Tobacco consumption and benzo(a)pyrene-diol-epoxide–DNA adducts in spermatozoa: in smokers, swim-up procedure selects spermatozoa with decreased DNA damage

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Objective: To analyze the distribution of benzo(a)pyrene-diol-epoxide (BPDE)–DNA adducts in spermatozoa selected and nonselected by a swim-up procedure with relation to smoking habits.
Design: Comparative study.
Setting: Public university and public university hospital.
Patient(s): Seventy-nine men (37 smokers and 42 nonsmokers) who visited an infertility clinic for diagnostic.
Intervention(s): Tobacco and environmental exposure assessment, semen sample analysis, swim-up procedure, BPDE-DNA adduct immunolabeling.
Main Outcome Measure(s): BPDE-DNA adduct quantification in selected (SEL-SPZ) and nonselected (NONSEL-SPZ) spermatozoa. Data were normalized by using a normalized fluorescence value (NFV).
Result(s): The mean NFV (±SD) in SEL-SPZ was significantly higher in smokers than in nonsmokers (18.9 ± 11.5 vs. 10.5 ± 10.4, respectively). Within smokers, a paired analysis (SEL-SPZ and NONSEL-SPZ) showed that NFV was significantly lower in SEL-SPZ than in NONSEL-SPZ (20.0 ± 11.3 vs. 31.5 ± 16.0, respectively). Conversely, within nonsmokers, the mean NFV was higher in SEL-SPZ than in NONSEL-SPZ (10.3 ± 10.6 vs 4.3 ± 7.1, respectively).
Conclusion(s): Tobacco consumption is associated with BPDE-DNA adducts in spermatozoa. In smokers, semen processing by swim-up recovers potentially fertilizing spermatozoa that show a significantly lower amount of BPDE-DNA adducts compared with NONSEL-SPZ. Further study is needed to improve the spermatozoa selection in smoking patients requiring assisted reproductive technologies. (Fertil Steril® 2011;95:2013–7. ©2011 by American Society for Reproductive Medicine.)

Key Words: DNA adducts, spermatozoa, polycyclic aromatic hydrocarbon, sperm selection technique, swim-up

Tobacco consumption exposes smokers to >4,000 toxic components. Benzo(a)pyrene (B[a]P) is formed during the combustion of tobacco and metabolized by cytochrome P450 enzymes into benzo (a)pyrene-diol-epoxide (BPDE), which has the potential to bind covalently to DNA to form BPDE-DNA adducts (1). BPDE-DNA adducts are premutational lesions that constitute a potential source of carcinogenic damage (2). In spermatozoa, BPDE-DNA adducts are increased by smoking (3). Because ejaculated spermatozoa have minimal repair capacity (4), formation of such adducts in spermatozoa is a potential source of transmissible prezygotic DNA damage. Indeed, parental transmission of BPDE-DNA adducts has been shown via preferential gametic transmission from smoking fathers to early human preimplantation embryos (5).

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The presence of DNA damage within spermatozoa is associated with fertilization impairment, early pregnancy loss, perinatal morbidity associated with assisted reproductive techniques (ART), and increased risk of childhood cancer (6–9). Various semen preparation techniques were developed by ART to isolate motile morphologically normal spermatozoa used for intrauterine insemination or oocyte in vitro fertilization (IVF). These techniques separate selected spermatozoa from nonselected (dead or immovable) spermatozoa, leukocytes, and bacteria, producing oxygen radicals that negatively influence the ability of the sperm to fertilize the ovum (10). The most commonly used methods are simple washing of spermatozoa (using various culture media), methods based on self-migration of spermatozoa (i.e., swim-up), and discontinuous gradient centrifugation (DGC) using preparations of different density gradients. The swim-up from a washed pellet is easy to perform and very cost effective. It is the oldest and most commonly used sperm separation method, still used in IVF laboratories around the world (11). A recent meta-analysis showed no difference in rates of pregnancy or miscarriage between the three semen preparation techniques described above (10).

In studies that quantify BPDE-DNA adducts, sperm are selected from either untreated semen samples (12) or semen treated by a swim-up (3, 5) or DGC (13) procedure. To our knowledge, no

study has been conducted to compare BPDE-DNA adduct levels in selected and nonselected sperm. Using a swim-up procedure, we analyzed the distribution of BPDE-DNA adducts in selected and nonselected spermatozoa in relation to smoking habits.

MATERIALS AND METHODS

Population

We recruited 79 men between January 2009 and May 2009 in the infertility clinic of the University Hospital La Conception (Marseilles, France). Exposure to polycyclic aromatic hydrocarbons (PAHs) by active smoking ($\geq$5 cigarettes per day for $\geq$3 months) and/or occupation was evaluated using a standardized questionnaire (14). Participants had no identifiable cause of male infertility and no current medication known to impair semen quality.

Sperm Sample Collection and Analysis

Ejaculate was obtained via masturbation into a sterile collection tube after a period of sexual abstinence of 2–6 days. Semen analysis was performed after 30 minutes of liquefaction at 37°C in accordance with the current World Health Organization (WHO) guidelines (15). Informed consent was obtained for inclusion of any remaining semen sample in the Germetheque Biobank, France. The sample was coded and then used for spermatozoa selection before immunostaining.

Spermatozoa Selection Procedure

Spermatozoa were selected by a swim-up technique. Semen was washed in culture medium (Ferticult; JCD, La Mulatière, France). The supernatant was discarded, and 200 μL culture medium was carefully applied so as not to dislodge the sperm pellet. The tube was disposed at an angle of 45° and the preparation incubated at 37°C under 5% CO_2 for 30 minutes to separate the selected and nonselected spermatozoa (16). The layered medium that contained the selected spermatozoa (SEL-SPZ) was then transfered to another tube. The nonselected spermatozoa remaining in the pellet (NONSEL-SPZ) were resuspended in 200 μL culture medium.

Spreading and Immunofluorescent Staining [Adapted from (13)]

The spermatozoa samples (SEL-SPZ or NONSEL-SPZ) were washed in 10 mmol/L Tris buffer followed by phosphate-buffered saline solution (PBS), then spread onto one Shandon Cytoslide (Thermo Fisher Scientific, Cergy Pontoise, France) by cytocentrifugation.

Slides were air dried and fixed with methanol:acetic acid (3:1) overnight at −20°C. Before staining, slides were washed with PBS, and treated with RNase (100 μg/mL for 1 hour at 37°C) and proteinase K (10 μg/mL for 10 minutes). The slides were washed again with PBS, incubated in 4 N HCl for 10 minutes, neutralized with 50 mmol/L Tris-base for 5 minutes, and washed with PBS.

The slides were incubated with 10% goat serum for 45 minutes at 37°C and then incubated at 4°C overnight with a mouse monoclonal primary antibody against BPDE-DNA (5D11; Santa Cruz Biotechnology, Santa Cruz, CA) that had been diluted 1:50 in blocking solution (5% goat serum in 10 mmol/L Tris-buffer, 0.1% Triton X-100).

After washing with PBS, the slides were incubated for 45 minutes at 37°C with a secondary Alexa Fluor 488 goat antimouse IgG antibody (Molecular Probes; Invitrogen, Carlsbad, CA) at a dilution of 1:200. The labeled BPDE-DNA adducts emit a green fluorescence. The nuclei DNA was counterstained with 4′,6-diamidino-2-phenylindole (DAPI) emitting blue fluorescence, and slides were mounted with Vectashield (Vectors Laboratories, Burlingame, CA).

Control Samples

Human lymphocytes were collected from one healthy nonsmoking donor after informed consent and used as an internal control for each batch of sperm samples during immunolabeling. Lymphocytes were either treated for 1 hour with 40 μmol/L BPDE (positive control; Midwest Research Institute, Kansas City, MO) (13) or left untreated (negative control), spread, and stained as described above.

BPDE-DNA Adduct Quantification

Slides were examined with an epifluorescent Olympus BX-60 microscope (Olympus France, Rungis, France) at a magnification of ×1,000. Spermatozoa were identified based on the size of the nucleus and the presence of a flagellum. Digital pictures were acquired with a highly sensitive CCD color digital camera (Sony, Tokyo, Japan) for both Alexa 488 and DAPI staining (Visilog 6.7; Noesis, Saint Aubin, France).

Nuclear areas for each image were selected by using ImageJ image analysis software (http://rsb.info.nih.gov) on the basis of DAPI fluorescence, then overlaid onto the corresponding Alexa 488–captured picture to localize the green fluorescence to specific nuclei and thus indicate the DNA damage. Fluorescence was expressed as arbitrary units of average gray value average (AGVA), i.e., the mean gray value of all of the pixels selected within each digital picture by the software.

DNA adducts were measured in nine different batches, containing a mixture of samples from smokers and nonsmokers. The results were normalized against the internal negative and positive control samples present in each batch to adjust for interbatch variability. For each sample, the fluorescence value (FV) corresponds to the mean FV of all analyzed cells (from 50 to 184 cells, depending on the samples). A normalized fluorescence value (NFV) was calculated as follows (arbitrary units):

$$\text{NFV} = (\text{FV of the sample} - \text{FV of the negative control})/(\text{FV of the positive control} - \text{FV of the negative control}) \times 100.$$

Statistical Analyses

The variables were normally distributed as determined by the Kolmogorov-Smirnov goodness-of-fit test. An independent-samples *t* test was used to compare data from smokers and nonsmokers, and comparisons between SEL-SPZ and NONSEL-SPZ were made by using a paired-samples *t* test. Associations between NFV and the number of cigarettes smoked per day were assessed by Pearson correlation coefficient. Statistical analyses were performed with SPSS Statistics 18 for Microsoft Windows (SPSS, Chicago, IL). Statistical significance was set at $P<.05$.

Institutional Review Board

All samples were provided to the authors by Germetheque Biobank. Germetheque obtains informed consent from each patient for inclusion of samples in the biobank and their use for studies on human fertility in accordance with the Helsinki Declaration of 1975 on human experimentation. The Germetheque Scientific Committee approved the present study design. Consequently, no IRB approval was sought.

RESULTS

Population

The population included 37 smokers and 42 nonsmokers of similar ages (34.5 ± 5.9 years and 35.8 ± 6.2 years, respectively; $P=.318$). The mean tobacco consumption in smokers was 16.0 ± 6.2 cigarettes per day (ranging from 5 to 30).

Only 3 patients (3.8%) showed normal semen parameters according to WHO criteria. Four patients (5.1%, 2 smokers and 2 nonsmokers) were potentially exposed to occupational PAHs.

Semen Analysis

Comparison of semen parameters between smokers and nonsmokers showed no statistical difference in ejaculate volume (4.0 ± 1.5 mL vs. 3.9 ± 1.4 mL, respectively; $P=.726$), total sperm count by ejaculate (250.3 ± 254.1 vs. 180.4 ± 236.4, respectively; $P=.210$), percentage of rapid progressive sperm (8.0 ± 10.6 vs. 5.8 ± 7.8,