by Evenson et al [32,33]. Briefly, $1\text{-}2 \times 10^6$ sperm cells per ml were treated with a low pH 1.2 detergent solution (0.08 M HC1, 0.15 M NaCl, 0.1% Triton X-100, pH 1.2). After 30 sec, the cells were stained for 3 min by adding 1.2 ml acridine orange stain in a phosphate-citrate buffer, pH 6.0. Then, the cell suspensions were analyzed using a flow cytometer. Sperm chromatin damage can be quantified by cytometric measurements of the metachromatic shift from green (native, double-stranded DNA) to red (denatured, single-stranded DNA) fluorescence using a FACScan flow cytometer (Becton-Dickinson, Mansfield, MA, USA). Flow cytometry measurements of 5,000 sperm per sample provided statistically robust data on the ratio of red to green sperm and the extent of the DNA denaturation. Histogram files were transferred and analyzed using FlowJO software, which included the calculation of alpha t (αt) parameters.

2.6. Detection of 8-oxo-dGuo adducts in sperm

Sperm DNA were isolated according to the procedure recommended by the European Committee for Standardization on Oxidative DNA Damage (ESCODD) [26,54], with modifications to minimize DNA oxidization during DNA isolation procedures. Briefly, sperm samples ($15\text{-}30 \times 10^6$ sperm^{-1}) were washed with 1% HSA in PBS and centrifuged at $3000 \times g$ for 5 min. The resulting pellet was added to 600 µl of ice-cold extraction buffer (10% (/v) sodium dodecyl sulfate and 1M dithiothreitol), 30 µl of proteinase K, 30 µl of

RNase A (1×10^{-2} mg/1) and 8 µl of RNase Tl (1 U/µl). 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 2 ml of 2-propanol were added. After centrifugation at 5,000 g for 5 min, the DNA pellet was washed with 1 ml of ice-cold 40% (v/v) 2-propanol, collected by centrifugation and dissolved in 200 µl of 0.1 mM desferrioxamine overnight. DNA concentration was measured by absorbance at 260 nm. Protein contamination was checked using the absorbance ratio A260/A280. An absorbance ratio over 1.6 was acceptable.

DNA hydrolysis was performed as described by Chao et al. with some modifications [34]. Briefly, sperm DNA samples (10-20 µg) were spiked with 2.82 pmol of $[^{15}N_5]$-8-oxo-dGuo and 84.3 pmol of $[^{15}N5]$-dG. Then, 5 µl of 0.2 U/µl nuclease P1 (in 300 mM sodium acetate and 1 mM $ZnSO_4$, pH 5.3) was added to the DNA solution, and the DNA was incubated at 37 °C for 2 h. Thereafter, 10 µl of 10× alkaline phosphatase buffer (500 mM Tris/HCl, pH 8, 1 mM EDTA) together with 4 µl of alkaline phosphatase was added and the incubation was continued at 37 °C for 2 h. Subsequently, 10 µl of 0.1 M HCl was added to neutralize the solution and the neutralized DNA hydrolysates were ready for 8-oxo-dGuo analysis.

The 8-oxo-dGuo adduct in sperm DNA was quantified using a LC-MS/MS with an on-line solid-phase extraction procedure as reported in recent studies [27]. After automatic sample cleanup, DNA samples were injected into an Agilent 1100 series HPLC system interfaced with a PE-SCIEX API 3000 triple quadrupole mass spectrometer with an electrospray ion source. Detection was performed in the positive ion multiple reaction monitoring mode for simultaneous quantification of 8-oxo-dGuo and dG Transition of the precursors to the product ions were as follows: 8-oxo-dGuo (m/z 284→168), $[^{15}N_5]$-8-oxo-dGuo (m/z 289→173), dG (m/z 268→152), and $[^{15}N_5]$-dG (m/z 273→157). With the use of isotopic internal standards and on-line SPE, this method exhibited a low detection limit of 1.8 fmol for 8-oxo-dGuo, which corresponds to 0.13 adducts 10^6 dG^{-1} when using 20 µg of DNA per analysis.

2.7. Detection of 8-oxo-dGuo adducts in urine

Urinary 8-oxo-dGuo concentrations in urine were also measured using a validated method of LC-MS/MS with on-line SPE as previously reported [27, 40]. Twenty µl of urine was diluted 10-fold with 5% methanol containing 0.1% formic acid. After the addition of 40 µl of $[^{15}N_5]$-8-oxo-dGuo solution (20 µg/l in 5% methanol/0.1% formic acid) as an internal standard, 100 µl of a prepared urine sample was injected directly into the same on-line SPE LC-MS/MS as described above. The precision of the present method was determined by performing replicate determinations of 8-oxo-dGuo in three different urine samples. The intra- and inter-day coefficient of variation (CV) were 2% - 3% and 4% - 5%, respectively [27]. The concentration of urinary 8-oxo-dGuo was adjusted to the urinary creatinine (ng 8-oxo-dG/mg creatinine) to control for variation in urinary output. Urinary creatinine was determined using the HPLC-UV method described by Yang [35].

2.8. Detection of bulky DNA adducts

The presence of bulky DNA adducts was determined by the ^{32}P-Postlabeling method [37]. Briefly, DNA (10 μg) was degraded enzymatically to normal and adducted by deoxyribonucleoside 3′-monophosphates with micrococcal nuclease and spleen phosphodiesterase at pH 6.0 and incubated at 37 °C for 3.5 h. After treatment of the mixture with nuclease P1 to convert normal nucleotides to nucleosides, adducted nucleotides were converted to 5′-^{32}P-labeled deoxyribonucleoside 3′,5′-bisphosphates by incubation with carrier-free [γ-^{32}P] ATP and polynucleotide kinase. Radioactivity labeled modified nucleotides were mapped by multidirectional anion-exchange thin-layer chromatography (TLC) on polyethyleneimine -cellulose sheets. ^{32}P-labeled I-compounds were visualized by screen-enhanced autoradiography at -80 °C using Kodak BioMax XAR film or with the aid of an InstantImager (Packard Instruments). The radioactivity of TLC fractions for each sperm samples was determined with the aid of an InstantImager electronic autoradiography system as described [38]. 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 according to Zhou *et al* [39].

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

Each set of samples analyzed included positive controls of liver DNA from mice treated with PAHs to ensure that labeling procedures worked appropriately.

2.9. Statistical analysis

Data analysis was performed using a SAS version 9.1 (SAS Institute Inc., Cary, NC) with the results expressed as the mean ± standard deviation (SE). The distributions of semen quality parameters were examined for normality to determine if transformation were needed. Measures were log-transformed, if their skewness prevented them from being normally distributed. For each response variable, means of semen quality parameters and sperm DNA integrity measurements were compared using Bonferroni adjusted t-tests at the 0.05 level of significance. To control for confounding factors, (i.e., age, alcohol consumption, and smoking), multivariate regression analysis was conducted to determine any significant difference. Also, multivariate regression analysis was used to assess the relationship between semen quality parameters and DNA integrity parameters, while controlling for confounding factors. All hypothesis testing was two-sided with a significant level of 0.05.

3. Results

We used the first sampling event to ascertain demographic information on study participants. We retained more than 90% of the participants for the two sampling events. Mean ages, mean years of work, and percentages of alcohol consumption were similar between the PAH-exposed group and the control. The PAH-exposed group consistently had lower percentages of sperm motility, vitality, and normal morphology compared to the

control, but semen quality parameters did not differ significantly between groups. However, percentages of individuals from the PAH-exposed group with motility < 40% and normal forms <4% were significantly higher than the control (P_s = 0.048 and 0.012, respectively). Average of urinary 1-OHP levels were 10 µg/g creatinine and 0.3 µg/g creatinine for the PAH-exposed group and the control group, respectively. Urinary 1-OHP verified that the PAH-exposed group was subjected to high concentrations of PAHs, while the control group was exposed to minimum concentrations of PAHs. The 1-OHP levels for the PAH-exposed group were significantly higher than the concentrations of the control (P = 0.02) (Table 1).

Table 2 summarizes biomarkers associated with sperm DNA integrity and DNA oxidative damage from repeated measurements. For Sampling I, the percentages of DNA fragmentation, expressed as mean ± SD, were 33.75 ± 21.10 and 29.06 ± 7.79 for the PAH-exposed group and the control, respectively (P = 0.23). Spermatogenic 8-oxo-dGuo levels, expressed as mean ± SD, were 24.65 ±20.18 and 12.14 ± 8.05 /10^6 dG for the PAH-exposed group and the control group (P = 0.49), respectively. Urinary 8-oxo-dG levels expressed as mean ± SD were 5.76 ± 3.31 and 4.23 ± 3.25 /creatinine (ng/mg) for the PAH-exposed group and the control (P = 0.34), respectively. Bulky DNA adducts were detected in sperm DNA. The mean levels of bulky DNA adducts ranged from 59.2 to 71.2 in 10^9 nucleotides for the PAH-exposed group, which was higher than the mean levels for the control group (29.2 – 33.1 in 10^9 nucleotides) (P = 0.01). There were slight changes in the readings of the DNA integrity endpoints between the two sampling events.

Table 3 shows that TUNEL, SCSA, 8-oxo-dGuo, and DNA adducts did not correlate with semen parameters, including concentration, motility, morphology, and vitality, after adjusting for potential confounders including smoking status, age, and drinking.

Table 4 shows that 8-oxo-dGuo positively correlated with the degree of DNA fragmentation, measured by both TUNEL and SCSA (P_s = 0.045 and 0.034, respectively). Levels of bulky DNA adducts did not significantly correlate with the levels of 8-oxo-dGuo, TUNEL and SCSA.

4. Discussion

This is the first study that employed the longitudinal study design to assess changes of DNA integrity enpoints throughtout the entire process of spermatogensis cycle, and examined the realtionsip between the sperm DNA integerity endpoints and semen quality parameters. The asessment of sperm DNA integrity thoroughly by measuring DNA fragmentation, denaturation, and bulky DNA adducts, could be more far reaching because spermatozoa could transmit abnormal genetic materials to offsprings. Damged DNA could incorporate into an embroyonic genome, thus leading to errors in replication, and transcription during spermatogenisis, contributing to a range of human diseases [41]. Features of the study design inlcude the accurate ascertainment of exposure, sufficient power to detect cahgnes in biomarker results from PAH expousre and contorl for confounding factors.

DNA fragmentation in sperm is a part of spermatogenesis and is repaired by a DNA repair system. However, unresolved DNA strand breakages can have dramatic consequences for the genomic integrity of the developing gamete and affect sperm quality afterwards. We

found that the coke-oven workers exposed to PAHs experienced an increased mean percentage of DNA fragmentation and denaturation measured by the TUNEL assay and SCSA, respectively. However, the increase of the percentage DNA fragmentation in the coke-oven workers did not reach the significant level as compared to the control in our current study. Nonetheless, it is alarming that the mean DNA fragmentation measured by the TUNEL assay of 36.5% is close to the cut-off value related to lower pregnancy rates (19%) for *in vitro* fertilization patients [42]. Other studies have reported similar results corroborating that PAH exposure is associated with an increase in the percentage of DNA fragmentation in coke-oven workers [7], and the general population of the Czech Republic who were exposed to air pollution with high PAH levels [9]. However, both Hsu's and Selevan's studies did not examine the implication of the DNA fragmentation increase on male fertilization.

The mechanisms that induced DNA damage in sperm included 1) strand breaks during chromatin remodeling during spermiogenesis, 2) DNA fragmentation induced by endogenous endonucleases, and 3) post-testicular DNA fragmentation induced by reactive oxygen species (ROS) [43]. DNA fragmentation measured by the SCAC and TUNEL was correlated with sperm 8-oxo-dGuo. Our research suggested that DNA fragmentation could be induced by the hydroxyl radicals from PAH metabolism in the formation of 8-OH-guanine and 8-OH-2′-deoxyguanosine in a first stage and single-stranded DNA fragmentation [44]. Upon entering a biological system, PAHs can be activated metabolically by cytochrome P450 and form electrophilic intermediates, such as semiquiones and quinones. These reactive intermediates could undergo redox cycling and generate reactive oxygen species including superoxide anions and hydrogen peroxide (H_2O_2). The single–stranded DNA fragmentation could be repaired by the oocytes or the embryo. In addition, the oxygen radicals could activate sperm caspases and endonucleases, which consequently induce double-stranded DNA fragmentation that is virtually unrepairable and incompatible with normal embryo and fetal development [45]. Urinary 1-OHP was weakly correlated with DNA fragmentation of sperm. Such results may be due to the fact that 1-OHP may not represent all PAH metabolites in the large family of compounds, which metabolize in testicular tissues of the male reproductive system. Urinary 1-OHP has served as a reliable biomarker for PAH exposure and its biological metabolism in the entire biological system. However, a recent study has suggested that urinary 1-OHP may not be sufficient to assess the effect of PAHs on sperm DNA integrity and associated reproductive capacity [55].

The 8-oxo-dGuo adducts have been successful used to assess oxidative damage in tissues and cells. In the present study, we employed ESCODD recommended DNA isolation procedures and our newly established LC-MS/MS with an on-line solid-phase extraction method for detecting 8-oxo-dGuo adducts in sperm [26, 40, 54]. This combination of methods minimized oxidation on sperm DNA during extraction and analytical detection procedures. Also, the stated method was very specific, sensitive, and required a relatively small amount of sperm DNA at 10 μg. We observed that coke oven workers experienced significantly higher 8-oxo-dGuo levels in both urine and sperm than did the control subjects ($P = 0.049$). However, levels of urinary 8-oxod-Guo were higher than those in sperm, suggesting that germ cells were protected against environmental factors. The projected

mechanisms could include effective-restriction of DNAreacting compounds via the blood-testis barrier and effective repair of DNA damage during the early stages of spermatogenesis [10,11]. Despite the protection, increased 8-oxod-Guo in sperm suggests that exposure to PAHs is likely to cause oxidative DNA damage. Also, the levels of 8-oxo-dGuo in sperm were significantly and positively correlated with the levels of DNA fragmentation. To date, the sources contributing to DNA strand breakage have not been explored fully. However, this study added evidence that 8-oxo-dGuo could be associated with DNA fragmentation. This result was similar to those reported in studies suggesting an association between the two types of DNA damage; it supports the notion that ROS can reach DNA nucleotides leading to DNA fragmentation [46, 47]. The proposed mechanism for ROS induced DNA fragmentation is such that formation of 8-oxo-dGuo leads to the creation of basic sites and destabilization of the DNA backbone [50]. The DNA fragmentation was found to discrete into smaller fragments in the 20-25 kb range rather than much larger 50-kb toroid fragments detected by other studies [48,49]. It has been suggested that spermatogenic oxidative damage alters APT production of sperm and decreased sperm motility and viability [56].

A lack of correlation between the 8-oxo-dGuo in urine and sperm suggested that 8-oxo-dGuo may be a reliable biomarker to reflect oxidative damage to sperm. The insignificant correlation may be due to the origination of the lesions from different pathways and to different repair mechanisms involved. The 8-oxo-dGuo concentrations in urine were evaluated as reflection of the overall oxidatively damaged DNA in a biological system. The oxidatively damaged DNA were initially released into the bloodstream and then excreted into the urine [51]. Urinary 8-oxo-dGuo could originate from enzymatic hydrolysis (e.g. via the nudix hydrolases) of oxidized guanine nucleoside $5'$-phosphates in the nucleotide pool as well as from the nucleotide incision repair (ENR) pathway or from the mismatch repair pathway [48]. Urinary 8-oxo-dGuo reflected the results of a cellular DNA excision repair process including ENR in the whole body, rather than the result of changes within specific tissues or cell types [38]. On the other hand, although DNA adducts in most germ cell stages could be repairable, DNA repair does not occur in condensed spermatids and sperm in epididymis where protamine has replaced somatic histone, rendering the DNA transcriptionally inert [49]. In addition, NER is limited to testicular cells, spermatocytes, and round spermatids [52].

The presence of DNA adducts in sperm supported the notion that PAHs and/or their metabolites could pass the blood-testis barrier to enter seminiferous tubules. As such, PAHs could insult sperm during spermatogenesis. Some PAHs possess genetic and carcinogenic properties that derive from their abilities to form PAH-DNA adducts. The adducts may be a potential source of transmissible prezygotic DNA damage in spermatozoa [53] and may not impair fertilization during intracytoplasmic sperm injection [13,16]. We observed that the cokeoven workers with higher urinary 1-OHP had a higher level of bulky-DNA adducts than the control group did. Exposure to PAHs could be the environmental factor that contributes to the increase of bulky DNA adducts in sperm. We further detected benzo(a)pyrene DNA (BaP) adducts, along with specific benzo(a)pyrene-7,8-diol-9,10-epoxide-N^2-deoxyguanosine (BPDE-dG) adduct [58], an indicator of potential mutation and cancer risk associated with PAH exposure. Only a small fraction of BPDE-dG was detected in sperm

(2.1 adducts/10^9 nucleotides), which links to an increased risk of mutation and cancer [57]. PAHs metabolites and active DNA-adducting metabolites could pass the blood-testis barrier. However, it is not clear whether the adducts occur in the early or late stage of spermatogenesis and how spermatogenic repair mechanism play a role on the detection of the bulky DNA adducts and PAH actively adduct metabolites. Like other epidemiological and animal studies, the detected DNA adducts mirror the exposure to PAHs. However, much less is known about the role of bulky DNA modification induced by endogenous sources, as shown by the detection of bulky DNA adducts in the control [20]. Among semen quality parameters, bulky DNA levels had a negative relationship with sperm motility and vitality; however the degree of correlation did not reach a significant level. This result was different from Horak et al study, reporting bulky DNA adducts linked to decreased motility of sperm from patients whose semen contained low leukocyte content [20]. Other studies also observed similar results, but explained that the bulky DNA modifications could be associated with hormonal imbalance [59-61]. The bulky DNA modifications could be contributed by exogenous (e.g. repair pathways, tissue, and hormones) and endogenous sources (e.g. radiation, smoking and PAHs) [20, 59-62]. Further studies examine how those factors and sources influence the bulky DNA modifications could be useful for a better understanding of DNA adducts in the etiology of infertility and risk of mutation and cancer in man.

5. Conclusions

In summary coke oven workers experienced decreased DNA integrity of sperm. Monitoring of sperm DNA integrity has become an important part of the assessment of the impact of occupational and environmental insults on sperm. Detecting such DNA damage in sperm could provide a new element besides semen parameters in assessing environmental toxins' effect on male reproductive health. There is a need for development of a number of different testing methods to investigate sperm DNA damage at different levels and different molecular sites.

Acknowledgments

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Abbreviations

TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling
PAHs	Polycyclic aromatic hydrocarbons
1-OHP	1-hydroxypyrene
LC-MS/MS	liquid chromatography-mass spectrophotography/mass spectrophotography
8-OHdG	8-hydroxy-2′-deoxyguanosine
SCSA	Sperm chromatin structure assay
8-oxodGuo	8-oxo-7,8-dihydro-2′-deoxyguanosine

Highlights

- Sperm DNA is the target of PAH insults

- Oxidative stress was the main mechanism associated with PAH-induced DNA damage in sperm

- Urinary 8-oxodGuo may not be a good biomarker to assess DAN oxidative damage in sperm

Table 1

Demographic characteristics, semen quality and 1-OHP concentrations of the PAH-exposed group and the control*

	PAH-exposed Group		Control		P
	N	Mean. ± SD	N	Mean ± SD	
Age (years)	112	41±10	67	38 ± 11	0.64
BMI (kg/m²)	112	24.1±2.6	67	23.7±2.9	0.84
Drinking status (%)	112	38	67	20	0.45
Smoking (%)	112	39	67	26	0.09
Semen Quality	112		67		
Total count (ml)		226.6 ±124.2		227.3 ±112.5	0.72
Concentration (10^6 /ml)		113.3 ±96.6		123.4 ±102.4	0.65
Motility (%)					
Progressive		14.8		16.8	0.23
Non-linear		28.2		36.5	0.08
Non-progressive		15.7		23.5	0.06
Morphology (%)					
Normal form		29.3±3.2		35.5±2.6	0.13
Head defects		54.3±7.4		49.9±6.1	0.19
Coiled tail		5.6±4.3		4.1±3.2	0.25
Vitality (%)		74.5 ±19.2		82.5 ±17.5	0.23
Sperm concentration $<15 \times 10^6$ (%)[a]	2	14.2±0.6	0	N/A	
Motility < 40% (%)[a]	11	16.8	4	5.2	0.048
Vitality < 58% (%)[a]	15	14.4	6	10.2	0.08
Normal forms < 4% (%)[a]	24	21.3	3	5.0	0.012
Urinary creatinine (mg/ml)	112	1.7± 1.3	67	1.8±1.3	0.26
1-OHP concentration (µg/g creatinine)	112	14.2 ±12.6	67	3.8±4.5	0.04

*
Data were generated from Sampling I

[a]
Percentages of semen quality parameters below cut-off values recommended by the WHO (WHO 2010)

Table 2

Sperm DNA integrity and urinary oxidative stress status of the PAH-exposed group and the control

	PAH-exposed Group				Control				P
	Sampling I		Sampling II		Sampling I		Sampling II		
	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	
Sperm									
TUNEL (%)	110	38.2 ± 22.4	99	39.2 ± 20.1	67	30.4 ± 8.6	58	27.8 ± 10.2	0.232
SCSA (%)	110	11.3 ± 10.3	99	12.5 ± 9.5	67	9.4 ± 8.9	58	9.0 ± 7.8	0.245
8-oxodGuo (/10^6 dG)	110	24.3 ± 19.9	98	22.6 ±16.3	67	12.1 ± 8.1	58	20.3 ± 9.1	0.030
Bulky DNA adducts (10^9 nucleotides)	109	71.2 ± 30.2	98	59.2 ± 25.9	66	33.1 ± 20.2	58	29.2 ± 19.8	0.045
Urine									
8-OHdG (/10^6 dG)	112	3.6 ± 1.2	99	3.4 ±1.0	67	2.5 ± 1.1	58	2.4 ± 1.2	0.046

SCSA indicates sperm chromatin structure assay; TUNEL, TdT-mediated-dUTP nick end-labeling

Table 3

Correlations between semen quality parameters and DNA integrity endpoints[*][a]

	8-oxodGuo	TUNEL	SCSA	Bulky DNA adducts
Concentration	-0.05 (0.68)	0.03 (0.79)	0.05 (0.87)	-0.10(0.59)
Motility				
Progressive	-0.18(0.12)	-0.19(0.09)	-0.23(0.12)	-0.17(0.34)
Non-linear	-0.18(0.12)	-0.19(0.09)	-0.23(0.12)	-0.17(0.34)
Non-progressive	-0.18(0.12)	-0.19(0.09)	-0.23(0.12)	-0.17(0.34)
Normal form	-0.01 (0.92)	-0.07(0.54)	-0.14(0.64)	-0.24 (0.30)
Vitality	-0.14(0.25)	-0.18(0.09)	-0.26(0.14)	-0.09 (0.20)

[*]r(P)

[a]All readings from Sampling I and Sampling II were used.

Table 4

Correlation coefficients among sperm DNA integrity endpoints as estimated by spearman correlation analysis [*],[a]

	8-oxodGuo	TUNEL	SCSA	Bulky DNA adducts
8-oxodGuo	--	0.23 (0.046)	0.12(0.034)	0.23 (0.078)
TUNEL	0.23 (0.045)	--	0.16(0.023)	0.34 (0.098)
SCSA	0.12(0.034)	0.16(0.023)	--	0.48(0.102)
Bulky DNA adducts	0.23 (0.078)	0.34 (0.098)	0.48(0.102)	--

[*] r(P)

[a] Data from Sampling I and Sampling II