

Evaluation of aneuploidy and DNA damage in human spermatozoa: applications in field studies

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Summary. With the goal of incorporating measures of sperm nuclear integrity in an epidemiology study, semen samples from young Czech men were analysed for sperm aneuploidy and sperm chromatin structure in addition to routine measures of sperm production and quality. The exposure in question was to high seasonal air pollution containing reactive polyaromatic hydrocarbons potentially capable of affecting spermatogenesis and damaging sperm DNA. The sperm aneuploidy assay uses fluorescence *in situ* hybridization to label selected sperm chromosomes; as applied in this study, the sex chromosomes (X,Y) and chromosome 8 were targeted. The sperm chromatin structure assay detects sperm nuclei with increased susceptibility to denaturation, a feature that is associated with DNA damage. Logistically, these assays were relatively easy to incorporate into the study design. The aneuploidy assay provided information suggesting that exposure to high levels of air pollution may increase the risk of sperm aneuploidy and that it is important to control for exposure to cigarette smoke and/or alcohol in such studies. The sperm chromatin structure assay provided valuable baseline information about Czech semen donors and data suggestive of an adverse effect of smoking and air pollution on spermatozoa that merits further investigation.

Introduction

Semen analysis is an established clinical tool for assessing the fertility potential of individual men

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(Jouannet *et al.*, 1988; Bartoov *et al.*, 1993; Ayala *et al.*, 1996; Zinaman *et al.* 2000), and alterations in semen quality after environmental exposures are considered to be indicators of increased reproductive risk (Wyrobek *et al.*, 1997). Typically, semen analysis evaluates both semen production (based on measuring sperm concentration and total spermatozoa per ejaculate) and sperm quality (based on measuring sperm motility and morphology). However, routine semen analysis provides little or no information about the genetic integrity of the spermatozoa. It is clear from animal studies that exposure to reactive chemicals and drugs capable of damaging DNA can result in impaired embryonic development and/or birth defects in resultant offspring (reviewed by Hales & Robaire, 1996). Thus, the need exists to evaluate the genetic integrity of human spermatozoa after exposure to similar drugs and chemicals. Although indirect assays for monitoring the nuclear stability of human spermatozoa have been applied in clinical studies over the years (reviewed by Perreault, 1993), specific tests of sperm nuclear/genetic integrity have become available only recently (Perreault, 1998; Evenson, 1999; Robbins *et al.*, 1999).

The objective of this paper is to present an example of a human semen study in which two contemporary tests of sperm genetic integrity were integrated into the study design, and to summarize the extent to which they contributed to the interpretation of the study results. The study described below was conducted to investigate potential adverse effects of exposure to environmental air pollution on male reproductive health in young Czech men (Sram *et al.*, 1996). In addition to routine semen analysis, the study

included two tests of genetic integrity: the sperm chromatin structure assay (SCSA, Evenson & Jost, 1994) and a sperm aneuploidy assay (Robbins *et al.*, 1993).

General study objective, design and results

The overall study objective was to evaluate male reproductive health in young Czech men residing in two communities that differ with respect to levels of seasonal air pollution (Sram *et al.*, 1996). Outcomes of interest included health information obtained by questionnaire, a routine physical examination and semen analysis, including two measures of the genetic integrity of spermatozoa.

In the industrial Teplice District of the Czech Republic air pollution levels are high during the winter months. This pollution results from the combustion of high sulphur coal for both home-heating and local industry. In the winter, weather conditions (temperature inversions) prevent dissipation of the pollution, resulting in episodes of very high pollution (Sram *et al.*, 1996). In the comparison district of Prachatice, a rural area with little industry, air pollution during the winter is significantly lower than in Teplice, but still somewhat higher than in the summer. Importantly, air pollution levels are low in both districts from spring to early autumn. Companion studies in Teplice have shown that the air pollution contains polycyclic aromatic hydrocarbons that can form DNA adducts in body tissues (reviewed by Sram, 1999), and thus could potentially damage germ cell DNA.

The final study design (excluding the results of a pilot study conducted in 1992), was cross-sectional in nature and involved sampling young men (physical examination and semen sample) at the end of winter in 1993, the end of summer in 1993 and the end of winter in 1994. The sampling periods in each district were each 5 days. With this strategy, the men sampled in Teplice at the end of winter represent the exposed group, while men sampled in Teplice at the end of summer and men sampled in Prachatice at both times of year together represent the unexposed (comparison) group. Air pollution monitoring in both districts confirmed that average pollution was significantly higher in Teplice during both winters than in Teplice in the summer or in Prachatice in the summer or both winters. However, it is noteworthy that pollution in Teplice differed by year, with levels being higher in 1993 than in 1994.

The study population consisted of young (18-year-old) men living in Teplice or Prachatice

and invited to participate in the study around the time of a scheduled physical examination at the respective district hygiene station. Analysis of data from the physical examination and from a health questionnaire showed that the men residing in Teplice were similar to those from Prachatice with respect to general health, life style and habits that could affect reproductive function (such as consumption of cigarettes, alcohol and caffeine), and occasional exposure to solvents or metals through work or hobbies. A total of 272 men (66% of those interviewed) agreed to contribute a single semen sample for analysis.

Standard semen measures (volume, sperm concentration, sperm morphology) were determined according to guidelines provided by WHO (1992). Samples were allowed to liquefy at room temperature for not more than an hour before aliquots were removed for the standard semen analyses and videotaped for later estimation of the percentage of motile spermatozoa and the quality of motility determined by computer-assisted sperm motion analysis (CASA). The remaining semen was loaded into straws or aliquoted into cryovials, immediately frozen (without cryopreservative) on dry ice, and stored below -70°C . Samples were later shipped on dry ice to collaborating laboratories for genetic analyses, with care taken to confirm that they remained frozen throughout.

Statistical analysis of the routine semen data had shown that exposure to high levels of air pollution (i.e. living in Teplice in the winter) was associated with significant decreases in the percentage of motile spermatozoa and the percentage of spermatozoa with normal morphology (Selevan *et al.*, 2000). Exposure was not associated with decrements in sperm motion parameters measured by CASA; however, seasonal differences in some measures of sperm velocity were evident. Neither district of residence nor exposure was associated with decrements in sperm concentration or numbers per ejaculate. Based on these findings, it appeared that exposure to high air pollution could have a negative impact on sperm quality. Therefore, it was of interest to evaluate the genetic integrity of the spermatozoa in these semen samples.

Evaluation of sperm aneuploidy

Exposure to potential reproductive toxicants could affect sperm chromosomes in two general ways: by inducing structural aberrations (breaks, translocations) or by affecting the number of chromosomes in a given sperm cell. The test employed in these studies evaluated chromosome numbers. It uses multi-colour fluorescence *in situ* hybridization

(FISH) to detect the sex chromosomes (X,Y) and a selected autosome (chromosome 8) in sperm nuclei (Robbins *et al.*, 1993). Fluorescent chromosome-specific probes are used to label a portion of each chromosome with a unique colour resulting in a fluorescent spot or domain on the sperm nucleus when viewed by fluorescence microscopy. Duplicate fluorescent spots for a single chromosome are indicative of hyperhaploidy (disomy) while duplicate spots for all three chromosomes are indicative of diploidy.

Air dried smears of thawed semen were first decondensed in order to enhance hybridization (Robbins *et al.*, 1993, 1999), and then taken immediately into the hybridization procedure described in detail elsewhere (Rubes *et al.*, 1998; Robbins *et al.*, 1999). Because sperm aneuploidy is an infrequent event, 10,000 sperm nuclei were scored for each sample, making this a labour-intensive assay. Strict scoring criteria were used to minimize misclassification of sperm cells or hybridization signals due to technical artefacts (Rubes *et al.*, 1998). Potential biases that could be introduced by laboratory factors, such as the order of scoring or the batch of probe utilized, were examined and controlled (if necessary) in the statistical analyses (Robbins *et al.*, 1999).

Two studies were undertaken using subsets of the frozen semen samples archived from the main study. First, the effects of smoking cigarettes on sperm aneuploidy were evaluated (Rubes *et al.*, 1998). Cigarette smoke contains some of the same components as air pollution, particularly with respect to polyaromatic hydrocarbons, and smoking has been associated with decreased semen quality (Vine *et al.*, 1994). Therefore, we were interested in determining the extent to which smoking could be a potential risk factor or confounder for sperm aneuploidy in air pollution studies. Samples obtained from men living in Teplice during the late winter of 1994 were selected since this was the largest group of specimens available from any single sampling period. This strategy allowed us to control for air pollution (all men in this group having the same environmental exposures) or other unknown factors that might be specific to district of residence. Of the 63 men in this group, samples from 10 men who reported smoking a pack of cigarettes per day and 15 nonsmokers who reported minimal passive exposure to cigarette smoke were selected for analysis. Smoking status was confirmed by urinary cotinine levels. In attempting to control for other health and life style factors that could potentially affect semen quality, it was found that the men who smoked cigarettes were also more likely to drink alcoholic beverages. In fact, all the smokers were

medium to heavy drinkers while many of the nonsmokers were also nondrinkers. Therefore, we could not control for alcohol consumption in this group of men. After controlling for sexual abstinence of less than 2 days, caffeine consumption, wearing of briefs and working with solvents or metals (at least 10 h per week through work or hobby), significant associations were found between smoking (drinking) and YY disomy (Rubes *et al.*, 1998). Although the sum of the aggregate grouping of sperm disomic for chromosome X, Y, or 8 was also significantly elevated in smokers, no other individual disomies (XX, XY, or 8-8) were elevated, nor was there an increased risk of diploidy for smokers. These findings, although based on a small sample size, suggested that smoking and/or drinking should be considered potential risk factors for sperm aneuploidy outcomes in epidemiology studies.

Accordingly, semen samples from nonsmokers were selected to examine the potential relationship between exposure to high air pollution and sperm aneuploidy (Robbins *et al.*, 1999). Again using only samples obtained from men living in Teplice, we employed the same aneuploidy assay and scoring criteria to evaluate spermatozoa from 19 nonsmokers sampled after the period of highest air pollution (winter 1993) and compared these results with those obtained from 13 different nonsmokers sampled after the season of low air pollution (late summer 1993). Interestingly, the results again showed a significant association between exposure to high levels of air pollution and YY disomy (Robbins *et al.*, 1999). No other frequencies of disomy or diploidy differed between exposure groups. These two studies were conducted in different laboratories but used the same methods (learned in the laboratory of Dr Andrew Wyrobek (Vysis Inc., Downers Grove, IL), and probes purchased from the same company, Lawrence Livermore National Laboratory, Livermore, CA. Looking across the four groups of men examined in these two studies, it is interesting to note that the mean frequency of YY-8 spermatozoa (per 10,000 cells) was highest (4.5) in smokers sampled in late winter 1994, a season of high air pollution, as compared with nonsmokers sampled at the same time (2.2) or nonsmokers sampled in late winter 1993 (3.5) when the pollution was even higher than in winter 1994, and lowest in nonsmokers sampled in later summer 1993 (0.6), the season of low air pollution.

Sperm chromatin structure assay (SCSA)

The SCSA measures the relative susceptibility of sperm nuclear DNA to denaturation by acid or

heat (reviewed in Evenson & Jost, 1994; 2000). It employs a metachromatic dye, acridine orange, that fluoresces green when intercalated into double-stranded DNA or red when associated with single-stranded (denatured) DNA or RNA. By using flow cytometry, thousands of sperm nuclei (which contain negligible RNA) can be evaluated individually for both red and green fluorescence. The relative amount of red and green fluorescence in each spermatozoon is expressed as the ratio of red to (red+green), termed α_t . In fertile men, α_t values for most spermatozoa fall within certain limits. The per cent of spermatozoa outside the main population for α_t is designated $\text{COMP}\alpha_t$. $\text{COMP}\alpha_t$ provides an estimate of the percentage of spermatozoa with abnormal chromatin structure. A recent prospective study of couples attempting pregnancy showed that the time to conception (a clinical measure of fertility) is highly correlated with SCSA data (Evenson *et al.*, 1999). Importantly, $\text{COMP}\alpha_t$ values $\geq 30\%$ were highly correlated with failure to conceive after attempting pregnancy for 1 year (the clinical definition of infertility). On the other hand, men with $\text{COMP}\alpha_t < 15\%$ typically conceived within 3 months while those with $\text{COMP}\alpha_t$ of 15% to $< 30\%$ typically conceived in 4–12 months. High values for $\text{COMP}\alpha_t$ have also been reported after exposure to reproductive toxicants in animal models (Evenson & Jost, 1994; 2000) and correlate with other measures of DNA damage in spermatozoa (Aravindan *et al.*, 1997).

Of the 272 samples provided by the Czech semen donors, 266 contained sufficient spermatozoa for SCSA analysis. Frozen semen samples were thawed, diluted, incubated with acid (pH 1.2) for 30 s, immediately stained with acridine orange and analysed by flow cytometry as described in detail elsewhere (Evenson & Jost, 1994; Evenson *et al.*, 1999; Evenson, 1999). All SCSA outcomes were analysed (mean red fluorescence, mean green fluorescence, mean α_t , the standard deviation of α_t , and $\text{COMP}\alpha_t$). Here the results for $\text{COMP}\alpha_t$ are emphasized as this apical outcome correlates with fertility (Evenson *et al.*, 1999).

In a multivariable linear regression analysis, values for $\text{COMP}\alpha_t$ did not differ by district of residence, but were higher in the Teplice donors sampled in late winter 1993 (Selevan *et al.*, 2000). However, after controlling for potential confounding by season, which is known to affect some semen measures, purportedly as a latent consequence of exposure to elevated ambient temperatures in summer (Levine, 1994), the difference was no longer significant. Nevertheless, the preliminary results were intriguing and prompted us to take a closer look at this outcome in our study population.

The distribution of $\text{COMP}\alpha_t$ for the 266 18-year-old Czech donors is shown in Fig. 1. According to the fertility criteria proposed by Evenson *et al.* (1999) and described above, a relatively high percentage (19%) of these donors would be predicted to be infertile (having $\text{COMP}\alpha_t \geq 30\%$). Figure 2 shows the distribution of samples in the $\text{COMP}\alpha_t$ fertility categories for the Czech donors compared with the fertility study donors described previously (Evenson *et al.*, 1999). While these two groups of men differ by age and recruitment criteria, and are not suitable for direct statistical comparison, it is apparent that the distribution of $\text{COMP}\alpha_t$ by fertility category is shifted toward lower predicted fertility in the young Czech donors.

Discussion

Inclusion of the two assays for sperm nuclear integrity (FISH for sperm aneuploidy and SCSA

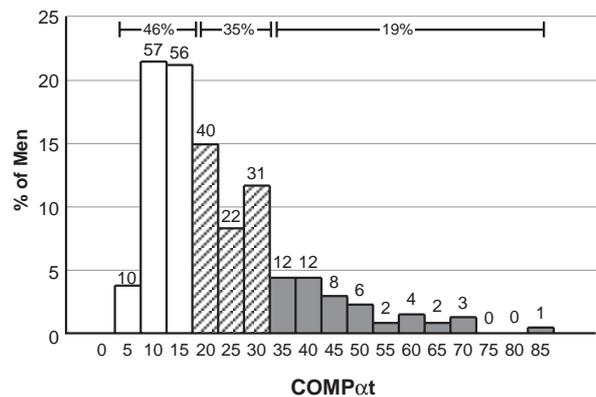


Figure 1. Distribution of the SCSA variable $\text{COMP}\alpha_t$ in semen samples from 266 Czech men. Shading is used to illustrate fertility category as defined by Evenson *et al.* (1999): open bars=probable high fertility; shaded bars=probable intermediate fertility; solid bars=probable infertility.

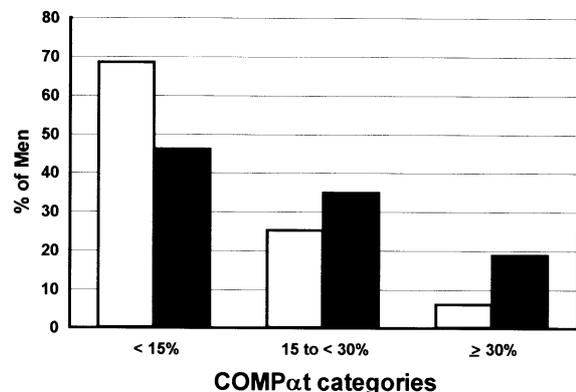


Figure 2. Percentage of semen donors in each of three fertility categories based on $\text{COMP}\alpha_t$ according to criteria of Evenson *et al.* (1999). Open bars=men in US fertility study (Evenson *et al.*, 1999); solid bars=men in Czech air pollution study.

as a reflection of DNA damage) in the epidemiology study described here was relatively easy from the standpoint of sample handling and study logistics. Indeed both of these tests lend themselves well to application in the field. Each test requires only a small aliquot of semen (0.1–0.2 ml semen for normospermic samples; ~0.5 ml for oligospermic samples), and these volumes are usually available except when semen volume is unusually low. When possible, requesting a sexual abstinence interval of at least 48 h will ensure sufficient semen from most donors. In the present study, we were unable to request the desired abstinence interval of at least 48 h and about 20% of the donors had shorter than desired abstinence. Nevertheless, sufficient semen was obtained to conduct the SCSA assay on almost all the men (266/272 samples, with two samples being excluded due to azoospermia). Furthermore, as long as the sample is frozen promptly and maintained at suitably low temperature, it can be stored for long periods (several years) before analysis (SCSA or FISH). This flexibility allows the study director to prioritize assays based on the results of the standard semen analyses or based on differing exposures in longitudinal studies.

Our finding with respect to an increased risk of YY aneuploidy in smokers/drinkers is based on a small sample size (25 men) and merits further exploration. It is important to point out that this is a new assay and while considerable effort has been put forward to evaluate the incidence of sperm aneuploidy by chromosome, age and clinical fertility status (Downie *et al.*, 1997; Hassold, 1998), very few studies have been undertaken to explore the potential relationship between sperm aneuploidy and life style or environmental exposures. Another small study (15 smokers of >15 cigarettes per day versus 15 nonsmokers) reported significantly increased YY1 sperm disomy in smokers (Potts *et al.*, 1999a). A somewhat larger smoking study (45 men) found a suggestive but 'unstable' association between smoking and XX18 sperm disomy (Robbins *et al.*, 1997). Interestingly this same study reported significant associations between alcohol consumption and XX18, diploidy XY18–18 and the duplication phenotype XX18–18 after controlling for caffeine, smoking and donor age, again suggesting that alcohol consumption may be a potential risk factor in studies relating other exposures to sperm aneuploidy. In the same study, significant associations were also found between caffeine consumption and XX18 and XY18 disomies, diploidy XY18–18 and the duplication phenotype YY18–18 after controlling for alcohol, smoking and donor age. Further work is needed to characterize the poten-

tial risks of these life style factors with respect to sperm aneuploidy.

A few recent epidemiology studies have examined potential relationships between other types of environmental exposures and sperm aneuploidy. Harkonen *et al.* (1999) used FISH to evaluate chromosomes 1 and 7 in 30 healthy Danish farmers before and after exposure to fungicides. Although exposure to fungicides was not associated with increased sperm aneuploidy, smoking was significantly associated with the incidence of sperm disomic for chromosome 1 and with diploid spermatozoa (as well as with the aggregate frequency of aneuploid spermatozoa). A recent study of 32 Chinese pesticide factory workers (exposed to organophosphate pesticides) did find an increased risk of sperm aneuploidy (examining chromosomes X, Y and 18) when compared with a group of 43 unexposed workers (Padungtod *et al.*, 1999).

The significance of these findings regarding the overall risk of sperm aneuploidy from exposure to cigarette smoke, air pollution or other exposures, is unclear. Most epidemiology studies published to date have evaluated aneuploidy in only 2 or 3 of the 23 human chromosomes. However, some clinical studies (examining the relationship of poor semen quality and sperm aneuploidy) are beginning to use probes to multiple chromosomes (Martin *et al.*, 1999; Pang *et al.*, 1999; Pfeffer *et al.*, 1999; Vegetti *et al.* 2000). Such an approach should improve the sensitivity of this assay for detecting sperm aneuploidy in future epidemiology studies. The clinical studies also introduce the possibility that men with severely abnormal semen quality may be at risk of higher levels of sperm aneuploidy; thus, in epidemiology studies, poor semen quality prior to exposure to compounds of interest may be an important factor when evaluating the potential relationship between exposure and sperm aneuploidy. Age is another such factor shown to be related to sperm aneuploidy (Robbins *et al.*, 1995) although it was not relevant in the studies described here since the Czech donors were all the same age.

The health significance of producing excess numbers of spermatozoa with YY disomy merits discussion. Individuals with 47,XYX karyotypes, as may arise if an oocyte is fertilized by a 24,YY spermatozoon, are usually fertile and do not exhibit phenotypic deficits. While such men appear to have an elevated risk of producing spermatozoa with YY disomy (Martin *et al.*, 1999) or spermatozoa with other disomies (Morel *et al.*, 1999), the risk is not as high as might be expected (based on a limited number of studies to date), suggesting that aneuploid germ cells may be eliminated during spermatogenesis. Nevertheless, other sex

chromosome disomies (XX, XY) in spermatozoa could give rise to individuals with both phenotypic and fertility defects, and of course autosomal sperm disomies can give rise to trisomic conceptuses with fatal or very serious developmental consequences (Hassold, 1998).

When estimating risks to fertility and pregnancy outcomes, it is important to note that although the increased frequencies of toxicant-induced sperm aneuploidy in studies reported to date may be statistically significant, the actual incidence (or percentage of spermatozoa affected) is still quite low. Nevertheless, at present, any significant relationship between an exposure and increased risk of sperm aneuploidy is potentially important and studies such as those summarized here will contribute to risk estimates as we learn more. In addition, recent progress has been made toward using two probes to different regions of one chromosome in order to identify structural (as well as numerical) aberrations, such as translocations in spermatozoa (Van Hummelen *et al.*, 1996; Baumgartner *et al.*, 1999). Since structural aberrations might be induced by different chemicals and/or different mechanisms, their detection in future epidemiological studies will be of interest.

Examination of the SCSA results for the young Czech donors raised several questions. First, the distribution of $\text{COMP}\alpha_t$ in these samples demonstrated a higher than expected percentage of men who might be at risk for infertility. Is this a consequence of growing up and going through puberty in a polluted environment, or could high $\text{COMP}\alpha_t$ simply be related to their young age? Currently there are no SCSA data for men of this age that can be used for comparison purposes. It would be informative to follow these men (or any cohort of adolescents) into adulthood to determine whether sperm chromatin structure varies by age or maturity. Second, there was some evidence of a seasonal effect for this outcome, with $\text{COMP}\alpha_t$ values being somewhat higher in the samples obtained in late winter versus late summer, even in the cleaner district. Other parameters of semen quality have been reported to show subtle seasonal changes, perhaps related to detrimental effects of heat in the summer becoming manifest by the late winter (reviewed by Levine, 1994). The possibility that SCSA varies by season deserves attention.

SCSA is thought to reflect DNA damage in spermatozoa (Evenson & Jost, 1994; Evenson, 1999). Unlike numerical and most structural aberrations in sperm chromosomes, which are likely to arise during meiosis, damage to germ cell DNA can arise at any point in spermatogenesis. Damage induced in spermatogonia, spermatocytes and round spermatids is likely to be repaired as these

cells have good DNA repair capability. In contrast, condensed spermatids and epididymal spermatozoa lack DNA repair capability, so damage to these cells is more likely to be transmitted to the zygote at fertilization. It is therefore of interest to measure DNA damage in spermatozoa in epidemiology studies. Several established tests for DNA damage in somatic cells have recently been applied to spermatozoa, including the terminal uridine nick end-labelling (TUNEL) assay (which detects DNA strand breaks), a related nick translation assay, and the single-cell gel electrophoresis (SCGE or 'comet') assay. In addition, measurement of various DNA adducts in spermatozoa may provide informative biological markers of both exposure and effect. A detailed description of these assays as applied to spermatozoa is beyond the scope of this article, but can be found in recent reviews (Perreault, 1998; Sakkas *et al.*, 1998; Evenson, 1999). Of relevance to this paper is preliminary information indicating a strong correlation between the results of these assays, which provide direct information about DNA breakage, and the SCSA which measures acid-induced sperm chromatin denaturation as an indirect measure of DNA damage (Sailer *et al.*, 1995; Aravindan *et al.*, 1997).

The SCSA has been included in several recent epidemiology studies in addition to the air pollution study discussed in this paper. For example, men who smoke cigarettes were found to be significantly more sensitive to acid-induced denaturation of sperm DNA as measured by SCSA (Potts *et al.*, 1999b). Of relevance to this discussion, the spermatozoa of smokers also possessed higher levels of DNA strand breaks than those of nonsmokers. Thus, both assays showed an association between smoking and DNA damage. Interestingly, in our analysis of Czech semen donors, smoking was retained in the multivariable linear regression model examining the relationship between exposure to air pollution and $\text{COMP}\alpha_t$ (unpublished observation). Therefore, it may be important to consider smoking status as a potential risk factor in epidemiology studies measuring SCSA, as it appears to be for sperm aneuploidy outcomes. SCSA has also been monitored in at least one study of pesticide applicators (Larsen *et al.*, 1998) and men exposed to solvents (Lemasters *et al.*, 1999), although statistically significant associations between the exposure in question and SCSA outcomes were not found in these studies. As SCSA and other assays detecting DNA damage are applied more widely, the risks for induction of toxicant-induced DNA damage in spermatozoa and inherent consequences with respect to fertility

and developmental potential should become better understood.

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