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Episodic air pollution is associated with increased DNA fragmentation in human sperm without other changes in semen quality

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BACKGROUND: This study examined potential associations between exposure to episodes of air pollution and alterations in semen quality. The air pollution, resulting from combustion of coal for industry and home heating in the Teplice district of the Czech Republic, was much higher during the winter than at other times of year with peaks exceeding US air quality standards. METHODS: Young men from Teplice were sampled up to seven times over 2 years allowing evaluation of semen quality after periods of exposure to both low and high air pollution. Routine semen analysis (sperm concentration, motility and morphology) and tests for sperm aneuploidy and chromatin integrity were performed, comparing measurements within each subject. Exposure was classified as high or low based on data from ambient air pollution monitoring. RESULTS: Using repeated measures analysis, a significant association was found between exposure to periods of high air pollution (at or above the upper limit of US air quality standards) and the percentage of sperm with DNA fragmentation according to sperm chromatin structure assay (SCSA). Other semen measures were not associated with air pollution. CONCLUSION: Exposure to intermittent air pollution may result in sperm DNA damage and thereby increase the rates of male-mediated infertility, miscarriage, and other adverse reproductive outcomes.

Key words: air pollution/computer-aided sperm analysis/human semen/sperm aneuploidy/sperm chromatin structure assay

Introduction

Since the 1950s, residents of the Teplice District in Northern Bohemia, Czech Republic have been exposed to high levels of air pollution generated from the combustion of high-sulphur coal used for local industry and home heating (Moldan and Schnoor, 1992; Sram *et al.*, 1996). This pollution is severe during the winter when coal is also burned for residential heating and when climatic conditions result in temperature inversions, trapping pollution in the valleys. Concern over potential adverse health effects of this air pollution motivated the Czech government to establish a comprehensive air pollution monitoring and health effects research programme, the Teplice Program, which began in 1991 (Sram, 2001; Sram *et al.*, 1996). Air pollution monitoring for the Teplice Program confirmed that pollution was elevated during winter months with intermittent peaks due to thermal inversions. During these peaks, values occasionally exceeded US and Czech 24 h air quality standards for SO_2 and PM_{10} (Sram *et al.*, 1996). The monitoring data from the early 1990s motivated the Czech government to take actions to ameliorate the air pollution, including closure of some power plants, and change-overs from coal to gas for home and institutional heating (Sram, 2001). As a consequence of these changes, pollution levels decreased significantly between 1993 and 1994 and thereafter (Pinto *et al.*, 1998; Benes *et al.*, 2001).

Among the health effects studies initiated early in the Teplice Program was a male reproductive health study. Young men (aged 18 years) living in Teplice in 1993 and 1994 (Selevan *et al.*, 2000) were sampled (one semen sample from each participant), either in the winter (when pollution was high) or the late summer (when pollution was low). This study found no significant associations between exposures to air pollution and measures of sperm production (sperm concentration or total sperm count). However, significant associations were found between the exposure to air pollution and some indicators of sperm quality, namely sperm morphology, sperm chromatin

structure (Selevan *et al.*, 2000) and sperm aneuploidy (Robbins *et al.*, 1999) in men evaluated in the winter of 1993. Also, significant associations were found between exposure to air pollution and sperm morphology and motility in men evaluated in the winter of 1994. The authors pointed out that differences in results between years could be due to differences in levels of pollution, differences in the interval between the peaks of pollution and the specific day of semen sampling, and differences in the two groups of men examined.

This preliminary study (Selevan *et al.*, 2000) had several limitations, including: data on only one semen sample per man, having only environmental exposure data (no internal markers of exposure), and having limited (infrequent) data on particulate material ≤10 μm in size (PM_{10}) and its constituent polycyclic aromatic hydrocarbons (PAH). Nevertheless, the study suggested that exposure to ambient air pollution was associated with decreased semen quality, a finding that could negatively impact fertility, especially when considered on a population basis.

In light of these preliminary findings, the Czech government became interested in monitoring semen quality in young Teplice residents over longer periods of time during which actions were taken to reduce the air pollution. Therefore, a second study was initiated in 1995, designed to overcome the limitations of the first study. Accordingly, a cohort of men living in Teplice was sampled multiple times over 2 years, both during the winter when pollution was high, and during late summer after an extended period of much lower air pollution. This longitudinal design with repeated measures allows each man to serve as his own control. The study protocol also specified collection of serum samples to measure metals that may be associated with altered semen quality (reviewed by Robbins and Cousins, 1998), and urine to measure cotinine and thereby confirm self-reported smoking status. Air pollution monitoring, including measurement of PM_{10} and PAH, continued during the study period (Watts *et al.*, 1994; Lenicek *et al.*, 2001).

Materials and methods

Study design and participant recruitment

The study protocol was reviewed and approved by the Institutional Review Board of the Czech Academy of Sciences. Eligibility requirements included age (born 1974–1976) and residence in the Teplice District for ≥3 months prior to the start of the study, with the intent to remain in Teplice for ≥1 year. Accordingly, men from Teplice who had participated in the first study (Selevan *et al.*, 2000) were mailed an invitation to participate in the longitudinal study.

At entry, all participants provided written, informed consent, and passed a routine physical examination. For each of seven sampling sessions, the cohort of men was scheduled for appointments over 5 consecutive days; thus, the cohort had essentially the same exposure preceding each sampling. Sample 1, obtained in September 1995, served as the first reference or baseline sample. To capture exposures to episodes of high air pollution, men were sampled monthly during the winter of 1996, i.e. in January (sample 2), February (sample 3) and March (sample 4). This was followed by a second reference sample in September 1996 (sample 5), a fourth winter sample in February 1997 (sample 6) and a final reference sample in September 1997 (sample 7). Having this series of seven samples, four samples during periods of high air pollution and three after periods of relatively low air pollution, provided longitudinal data from which to examine potential associations between exposure to high versus low air pollution across two different winters, and to use each man as his own control. To remain in the final analysis, a participant had to have contributed at least one reference sample and two winter samples during the first year of the study.

Questionnaire

A structured questionnaire, similar to that used in the first study (Selevan *et al.*, 2000), was administered at entry (September 1995). It collected information about each participant's reproductive and general health, and about specific factors that might potentially impact semen quality, including: recent fever, use of medications and vitamins, smoking habits, alcohol and caffeine consumption, type of underwear worn (boxers or briefs), and exposures to solvents, pesticides or metals through work or hobby. The specific factors were asked with respect to the 90 days preceding semen sampling. This is considered a time span relevant to a given semen sample, since it encompasses the entire process of spermatogenesis, including epididymal maturation (Heller and Clermont, 1964; Selevan *et al.*, 2000). A brief questionnaire was administered at each subsequent cycle to update information on lifestyle habits and medical/occupational exposures.

Semen collection and routine analyses

Semen samples were collected on-site by masturbation into clean glass containers (Kavalier, Sazava, CZ), and the abstinence interval recorded (an interval of 2–7 days having been requested). The semen was allowed to liquefy at room temperature for 30 min and processed within 1 h of collection. Sample volume was measured in a 15 ml graduated centrifuge tube and an aliquot removed to determine sperm concentration by haemocytometer (World Health Organization, 1992). For motility analysis, aliquots of semen were loaded into 20 μm deep chambers (Microcell; Fertility Technologies, Inc., Natick, MA, USA), mounted on a heated (37°C) microscope stage and videotaped (Selevan *et al.*, 2000). Samples with high sperm concentrations were diluted with homologous seminal fluid to achieve a concentration suitable for computer-aided sperm analysis (CASA). Aliquots of semen (7 μl) were smeared onto slides (four slides per sample), air-dried, fixed in 95% ethanol (15 min) and later stained with Papanicolaou stain for morphological evaluation (World Health Organization, 1992). Finally, aliquots of semen were loaded into straws (0.5 ml) and frozen (–80°C) without cryoprotectant for later analysis of genetic outcomes.

After all samples were obtained, videotapes were analysed for motility and motion characteristics by a single trained technician. The percentage of progressively motile cells, defined as World Health Organization (1992) grades 'a' + 'b', was determined visually from the videotapes, based on ≥100 sperm in several fields. Videotaped sperm were analysed by CASA using Cell Motion Analyser (Version 2.0; Medical Technologies, Montreux SA, Switzerland) to evaluate the quality of sperm motion. Analyses were conducted at 60 Hz with maximum/minimum number of frames = 32/15. Sperm were considered motile when average path velocity was >10 μm/s. Typically, 100–200 sperm tracks were analysed per sample, although a small number of samples (n = 11) had only 51–98 sperm tracks on the videotape. For each sample, three CASA outcomes were reported: straight-line velocity, an indication of sperm progression; curvilinear velocity, an indication of sperm vigour; and linearity, an indicator of the straightness of the sperm track. Of routinely obtained CASA outcomes, these measures are the least dependent upon instrument brand or software version (reviewed in Perreault, 2002) and therefore most comparable with data in the preliminary study (Selevan *et al.*, 2000) and in the literature.

The percentage of morphologically normal sperm was determined by examining 300 sperm per sample at ×1000 magnification under oil immersion and classifying them according to strict criteria as described by the World Health Organization (1992). The percentage of sperm with morphologically normal heads was also recorded. Two experienced technicians scored pre-coded slides so that they were read blind. Slides were pre-sorted so that all samples from a given participant were scored by one technician to preclude within-participant error due to inter-technician variability in scoring.

Sperm chromatin structure assay

Straws containing frozen semen were shipped to South Dakota State University for analysis of sperm chromatin structure using SCSA® (Evenson *et al.*, 2002). Briefly, semen was thawed, diluted in acid buffer (pH 1.2) to potentially denature damaged nuclear DNA, stained with acridine orange and immediately evaluated by flow cytometry. Acridine orange is a metachromatic dye that fluoresces green when intercalated into double-stranded native DNA, but red when complexed with single-stranded DNA. Dual band flow cytometry was used to detect both green (515–530 nm band pass filter) and red (630 nm long pass filter) fluorescence in ≥5000 sperm per sample. DNA denaturation was detected by a shift from green to red fluorescence and quantified by the expression 'DFI', defined as the ratio of red/(red + green) fluorescence. Cells with DNA fragmentation index (DFI) values above a threshold are considered abnormal and the percentage of such cells is termed %DFI.

Sperm aneuploidy

The sperm aneuploidy assay was conducted as previously described (Robbins *et al.*, 1995; Rubes *et al.*, 1998, 2002) for 15 of the participants selected because they contributed all seven samples and were either non-smokers or smoked <20 cigarettes/day. Briefly, air-dried smears of sperm were decondensed and hybridized immediately using fluorescent chromosome-specific α-satellite DNA probes for chromosomes X and 8, and satellite III DNA probe for chromosome Y (Vysis, Downers Grove, IL, USA). This method allows distinction between diploid and disomic sperm nuclei, and meiosis I and meiosis II errors in sex-chromosomal aneuploidy and diploidy. Slides were randomized and 10,000 sperm were scored per sample (~70 000 sperm total per donor), using strict scoring criteria previously validated against human sperm/hamster oocyte data (Robbins *et al.*, 1993). The number of disomic sperm (exhibiting signals for XX8, YY8, XY8, X88 or Y88) per sample was recorded and the total number of disomic sperm per sample (per 10,000 cells) was calculated. The same was done for the number of diploid sperm (exhibiting signals for XX88, YY88 or XY88). Finally total disomies and total diploidies were summed.

Biomarkers of exposure to cigarette smoke and metals

Urine samples were obtained with samples 1 (September 1995), 2 (January 1996) and 4 (March 1996), and assayed for cotinine to confirm self-reported smoking status (Langone and Van Vunakis, 1982). Blood was collected by venipuncture with samples 1 (September 1995), 3 (February 1996), 5 (September 1996) and 6 (February 1997) and sent to the National Institute of Public Health (Prague, Czech Republic) for analysis of lead, mercury and cadmium, as an indication of possible exposure to metals from the air pollution or from occupational exposures or hobbies.

Ambient air pollution monitoring

Air pollution data, collected for 24 h periods as described (Pinto *et al.*, 1998), included sulphur dioxide (SO_2), nitrogen oxides (NOx), and particulate matter <10 μm in aerodynamic diameter (PM_{10}). These data were collected daily except in summer months when PM_{10}, and therefore PAH extracted from it, was measured only once or twice per week. Because air pollution is consistently low in the summer, as indicated by historic and ongoing monitoring, values obtained during the summer were considered representative of all days. PAH was extracted from the particulate matter, fractionated and analysed by gas chromatography–mass spectroscopy to identify carcinogenic and non-carcinogenic species and to calculate the concentration of total PAH (Lenicek *et al.*, 2001). For each pollutant, the average value for the 90 days preceding each semen sampling was calculated in order to represent the average exposure to which those sperm had been exposed during their development from stem cells, through meiosis, spermiogenesis and epididymal transit (Clermont, 1963; Heller and Clermont, 1964).

Statistical analysis

Semen data were transformed when necessary to optimize distribution normalcy. Each semen outcome was then tested for association with exposure using Mixed models (PROC MIXED, SAS, 8.01, Cary, NC, USA) for repeated measures. This procedure permits inclusion of participants with incomplete data (fewer than seven semen samples). Exposure was dichotomized as low (relevant to the reference samples 1, 5 and 7) or high (relevant to the winter samples 2, 3, 4 and 6). Factors potentially associated with semen measures and categorized as previously described (Selevan *et al.*, 2000) were considered in the regression model. These included: abstinence interval (<2 days versus longer); high fever (>38°C) within the last 3 months; wearing briefs versus loose-fitting underwear; alcohol consumption (0–25, 25–199 or ≥200 ml ethanol/week); cigarette smoking (none, <1 or ≥1 pack/day); caffeine consumption (<$^1/_2$, $^1/_2$–3 or ≥3 coffee cup equivalents/day); and working with solvents or metals (≥10 h/week versus less). If a variable in the model was either of borderline statistical significance ($P < 0.1$) or a confounder (i.e. the associated β in the mixed model changed by >10%), it was retained in the final model. Because cigarette smoke contains chemicals also found in the air pollution, and has been associated with altered semen quality, including sperm chromatin structure (Potts *et al.*, 1999), all models were tested with smoking included. $P < 0.05$ was considered statistically significant.

Results

Air pollution

Figure 1 illustrates the air pollution levels for SO_2, NOx, PM_{10} and PAH, shown as means for the 90 days preceding semen sampling. These 90 day mean values approached or exceeded the upper limits for annual US air quality standards for SO_2 (80 μg/m^3/day), NOx (100 μg/m^3/day) and PM_{10} (50 μg/m^3/day). Furthermore, individual daily values exceeded the US 24 h limit (365 μg/m^3/day for SO_2 and 150 μg/m^3/day for PM_{10}) >1 day per year and were therefore out of compliance with US air quality standards (data not shown). With respect to the previous study, mean 90 day values for winter 1996 and 1997 were similar to those calculated for winter 1994, and lower than those calculated for winter 1993 (Selevan *et al.*, 2000).

Participants

Men from Teplice who had participated in the first study ($n = 183$) were mailed an invitation to participate in this study. Eighty-nine men were subsequently contacted by phone, while the remainder either could not be located ($n = 47$) or had moved out of the district ($n = 45$). Of those contacted, 60 agreed to participate

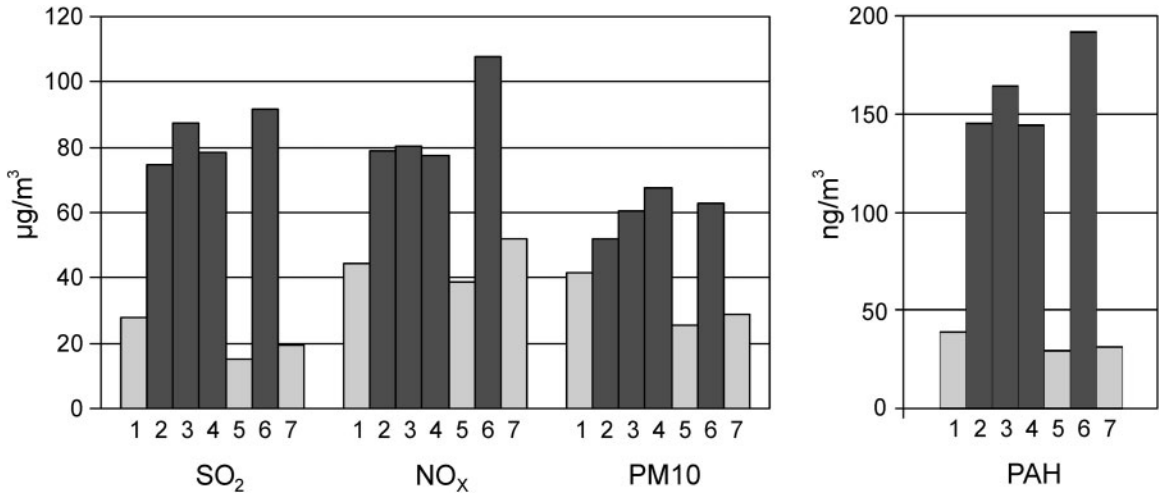

Figure 1. Air pollution data summary. Bars are means of available data for the 90 days preceding semen sampling and are labelled 1–7 to correspond with the longitudinal semen samples. Data relevant to the reference samples (1, 5 and 7), i.e. semen collected in September 1995, 1996 and 1997 respectively are lightly shaded to indicate times of low air pollution. Data relevant to the semen collected in the winter (2, 3, 4 and 6, i.e. January, February or March 1996 and February 1997 respectively) are more darkly shaded to indicate times of higher pollution. Air pollution data were collected daily except that PM_{10} (and therefore PAH extracted from PM_{10}) was collected once or twice per week in the summertime (bars 1, 5 and 7 only) when PM_{10} was uniformly low.

in the study and 29 refused. Of the 60 who agreed, 12 did not show up for their initial appointment, leaving 48 men who were enrolled in the study. During the course of the study, 11 men were excluded because they moved or travelled out of the district ($n = 6$), or were lost to follow-up ($n = 5$). Also, one man was disqualified on medical grounds, because he had a varicocelectomy midway through the study. Of the 36 men who completed the study, 21 gave seven samples, 10 gave six, two gave five, two gave four and one gave three samples.

Characteristics of the study participants were similar to those in the preliminary study (Selevan *et al.* 2000), with only slight increases in cigarette, alcohol and caffeine consumption (Table I). About half of the participants were self-reported smokers, and all but one of these smoked less than a pack a day. Self-reported 24 h smoking agreed with urinary cotinine levels, e.g. Spearman correlation = 0.64, $P < 0.001$, for sample 1. No participants reported working with solvents and only three men reported working with metals.

Blood levels of lead, cadmium and mercury did not differ comparing reference samples with those obtained mid-winter (reflecting exposure to higher air pollution) ($P > 0.05$, Wilcoxon test). Mean lead concentration (CI) in blood collected with sample 1 was 4.3 µg/dl [95% confidence intervals (CI): 3.2, 5.3] and only one participant had a blood lead >10 µg/dl. Mean cadmium concentration for these samples was 1.0 µg/l (95% CI: 0.7, 1.2) and individual values correlated with smoking status ($r = 0.64$, $P < 0.001$) as expected (reviewed by Robbins and Cousins, 1998). Mean mercury concentration for these samples was 0.7 µg/l (95% CI: 0.6, 0.9).

Table I. Characteristics of study participants compared with semen donors in study 1[a]

	Study 1, Teplice[a] ($n = 54$)	Study participants[b] ($n = 36$)
Age (years)	18	19–21
Smokes cigarettes		
None	59.1	50.0
1–19/day	29.9	47.2
≥20/day	11.0	2.8
Drinks alcohol		
<25 ml/week	44.8	36.1
25–199 ml/week	44.2	56.6
≥200 ml/week	11.0	8.3
Consumes caffeine[c]		
None (<0.5)	31.2	22.2
0.5–<3 cups/day	57.8	52.8
≥3 cups/day	11.0	25.0
Abstinence		
≥2 days	78.0	83.3
<2 days	22.0	17.7
Fever >38°C		
Yes	10.4%	8.3%
Briefs?[d]		
Yes	85.2%	83.3%
No	15.8%	16.7%
Work/hobbies <10 h/week	85.1	91.4
with metals ≥10 h/week	14.9	8.3
Work/hobbies <10 h/week	85.1	100
with solvents ≥10 h/week	14.9	0

Values are percentages unless otherwise specified.
[a]Study 1 = Selevan *et al.* (2000).
[b]From first questionnaire at entry into study.
[c]Coffee cup equivalents calculated as in Pastore and Savitz (1995).
[d]Yes = always; no = never or sometimes.

Semen outcomes and their association with air pollution

Descriptive statistics for semen data (means and 95% CI) are given in Table II, showing the time periods when air pollution levels were higher (winter: sampling periods 2, 3, 4 and 6) versus the reference sample periods (1, 5 and 7). Examination of values for the reference samples reveals that this cohort of young men consisted largely of normozoospermic individuals (reference values, World Health Organization, 1999) for semen volume, sperm concentration, and sperm motility. Mean values for percentage normal morphology, percentage normal head morphology

Table II. Descriptive statistics for semen outcomes obtained by repeated sampling of 36 men seven times over 2 years[a]

	Semen endpoints						
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
	September 1995	January 1996	February 1996	March 1996	September1996	February 1997	September 1997
	Low	High	High	High	Low	High	Low
Sperm count ($\times 10^6$)	293.5 (223.0–364.0)	253.7 (167.4–340.0)	270.4 (181.2–359.6)	300.5 (211.8–389.1)	278.1 (197.9–358.2)	234.2 (188.1–280.3)	262.2 (178.0–346.3)
Sperm concentration ($\times 10^6$/ml)	98.6 (74.9–122.4)	78.5 (57.1–99.9)	79.9 (60.9–99.0)	103.1 (76.6–129.6)	92.1 (66.3–.117.9)	81.6 (67.9–95.4)	103.6 (65.4–141.7)
Semen volume (ml)	3.3 (2.7–3.9)	3.1 (2.4–3.8)	3.4 (2.7–4.1)	3.2 (2.5–3.9)	3.3 (2.8–3.8)	3.0 (2.5–3.6)	2.8 (2.4–3.1)
Motile sperm (%)	58.5 (52.0–65.0)	55.0 (47.2–62.9)	59.1 (52.0–66.3)	66.3 (61.8–70.7)	62.7 (55.7–69.8)	68.3 (64.4–72.3)	56.2 (50.9–61.6)
Normal sperm head morphology (%)	29.5 (25.8–33.1)	29.0 (26.6–31.3)	26.4 (23.7–29.1)	26.8 (23.6–29.9)	26.0 (23.4–28.6)	24.5 (23.1–26.0)	27.8 (26.4–29.2)
Normal sperm morphology (%)	17.5 (14.9,20.1)	14.8 (13.1,16.6)	15.8 (11.8–19.7)	12.7 (10.7,14.6)	11.3 (9.3–13.3)	8.4 (7.5–9.2)	7.9 (6.8–8.9)
Straight line velocity (μm/s)	33.6 (31.4–35.7)	35.8 (31.8–39.9)	35.1 (32.9–37.2)	35.5 (33.7–37.3)	35.0 (33.0–37.1)	36.4 (34.9–38.0)	35.1 (33.2–37.1)
Curvilinear velocity (μm/s)	69.4 (66.6–72.1)	76.7 (68.4–84.9)	69.5 (65.0–74.0)	71.5 (68.2–74.7)	70.9 (66.6–75.2)	72.8 (69.1–76.5)	71.0 (67.0–75.0)
Linearity	50.9 (48.4–53.4)	51.1 (48.1–54.1)	53.8 (51.0–56.7)	52.2 (49.8–54.6)	52.2 (49.1–55.3)	52.4 (49.8–55.1)	51.6 (49.4–53.8)
SCSA–DFI (%)	15.1 (12.4–17.8)	20.3 (16.0–24.6)	15.8 (11.8–19.7)	17.4 (13.0–21.7)	13.5 (10.3–16.7)	15.4 (11.3–19.5)	12.2 (9.5–14.8)
Total aneuploidy[b] (no./10,000 sperm)	21.2 (16.0, 26.6)	24.2 (17.3–31.0)	22.6 (13.7–31.6)	20.1 (13.0–27.2)	21.3 (13.8–28.7)	18.5 (14.0–23.0)	24.0 (15.6–32.5)

[a]Values are means (95% confidence interval) for each group of samples. Pollution is designated as 'low' (columns 1, 5 and 7) or 'high' (columns 2, 3, 4 and 6) for the 3 months preceding each sample (see Figure 1).
[b]Values for total aneuploidy represent the sum of total disomy and total diploidy and are based on 15 men who contributed seven samples each.
SCSA–%DFI = sperm chromatin structure assay–DNA fragmentation index.

and sperm motion characteristics (measures for which World Health Organization reference values are not provided) were comparable to those recorded in the first study (Selevan *et al.*, 2000). Mean values for SCSA–%DFI were within the range associated with good fertility potential (Evenson *et al.*, 1999, 2002) and values for total aneuploidy were within the range of those reported in the literature (reviewed by Shi and Martin, 2002).

Results of the repeated measures analysis categorized air pollution as low (all reference samples) versus high (all winter samples), and controlling for various factors other than exposure that might affect semen quality (Table III). β is the slope of the regression line for the relationship between exposure and outcome, and is significant in the model when the 95% CI of the β does not include 0. Only mean SCSA–%DFI was significantly associated with exposure (Table III). No significant associations were found between exposure to air pollution and any of the routine semen measures (volume, concentration, total count, percentage motile, or percentage normal morphology considering whole sperm or sperm head shape). Similarly, no significant associations were found between exposure and any of the three selected CASA measures, or total aneuploidy

Table III. Results of repeated-measures statistical analysis by semen outcome, with exposure categorized as low or high

Outcome	Transformation	β (95% CI)	Adjusted for:
Semen volume	Log	0.05 (–0.20, 0.10)	Smoking, alcohol, briefs
Sperm concentration	Square root	–0.46 (–1.40, 0.48)	Abstinence, smoking, alcohol, caffeine, briefs
Total sperm count	Square root	–0.69 (–2.41, 1.19)	Abstinence, smoking, alcohol, caffeine, briefs
Percent motile sperm	Arc sin	–0.04 (–0.11, 0.02)	Abstinence, briefs, smoking
Straightline velocity	Log	0.03 (–0.27, 0.08)	Smoking
Curvilinear velocity	Log	0.025 (–0.02, 0.07)	Briefs
Linearity	none	0.65 (–1.56, 2.86)	Smoking, alcohol, caffeine, briefs
Percentage normal morphology	Arc sin	–0.01 (–0.03, 0.01)	Abstinence, smoking, caffeine, briefs
Percentage normal head morphology	None	–1.16 (–3.33, 0.01)	Abstinence, smoking, caffeine
SCSA–%DFI	Log	0.19 (0.02, 0.36)*	Smoking, briefs
Total aneuploidy	Square root	–0.17 (–0.79, 0.46)	Abstinence, smoking, alcohol, caffeine, briefs

*$P < 0.05$.
CI = confidence interval; SCSA–%DFI = sperm chromatin structure assay–DNA fragmentation index.

(Table III). Separating total aneuploidy into broad categories (total disomy or total diploidy) did not change the non-significant findings, nor did examination of frequencies of individual types of disomy or diploidy.

Discussion

The objective of this study was to test whether exposure to intermittent high levels of air pollution was associated with decrements in semen quality. Based on a preliminary study that sampled men at only one time (Selevan *et al.*, 2000), the present study used repeated sampling to increase the power to detect changes in a relatively small cohort of 36 young men, monitoring semen quality during periods of both low and high air pollution. Accordingly, men living in the Teplice district of the Czech Republic and exposed to relatively high levels of air pollution during the winter months were sampled seven times, three in late September (1995, 1996 and 1997) when air pollution had been low for ≥3 months, and four times in the winter (three in 1996 and once in 1997) when pollution was much higher. Of an array of semen outcomes tested, only sperm chromatin structure (SCSA–%DFI) was significantly associated with exposure to high levels of air pollution.

Consistent with the earlier study (Selevan *et al.*, 2000), the present study found no significant associations between exposure and sperm concentration or total sperm counts, measures of sperm production. Although inter-individual variability in sperm concentration was high in this cohort, as is typical for humans, the repeated measures study design would be expected to detect consistent changes in this measure. It is likely therefore that intermittent exposure to air pollution, including the high levels encountered during thermal inversions in Teplice in the winter, is not a risk factor for decreased sperm concentrations or sperm counts. These results do not rule out an impact of continuously high air pollution on sperm production. Adamopoulos *et al.* (1996) reported decreasing sperm concentrations in Athenian men during a period when air pollution was increasing and hypothesized that air pollution might contribute to secular trends in declining sperm counts.

Also consistent with the earlier study (Selevan *et al.*, 2000), this study found a significant association between exposure to air pollution and the percentage of sperm with fragmented DNA (SCSA–%DFI) thereby increasing the weight of evidence that exposure to high levels of air pollution may have damaging effects on sperm DNA. That this effect occurred in the absence of other changes in semen quality is not unexpected because SCSA is considered an independent measure of sperm function, namely sperm genetic integrity (Evenson *et al.*, 1980, 1991, 1999, 2002). In clinical populations, routine semen measures of sperm production and physiology (sperm concentration, motility, morphology) are not strong predictors of SCSA. A biologically plausible hypothesis regarding the aetiology of the observed association between exposure to air pollution and increased SCSA–%DFI is that reactive metabolites of PAH might reach the testes and react with sperm DNA to form adducts. Previous studies in the Teplice Program have shown that PAH found in the PM_{10} fraction can enter the body and form DNA adducts in at least two tissues, blood and placenta (Binkova *et al.*, 1995; Topinka *et al.*, 1997). Although DNA adducts in most germ cell stages should be repairable, DNA repair does not occur in condensed spermatids and epididymal sperm in which protamine has replaced somatic histones, rendering the DNA transcriptionally inert (reviewed by Baarends *et al.*, 2001). Thus, toxicant-induced DNA damage in this repair-deficient period of late spermiogenesis and epididymal sperm maturation, or about the last 10 days before ejaculation, would not be repaired and may be manifest as increased SCSA–%DFI. A recent study documenting the presence of benzo(a)pyrene diol epoxide–DNA adducts in sperm of men who smoke cigarettes (Zenzes *et al.*, 1999) lends further plausibility to this hypothesis. Furthermore, exposure to particulate matter containing PAH has been associated with increased rates of germ line mutation at expanded-simple-tandem-repeat DNA loci in mice (Somers *et al.*, 2004). The latter report suggests that PAH metabolites may reach the testes and induce mutations in pre-meiotic germ cells. The present results, with transient changes in SCSA–%DFI associated with intermittent air pollution, are consistent with DNA damage to post-meiotic, late stage spermatids.

The change in SCSA–%DFI associated with exposure in this study, although statistically significant, was relatively modest in magnitude. Mean baseline values for SCSA–%DFI for this cohort were within a range considered normal (12–15%) and increased to 15–20% after exposure, which is still considered indicative of good fertility potential (Larson *et al.*, 2000). Based on clinical studies, however, when SCSA–%DFI approaches and exceeds 30% the risk for infertility and spontaneous miscarriage is considerable, even in men with otherwise good semen quality (Evenson *et al.*, 1999; Larson *et al.*, 2000; Spano *et al.*, 2000; Zini, 2002; Larson-Cook *et al.*, 2003; Saleh *et al.*, 2003; Virro *et al.*, 2004). Although the change in average SCSA–%DFI observed in this study may not have affected the fertility potential of these men, changes of this magnitude could impact fertility in men with higher baseline SCSA–%DFI. Thus, when evaluating environmental risks to the general population, even modest increases in SCSA–%DFI may impact fertility in those men at the higher end of the distribution of SCSA–%DFI.

In contrast to the SCSA findings, associations between exposure to intermittent air pollution and sperm morphology or motility (percentage motile sperm) found in the earlier study were not replicated in the present study. Inconsistencies between studies, in which essentially the same laboratory methods were used, could be due to differences in the exposures. Indeed, remedial actions by the Czech government resulted in a decline in air pollution between 1993 and subsequent years (Pinto *et al.*, 1998; Benes *et al.*, 2001). With specific reference to the two semen studies being discussed, mean SO_2 levels for comparable 90 day intervals (late December to late March) were notably higher in 1993 (164.0 $\mu g/m^3$, Selevan *et al.*, 2000) compared with 1996 (78.5 $\mu g/m^3$, from Figure 1, this study). The same was true for PM_{10} where the comparable 1993 mean was 184.7 $\mu g/m^3$ (Selevan *et al.*, 2000) compared with 67.8 $\mu g/m^3$ for 1996 (from Figure 1, this study). Other differences that could affect the consistency of results include differences in the timing of peak exposures (during thermal inversions) with respect to semen sampling, and differences in

the components of the particulate fraction which can change from year to year (Pinto *et al.*, 1998). Nevertheless, the lack of consistency between these two studies coupled with the lack of association with CASA outcomes (i.e. the quality of sperm motion) in both studies, weakens the weight of evidence for a convincing association between exposure to air pollution and these specific measures of sperm quality.

Exposure to 'high' air pollution was not associated with increased sperm aneuploidy in this study, although an earlier study had found an association with increased incidence of YY disomy in a small group of non-smoking men sampled in Teplice in the winter of 1993 when exposures were higher (Robbins *et al.*, 1999). This discrepancy suggests that intermittent air pollution may not be a significant risk factor for sperm aneuploidy. However, these results do not rule out possible interactions between exposures to mutagens in air pollution (intermittent or continuous) and in cigarette smoke that could contribute to genetic damage in sperm (such as increased aneuploidy or DNA damage). The present cohort included both non-smokers and smokers, although the latter were not heavy smokers, all but one smoking less than a pack a day. Smoking alone was not associated with SCSA–%DFI (data not shown), but SCSA–%DFI remained significantly associated with air pollution whether or not smoking was included in the model. Previous reports have suggested that smoking ≥1 pack of cigarettes a day may be associated with increased sperm aneuploidy (Rubes *et al.*, 1998), and smoking ≥10 cigarettes a day may be associated with DNA damage as measured by a variant of the SCSA (Potts *et al.*, 1999).

Despite the lack of association between air pollution and sperm aneuploidy in this study, these longitudinal data were useful for examining the stability of sperm aneuploidy (disomy and diploidy) in individuals over an extended period. Analysis demonstrated that three men in this cohort consistently exhibited unusually high levels of sperm aneuploidy over the 2 years (independent of exposure), suggesting that a common genetic defect may influence endogenous levels of sperm cell aneuploidy (Rubes *et al.*, 2002). In the present study, the use of a repeated measures design allowed examination of the risks of exposure to air pollution in a small group of men despite this inter-individual variability for the aneuploidy measures.

The present study included measurement of blood lead, cadmium and mercury, as an indicator of possible occupational or environmental exposures, and because relatively high levels of lead (>40 μg/dl blood) have been associated with poor semen quality (reviewed by Apostoli *et al.*, 1998; Robbins and Cousins, 1998). Results indicated that the levels of these metals in the participants were within the range expected for the general US population (Centers for Disease Control, 2003), and were well below those expected to be associated with poor semen quality (Apostoli *et al.*, 1998; Robbins and Cousins, 1998; Bonde *et al.*, 2002). Furthermore, these blood metals were not associated with air pollution. Therefore, blood metal data do not suggest significant occupational or environmental metal exposures relevant to the study findings. To our knowledge, these studies in the Czech Republic (Selevan *et al.*, 2000 and the present study) are the only environmental epidemiology studies to date reporting associations between exposure to ambient air pollution and altered semen quality in humans. However, a recent occupational health study in Italy found changes in semen quality in motorway tollgate workers exposed continuously (6 h per work day) to automobile exhaust (De Rosa *et al.*, 2003). Compared to an age-matched control group, these men had lower sperm viability, motility and velocity (measured by CASA), and fewer sperm with normal chromatin evaluated microscopically via acridine orange staining. Unlike our participants, however, these men had elevated blood lead levels (averaging 20.1 μg/dl) to which the authors attributed the seminal deficiencies. Although automobile exhaust contributed to the air pollution in Teplice, source signature models showed that combustion products of coal used for industry and home heating were the major components (Pinto *et al.*, 1998). Thus the present study differs from that of De Rosa *et al.* (2003) with respect to both exposure composition and duration.

Taken together, this study and the previous study (Selevan *et al.*, 2000) provide novel evidence that exposure to episodes of relatively high air pollution may have adverse effects on semen quality, specifically on sperm chromatin integrity. Because high SCSA–%DFI (>30%) has been associated with clinical infertility and increased risk of spontaneous abortion (Evenson *et al.*, 1999; Larson *et al.*, 2000; Spano *et al.*, 2000; Zini *et al.*, 2002; Larson-Cook *et al.*, 2003; Saleh *et al.*, 2003; Virro *et al.*, 2004), the present findings may have implications for fertility on a population basis. Confirmation of similar changes in other study groups exposed to episodic or continuous air pollution, especially at levels that approach or exceed US air quality standards, would be of value for more detailed risk characterization.

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